Immunization of Mice with Single PspA Fragments Induces Antibodies Capable of Mediating Complement Deposition on Different Pneumococcal Strains and Cross-Protection

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PspA is an important candidate for a vaccine with serotype-independent immunity against pneumococcal infections. Based on sequence relatedness, PspA has been classified into three families comprising six clades. We have previously addressed the cross-reactivity of antibodies against PspA fragments containing the N-terminal and proline-rich regions of PspA from clades 1 to 5 (PspA1, PspA2, PspA3, PspA4, and PspA5) by Western blot analysis and reported that anti-PspA4 and anti-PspA5 were able to recognize pneumococci expressing PspA proteins from all of the clades analyzed. We have now analyzed the functional capacity of these antibodies to bind and to mediate complement deposition on intact bacteria in vitro. Our results show that both PspA4 and PspA5 elicited antibodies that are able to bind and to mediate complement deposition efficiently on pneumococcal strains bearing PspA proteins from clades 1 to 5. Moreover, mice immunized with PspA4 and PspA5 were protected against an intranasal lethal challenge with strains expressing PspA proteins from the two major families. PspA4 and PspA5 are thus able to induce antibodies with a high degree of cross-reactivity in vitro, which is reflected in cross-protection of mice. We have also analyzed the contribution of the nonproline (NonPro) block within the conserved proline-rich region to the reactivity of anti-PspA antibodies, and the results indicate that N-terminal α-helical region, the blocks of proline repeats, and the NonPro region can influence the degree of cross-reactivity of antibodies to PspA.
PspA-expressing strains. While anti-PspA2 showed reaction restricted to the same clade. Within Fam2, anti-PspA3 serum also showed reactivity restricted to PspA3-expressing strains, while anti-PspA5 and, more strikingly, anti-PspA4 sera showed a broad recognition capacity, being able to react with strains expressing PspA proteins from clades 1 to 5 (7). The ability of sera to recognize a pneumococcal strain by Western blot analysis does not necessarily correlate with their capacity to induce protection in vivo though. In fact, the levels of antibodies to PspA detected by enzyme-linked immunosorbent assay (ELISA) or through surface staining of the bacteria failed to provide a useful correlate of protection (22). Based on the strong evidence supporting the importance of complement in protection against pneumococcal disease, it was proposed that in vitro complement deposition mediated by antibody may be used as a surrogate assay for the prediction of protection induced by surface antigens of pneumococci (15). This work aimed at further characterizing antibodies against the PspA1, PspA2, PspA3, PspA4, and PspA5 N-terminal fragments in terms of their capacity to mediate C3 deposition on the surface of pneumococci expressing PspA proteins from different clades. Moreover, protection of mice against a lethal intranasal challenge with strains expressing PspA from Fam1 or Fam2 was also analyzed. The basis for the broad reactivity observed in the anti-PspA4 serum by Western blot analysis was also further investigated. Of the five PspA fragments analyzed, PspA4 was the only one containing a nonproline (NonPro) block within the proline-rich region. Not all native PspA proteins include this region: of 24 PspA sequences analyzed by Hollingshead and collaborators (10), 14 were shown to have this NonPro block. We have thus examined whether this region would be responsible for increased cross-reactivity.

**MATERIALS AND METHODS**

**Construction of PspA fragments.** All cloning procedures were performed with *Escherichia coli* DH5α grown in Luria-Bertani medium supplemented with ampicillin (100 μg/mL). The plasmids encoding the N-terminal regions of PspA proteins from clades 1 to 5 (PspA1, PspA2, PspA3, PspA4, and PspA5) were previously described (7). Two new plasmids encoding PspA4 fragments were constructed: while the original construct (PspA4) encoded the complete N-terminal region plus the proline-rich region (containing a NonPro block within this region), PspA4AB contains only the N-terminal α-helical region without the entire proline-rich region, and PspA4Pro contains the N-terminal α-helical region plus the first block of prolines only, lacking both the NonPro and second proline blocks (Fig. 1). Both fragments were amplified by PCR from the original PspA4 construct using primers 5’ TAGCTCGAGACCATGGTAAGAGCAGA GAAAGCC 3’ (forward) and 5’ GTGACTTTAGCTTCTTCATCTC CATC 3’ (PspA4AB-reverse) or 5’ GTGACTTTATGTITTGGTGCTGCTG GCT 3’ (PspA4Pro-reverse). The gene products were cloned into the pGEMT-easy vector (Promega), and the sequences were confirmed by DNA sequencing. The pGEMT-easy-pspA4 constructs were digested with XhoI and KpnI, and the resulting fragments were subcloned into the pAE 6×His vector (18), generating pAE-pspA4AB and pAE-pspA4Pro. A plasmid encoding a fusion between PspA3 and the proline-rich region (containing the NonPro block) of PspA4 was constructed by amplification of the proline-rich region of PspA4 using primers 5’ TAGCTCGAGACCATGGTAAGAGCAGA GAAAGCC 3’ (forward) and 5’ TAGGGTT ACTTATGGTTTGCTGCTGAAAGC 3’ (NonPro R). The gene product was cloned into the pGEMT-easy vector (Promega), and the sequence was confirmed by DNA sequencing. The pGEMT-easy-NonPro construct was digested with XhoI and KpnI, and the resulting fragment was subcloned into the 3’ end of *pspA3* in pTG-pspA3NS (13). The fragment encoding the PspA3-NonPro fusion (Fig. 1) was digested with XhoI and KpnI and cloned into pAE 6×His, generating pAE-pspA3-NonPro.

**PspA expression and purification.** The pAE 6×His vectors containing the *pspA* constructs were used to transform BL21(DE3) *E. coli* competent cells (Invitrogen). Protein expression was induced in mid-log-phase cultures by the addition of 300 mM NaCl. The recombinant proteins, bearing an N-terminal 6×His tag, were purified from the soluble fraction through affinity chromatography using Ni²⁺-charged resin (His Trap HP; GE HealthCare) in an Äkta Prime apparatus (GE HealthCare). Elution was carried out with 250 mM imidazole. The purified fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, dialyzed against 10 mM Tris-HCl (pH 8.0)–20 mM NaCl–0.1% glycerol, and stored at −20°C.

**Pneumococcal strains.** All of the strains used in this study were maintained as frozen (−80°C) stocks in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) in 20% glycerol. The serotypes, PspA clades, and sources of the strains are given in Table 1.

**Animal immunization and challenge.** Animal experimental protocols were approved by the Ethics Committee of the Instituto Butantan (São Paulo, Brazil). Five- to 7-week-old female BALB/c mice from the Instituto Butantan (São Paulo, Brazil) were immunized intraperitoneally with 5 μg of PspA1, PspA2, PspA3, PspA4, or PspA5 adjuvanted with Al(OH)₃ (50 μg Al³⁺). Animals were given three doses of protein at 14-day intervals. For the experiments with PspA4AB, PspA4Pro, and PspA4 or PspA3 and PspA3-NonPro, animals were immunized subcutaneously with three doses of protein (5 μg) adjuvanted with Al(OH)₃ at 14-day intervals. The adjuvant alone was used as a control. sera were collected from mice by retro-orbital bleeding 1 day before each dose and 14 days after the final dose. Mice were challenged 15 days after the last immunization. Animals were anesthetized through the intraperitoneal route with 200 μl of a 0.2% xylazine−1.0% ketamine mixture and then challenged through the inoculation of 50 μl of a suspension of strain A66.1 (4 × 10⁸ CFU/animal) or ATCC
representative of two experiments using sera from independent immunizations. For complement deposition, bacteria were then washed with PBS, resuspended in 2% formaldehyde in PBS, and analyzed using a FACScalibur (BD Biosciences). Ten thousand gated events were acquired and analyzed in fluorescence intensity histograms.

RESULTS

PspA4 and PspA5 elicit antibodies with a high degree of cross-reactivity. Sera collected from individual mice immunized with PspA1, PspA2, PspA3, PspA4, or PspA5 were analyzed by ELISA for reactivity against each recombinant PspA fragment. Data from sera collected after the second immunization are shown in Fig. 2. The pattern of cross-reactivity followed the classification of the different clades into the families, with the induction of significantly higher titers of anti-Psa antibodies against PspA proteins from the same family in all of the fragments tested compared to sera from animals inoculated with alum only. Reactivity with fragments from the other family was not always observed: anti-PspA1 (Fam1) did not elicit higher titers of antibodies reacting with PspA5 (Fam2), anti-PspA2 (Fam1) did not show reactivity with either PspA4 or PspA5 (Fam2), and anti-PspA3 (Fam2) did not react with either PspA1 or PspA2 (Fam1). Both anti-PspA4 and anti-PspA5 showed increased reactivity with all five PspA fragments examined compared to sera from animals injected with alum.

Anti-PspA4 and PspA5 elicit antibodies that bind and mediate C3 deposition on strains expressing different PspA proteins. We further tested the ability of the anti-PspA antibodies to bind to the surface of pneumococci bearing different PspA proteins, and again the pattern of increased reactivity between clades of the same family was generally observed. Anti-PspA1 and anti-PspA2 (Fam1) sera showed increased binding to St245/00 (clade 1, Fam1) and D39 (clade 2, Fam1), while binding to 3JYP2670 (clade 4, Fam2) and ATCC 6303 (clade 5, Fam2) was only slightly enhanced and no binding to M10 (clade 3, Fam2) at all was seen (Fig. 3A). Anti-PspA3 (Fam2) showed the narrowest cross-reactivity, being able to bind efficiently only to M10 (clade 3, Fam2). Restricted binding was seen for anti-PspA3 even to Fam2 strains 3JYP2670 (clade 4) and

![FIG. 2. Cross-reactivity of sera against PspA. Sera from four BALB/c mice immunized with PspA1, PspA2, PspA3, PspA4, or PspA5 were collected after the second immunization and analyzed individually by ELISA against each PspA fragment. The results are shown as the log_{10} of the titer. An asterisk indicates a statistically significant difference from animals immunized with alum (Student’s t test, P ≤ 0.05). Results are representative of two experiments using sera from independent immunizations.](image-url)
TABLE 2. Survival after intranasal challenge with A66.1 (clade 2, Fam1)

| Immunizing antigen | No. alive/total | % Survival | P value |
|--------------------|----------------|------------|---------|
| Alum               | 0/6            | 0          |         |
| Fam1               |                |            |         |
| PspA1              | 3/6            | 50         | 0.09    |
| PspA2              | 5/6            | 83*        | 0.008   |
| Fam2               |                |            |         |
| PspA3              | 2/6            | 33         | 0.23    |
| PspA4              | 6/6            | 100*       | 0.001   |
| PspA5              | 4/6            | 67*        | 0.03    |

* Statistically significant difference from animals inoculated with alum (Fisher exact test).

ATCC 6303 (clade 5), and no binding at all to Fam1 strains St245/00 (clade 1) and D39 (clade 2) was observed (Fig. 3B). Both anti-PspA4 and anti-PspA5 (Fam2) showed efficient binding to all of the strains tested (Fig. 3B). It is important to stress that the degree of binding varies with each individual pneumococcal isolate, so that we have only compared the median fluorescence values of the same strain with different sera.

As for complement deposition, anti-PspA1 and anti-PspA2 (Fam1) sera were able to efficiently mediate the deposition of C3 only on Fam1-bearing strains (St245/00 and D39) (Fig. 3C). Anti-PspA3 (Fam2) showed efficient deposition only on M10 (clade 3, Fam2) (Fig. 3D), while anti-PspA4 and anti-PspA5 were capable of efficient mediation of C3 deposition on all Fam2 strains (Fig. 3D). As for Fam1 strains, anti-PspA4 was able to mediate C3 deposition on St245/00 (clade 1) and anti-PspA5 on D39 (clade 2) at levels similar to those observed for the homologous Fam1 antibodies (Fig. 3D). Again, only median fluorescence values of the same strain with the different sera were compared.

**PspA4 and PspA5 induce protection against challenges with Fam1- and Fam2-bearing strains.** Immunized mice were then submitted to a lethal intranasal challenge with pneumococci expressing PspA from Fam1 or Fam2. As shown in Table 2, animals immunized with PspA2 (Fam1) showed significant protection against a challenge with A66.1 (clade 2, Fam1) (P = 0.008). Moreover, PspA4 and PspA5 (Fam2) were also able to induce protection against A66.1, even though the strain expresses a PspA protein from a different family (P = 0.001 for PspA4 and P = 0.03 for PspA5). A66.1 was first reported to express only PspA2 (1), but subsequent work has shown it to express both PspA1 and PspA2 (3). We have amplified a single psaA band from this strain using primers LSM12 and SKH2 (17), and sequencing of cloned fragments confirmed only pspA2 clones. The expression of only PspA2 by A66.1 would be in accordance with the data showing protection after immunization with PspA2, but not with PspA1. Challenge with ATCC 6303 (clade 5, Fam2) was also performed, and as shown in Table 3, only protection within the same family was seen for PspA4 (P = 0.005) and PspA5 (P = 0.0007).

**Contribution of the NonPro region to the reactivity to PspA.** Of the five PspA fragments analyzed, PspA4 was the only one containing a NonPro region within the C-terminal proline-rich region. We have analyzed the contribution of both proline and NonPro regions to the cross-reactivity of PspA4. While the original PspA4 fragment contained the complete proline-rich region, PspA4AB contains only the α-helical region without the entire proline-rich region, and PspA4Pro contains the N-terminal α-helical region plus the first block of prolines only, lacking both the NonPro region and the second block of prolines (Fig. 1). We have analyzed the binding of anti-PspA4AB, anti-PspA4Pro, and anti-PspA4 antibodies to intact M10 (clade 3, lacks NonPro) and ATCC 6303 (clade 5, has Non-Pro). The capacity of antibodies to PspA4AB and PspA4Pro to bind to M10 was enhanced compared to that of antibodies to PspA4. Since M10 does not contain the NonPro region, reduced binding could be explained by a considerable amount of the antibodies induced after immunization with PspA4 being directed toward the NonPro region. Binding of antibodies to PspA4Pro was also slightly better than that of antibodies to PspA4AB, showing that the proline block is probably also immunogenic. Binding to ATCC 6303 followed a similar pattern, with higher binding for antibodies to PspA4Pro, followed by PspA4AB, but differences were not so evident (Fig. 4A). We next tested whether the addition of NonPro to PspA3 (which induced antibodies with very restricted cross-reactivity) would increase the recognition of different PspA proteins. Anti-PspA3 and anti-PspA3-NonPro showed similar binding to M10 (lacks NonPro), and the inclusion of the NonPro region clearly enhanced the cross-reactivity to ATCC 6303 (has NonPro) (Fig. 4B).

**FIG. 3.** Binding of antibodies and complement deposition in the presence of the different anti-PspA sera. Sera from mice immunized with PspA from Fam1 (PspA1 or PspA2) (A and C) or from Fam2 (PspA3, PspA4, or PspA5) (B and D) were tested for the ability to bind (1% serum, A and B) and to mediate deposition of C3 (10% serum, C and D) on *S. pneumoniae* strains bearing PspA proteins from clades 1 to 5. Serum from mice immunized with alum was used as a control for each strain and is represented by the gray area in each graph. Results are shown as fluorescence intensity histograms, and the median fluorescence intensity is indicated for each sample. Results are representative of two experiments using sera from independent immunizations.

TABLE 3. Survival after intranasal challenge with ATCC 6303 (clade 5, Fam2)

| Immunizing antigen | No. alive/total | % Survival | P value |
|--------------------|----------------|------------|---------|
| Alum               | 0/12           | 0          |         |
| Fam1               |                |            |         |
| PspA1              | 3/12           | 25         | 0.11    |
| PspA2              | 2/12           | 17         | 0.24    |
| Fam2               |                |            |         |
| PspA3              | 3/11           | 27         | 0.09    |
| PspA4              | 6/11           | 55*        | 0.005   |
| PspA5              | 8/12           | 67*        | 0.0007  |

* Statistically significant difference from animals inoculated with alum (Fisher exact test).
DISCUSSION

Since PspA is a highly polymorphic antigen, the correct choice of fragments to be included in a vaccine formulation is crucial. We have tested the ability of sera raised against PspA fragments from clades 1 to 5 to bind to the surface of and to mediate complement deposition on pneumococci bearing different PspA proteins. Complement deposition assays have been proposed as a surrogate for prediction of protection (15). Previously, our group has also analyzed antibodies against PspA1, PspA3, and hybrid molecules, showing that binding and complement deposition mediated by anti-PspA1 and anti-PspA3 antibodies were restricted to the same family, whereas the hybrids were able to broaden this recognition to some extent (6). Subsequent work has shown that PspA4 and PspA5 were able to elicit antibodies with broad cross-reactivity, being able to recognize extracts from several strains expressing PspA proteins from clades 1 to 5 by Western blot analysis (7). We now show that anti-PspA4 and anti-PspA5 are also able to efficiently bind to the surface of pneumococci expressing PspA proteins from clades 1 to 5. As for C3 deposition, both sera were able to efficiently mediate complement deposition on pneumococci from the same family (clades 3, 4, and 5), whereas for Fam1 strains, anti-PspA4 was able to mediate C3 deposition on a clade 1-bearing strain and anti-PspA5 on a clade 2-bearing strain at levels similar to those observed for the homologous Fam1 antibodies. PspA3 elicited antibodies with the narrowest cross-reactivity, which is in accordance with our previous Western blot analysis results. These results show that the previously proposed clade-dependent immunity in Fam2 (6) may not be characteristic of all of the representatives of this family and is probably restricted to clade 3.

We have performed an intranasal lethal challenge of immunized mice, and the groups that received PspA4 or PspA5 showed statistically significantly higher survival after a challenge with A66.1, which expresses PspA2 (Fam1), and ATCC 6303, which expresses PspA5 (Fam2). These results thus show that both fragments would be able to confer broad protection against pneumococci expressing PspA proteins from the two major families. Moreover, these results are in accordance with the broad reactivity of antibodies to PspA4 and PspA5 with pneumococcal strains bearing different PspA proteins detected both in Western blot experiments and in functional assays of
binding and complement deposition on intact bacteria. Fusion proteins composed of PspA fragments from Fam1 and Fam2 have been proposed as an alternative for the induction of broad protection using recombinant protein (6) or recombinant attenuated Salmonella (26). In the latter work, oral immunization of mice with Salmonella strains expressing PspA fusion proteins was shown to induce serum antibodies that were able to bind and to mediate complement deposition on strains expressing PspA proteins from clades 1 to 5 and also to provide protection against challenges with multiple pneumococcal strains. Our results show the potential of immunization with a single PspA fragment to induce cross-protection.

We have also analyzed the contribution of the NonPro region to the cross-reactivity of antibodies to PspA. The exclusion of the NonPro region of PspA4 led to the induction of antibodies with a higher capacity to bind to a strain that lacks this region, which indicates that a considerable amount of the antibodies induced by immunization with PspA4 might be directed to the NonPro region. The block of proline repeats also seemed to increase binding to the strains tested. Moreover, fusion of the entire proline-rich region containing NonPro to PspA3 (which was previously shown to induce antibodies with reduced cross-reactivity) was able to enhance the binding of the antibodies to a strain that expresses PspA containing the NonPro region. Though it was not possible to define precisely the exact contribution of each region to the cross-reactivity of the antibodies, these results indicate that the N-terminal α-helical region, the blocks of proline repeats, and also the NonPro region can influence the degree of cross-reactivity, depending on whether the strain tested expresses a PspA protein containing the NonPro region or not. It has been previously suggested that the proline-rich region may protect mice against a pneumococcal challenge (5).

In conclusion, our results show that immunization with both PspA4 and PspA5 elicits antibodies with a functional capacity to recognize and to mediate complement deposition on a broad range of pneumococcal isolates. More importantly, PspA4 and PspA5 were able to induce protection against a strain with one expressing PspA from Fam1 and one strain from Fam2 (the two major families), indicating that these antigens have potential to be used as vaccine antigens to induce broad protection against different pneumococcal strains.

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