Recognition of pneumococcal isolates by antisera raised against PspA fragments from different clades

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Pneumococcal surface protein A (PspA) is an important vaccine candidate against pneumococcal infections, capable of inducing protection in different animal models. Based on its structural diversity, it has been suggested that a PspA-based vaccine should contain at least one fragment from each of the two major families (family 1, comprising clades 1 and 2, and family 2, comprising clades 3, 4 and 5) in order to elicit broad protection. This study analysed the recognition of a panel of 35 pneumococcal isolates bearing different PspAs by antisera raised against the N-terminal regions of PspA clades 1 to 5. The antiserum to PspA clade 4 was found to show the broadest cross-reactivity, being able to recognize pneumococcal strains containing PspAs of all clades in both families. The cross-reactivity of antibodies elicited against a PspA hybrid including the N-terminal region of clade 1 fused to a shorter and more divergent fragment (clade-defining region, or CDR) of clade 4 (PspA1–4) was also tested, and revealed a strong recognition of isolates containing clades 1, 4 and 5, and weaker reactions with clades 2 and 3. The analysis of serum reactivity against different PspA regions further revealed that the complete N-terminal region rather than just the CDR should be included in an anti-pneumococcal vaccine. A PspA-based vaccine is thus proposed to be composed of the whole N-terminal region of clades 1 and 4, which could also be expressed as a hybrid protein.

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INTRODUCTION

Streptococcus pneumoniae is responsible for millions of deaths worldwide every year (Cherian, 2007). The increase in antibiotic resistance, together with the high cost and limited coverage of the current conjugate vaccine, reinforce the need for cost-effective strategies. Recent reports on strain replacement in pneumococcal disease further support the necessity of developing alternative vaccines (Hicks *et al.*, 2007; Singleton *et al.*, 2007). Several proteins have been investigated as vaccine candidates against *S. pneumoniae* infection, including pneumococcal surface protein A (PspA), an immunogenic virulence factor (Tai, 2006) that is able to elicit high antibody levels in humans (Nabors *et al.*, 2000) and to protect mice passively against challenge with virulent pneumococcal strains (Briles *et al.*, 2000).

PspA has five domains: (i) a signal peptide, (ii) an α -helical and charged N-terminal domain, (iii) a proline-rich region, (iv) a choline-binding domain and (v) a short hydrophobic tail (Yother & Briles, 1992; Yother & White, 1994) (Fig. 1).

The N-terminal moiety is a well-exposed part of the molecule (Daniels *et al.*, 2006; McDaniel *et al.*, 1994), and also its functional fragment, interacting with the immune system (Shaper *et al.*, 2004).

The N-terminus of PspA exhibits serological variability and can be divided into three regions, A, B and C. Region A is followed by an even more divergent region of about 100 aa, region B (McDaniel *et al.*, 1994). This N-terminal region is followed by a proline-rich region, known as C. The B region formed the basis for the classification of PspA into six clades and three families, and is thus also referred to as the clade-defining region (CDR) (Hollingshead *et al.*, 2000). Surveillance studies have shown that PspA families 1 and 2 are present in at least 90 % of isolates, with prevalence rates of about 50 % each (Beall *et al.*, 2000; Brandileone *et al.*, 2004; Heeg *et al.*, 2007; Hollingshead *et al.*, 2006; Ito *et al.*, 2007; Mollerach *et al.*, 2004; Payne *et al.*, 2005; Pimenta *et al.*, 2006; Sadowy *et al.*, 2006; Vela Coral *et al.*, 2001). Previous studies have shown that the level of cross-reactivity among different PspAs roughly follows the degree of similarity among the amino acid sequences, with a tendency for a higher cross-reactivity within the same family (Nabors *et al.*, 2000). This suggests that a

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Abbreviation: CDR, clade-defining region.

Fig. 1. Schematic diagram of PspA fragments obtained in this study. At the top is the whole PspA molecule, containing the N-terminal α -helical domain (including regions A and B), the proline-rich region (C) and the choline-binding domain. The recombinant fragments are represented showing the distinct domains. The hybrid PspA1ABC-4B contained the N-terminal domain plus the proline-rich region of a PspA clade 1 fused to the CDR (B region) of a clade 4 PspA. The initial and final amino acids are shown for each fragment.

PspA-based vaccine should contain at least one fragment from each major family (family 1, comprising clades 1 and 2, and family 2, comprising clades 3, 4 and 5) (Hollingshead *et al.*, 2000). To investigate the level of cross-reactivity within PspA families and clades, we analysed the recognition of a panel of 35 pneumococcal isolates collected during a surveillance study in Brazil (Pimenta *et al.*, 2006) by antisera raised against PspA fragments from clades 1 to 5. We also investigated the contribution of different regions of the N-terminus of PspA to its immunogenicity in order to elucidate the best composition for a PspA-based anti-pneumococcal vaccine.

METHODS

Construction of PspA fragments and hybrids. All cloning procedures were performed in *Escherichia coli* DH5 α grown in Luria–Bertani medium supplemented with ampicillin (100 μ g ml⁻¹). DNA fragments encoding the N-terminal region of PspA clades 1 to 5 (A, B and C regions) were amplified by PCR from the genomic DNA of pneumococcal strains 435/96, 371/00, 259/98, 255/00 and 122/02, respectively (GenBank accession nos AY082387, EF649968, AY082389, EF649969 and EF649970). The forward (F) and reverse (R) primers used for amplification of the PspA fragments were: 5'-CTCGAGGAAGAAGCGCCCGTAGC-3' (PspA1F and PspA3F); 5'-TAGTTATCTAGATGGTTGTGGTGCTGAAG-3' (PspA1R, PspA3R and PspA4R); 5'-TAGCTCGAGACCATGGTAAGAGCAGAAGAAGCC-3' (PspA2F, PspA4F and PspA5F); 5'-AGTTATCTAG-ATTTTGGTGCAGGAGCTGG-3' (PspA2R and PspA5R). The gene products were cloned into a pGEMT-Easy vector (Promega) and the correct sequences were confirmed by DNA sequencing. The pGEMT-Easy/PspA constructs were digested with the appropriate restriction endonucleases and the resulting fragments were subcloned into linearized pAE-6 $\times$ His vector (Ramos *et al.*, 2004). The hybrid PspA1ABC-4B (Miyaji *et al.*, 2002) and its expression vector (Darrieux *et al.*, 2007) have been described previously. Fig. 1 shows a scheme of the resulting PspA fragments.

PspA expression and purification. The pAE-6 $\times$ His vector containing the *pspA* constructs was used to transform BL21(DE3)SI *E. coli* competent cells (Invitrogen). Protein expression was induced in mid-exponential-phase cultures by the addition of 300 mM NaCl. The recombinant proteins, bearing an N-terminal histidine tag, were purified from the soluble fraction by affinity chromatography using Ni²⁺-charged resin (HisTrap HP; GE Healthcare) in an AKTApurifier apparatus (GE Healthcare). Elution was carried out with 250 mM imidazole. The purified fractions were analysed by SDS-PAGE, dialysed against 10 mM Tris/HCl (pH 8)/20 mM NaCl/0.1% glycine and stored at -20 °C.

Pneumococcal strains. All strains used in this study were maintained as frozen stocks (-80 °C) in Todd–Hewitt broth supplemented with 0.5% yeast extract (THY) with 20% glycerol, and are listed in Table 1. These strains have been classified previously by sequencing of the CDR (Pimenta *et al.*, 2006).

Animals and immunization. Female BALB/c mice from Instituto Butantan (São Paulo, Brazil) were immunized subcutaneously with 5 μ g recombinant PspA1ABC, PspA2ABC, PspA3ABC, PspA4ABC, PspA5ABC or the hybrid PspA1ABC-4B in Ringer's lactate solution using 50 μ g Al(OH)₃ as an adjuvant (final volume of 100 μ l per mouse). Adjuvant alone was used as a control. The animals were given three doses of protein at 7-day intervals. Sera were collected from mice by retro-orbital bleeding 1 week after the first immunization (for ELISA) or 2 weeks after the third immunization (for Western blotting).

Western blot analysis of anti-PspA sera. Pneumococcal isolates were plated on blood agar, transferred to THY and grown to an OD₆₀₀ of 0.6. The cell pellets were lysed by sonication and the soluble protein extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes. Pooled anti-PspA sera (six mice per group) made against the recombinant PspA fragments of clades 1 to 5 or the clade 1–4 hybrid were added at a dilution of 1:1000 (sera collected after the third immunization), followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (diluted 1:1000; Sigma). Detection was performed with an ECL kit (GE Healthcare).

Table 1. PspA clades and serotypes of the pneumococcal strains used in this study

Family 1				Family 2					
Clade 1		Clade 2		Clade 3		Clade 4		Clade 5	
Strain	Serotype	Strain	Serotype	Strain	Serotype	Strain	Serotype	Strain	Serotype
435/96*	1	371/00*	6B	259/98*	14	255/00*	14	122/02*	23F
M12	6B	M8	6B	M10	3	M2	6B	P255	6A
P13	9V	P83	19A	P5	11A	M15	23A	P865	23F
P105	6A	P174	23A	P24	6A	P46	6B		
P195	18A	P176	9N	P148	23F	P71	23F		
P266	23F	P247	6B	P212	23F	P101	19F		
P468	6A	P278	12	P539	19F	P198	18A		
P1151	11A	P339	6B	P762	14	P414	10A		
P1161	14	P755	14	P783	19F	P1149	19F		

*Strains from which the PspA fragments were cloned.

Analysis of serum reactivity with different PspA regions.

Recognition of the different PspA regions by antibodies to the recombinant proteins was analysed by ELISA. PolySorp 96-well plates (Nunc) were coated with fragments containing PspA regions A, AB or ABC from clade 1 ($1 \mu\text{g ml}^{-1}$), washed three times with PBS containing 0.1 % Tween 20 and blocked with 10 % non-fat dried milk in PBS. The plates were then incubated with serial dilutions of individual sera from mice immunized with PspA1ABC or PspA1ABC-4B (sera collected after the first immunization) in 1 % BSA in PBS at 37 °C for 1 h. The plates were washed again and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (diluted 1 : 15 000; Sigma) in 1 % BSA in PBS at 37 °C for 1 h. Following three more washes, antibodies were detected by adding OPD substrate [0.04 % *o*-phenylenediamine in citrate/phosphate buffer (pH 5) containing 0.01 % H_2O_2]. After colour development (10 min), the reaction was stopped with 1.25 M H_2SO_4 and the A_{492} was determined. The reciprocal titre was considered to be the inverse of the last dilution of serum that registered an A_{492} of 0.10 (sera from mice immunized with adjuvant only yielded negligible absorbance). Differences between groups were analysed by Student's *t*-test.

RESULTS AND DISCUSSION

This work investigated the level of cross-reactivity within PspA clades and families using a panel of 35 pneumococcal isolates (eight PspA1 strains, eight PspA2, eight PspA3, eight PspA4 and three PspA5 strains) and polyclonal sera made against PspA fragments of the five prevalent clades (clades 1 to 5). Western blot analysis revealed considerable variation in the recognition of isolates by the antibodies to the different clades. In general, there was strong recognition of isolates containing PspAs of the same clade as that used for production of the sera, and recognition decreased at the same rate as the level of similarity between the clades (Fig. 2).

Anti-PspA1ABC antibodies were able to recognize PspA in all clade 1-containing strains and half of the clade 2-containing strains tested, whilst no reactivity with family 2 (clades 3–5) was observed (Fig. 2a). Anti-PspA2ABC antibodies revealed the narrowest recognition capacity,

with positive reactions seen with only half of the PspA clade 2-containing strains, two clade 1 isolates and none of the other PspA clades (Fig. 2b). Anti-PspA3ABC antibodies were strongly reactive with all clade 3- and two clade 2-containing isolates; interestingly, there was no reactivity with the other PspA clades of family 2 (clades 4 and 5) (Fig. 2c). Anti-PspA4ABC and anti-PspA5ABC antibodies showed the broadest recognition capacity; anti-PspA4ABC reacted with all but four pneumococcal extracts (P174 and P278 from clade 2 and M10 and P5 from clade 3; Fig. 2d), whilst anti-PspA5ABC antibodies recognized six clade 1, four clade 2, all clade 3 and 5, and six clade 4 PspA-containing strains (Fig. 2e). These results showed that, among the PspA fragments analysed, PspA4ABC would be the most suitable for the induction of broad reactivity and that the inclusion of one fragment from family 1 (PspA1ABC) and another from family 2 (PspA4ABC) would be an even more effective strategy. Furthermore, the low reactivity shown by the anti-PspA2ABC antiserum indicated that the inclusion of more representative sequences is also an important issue, as sequence analysis has shown that the PspA clade 2 fragment used in this work is more divergent from other clade 2 PspAs sequenced by Hollingshead *et al.* (2000) compared with the clade 1, 3, 4 and 5 fragments. Another relevant aspect could be the fragment size: whilst the smaller fragments (from PspA clades 1, 2 and 3) induced antibodies with reduced cross-reactivity, the longer fragments (from PspA clades 4 and 5) had a much broader recognition.

Previous studies using monoclonal antibodies (mAbs) generated by immunization with a pool of heat-killed pneumococci have revealed that a combination of only two Abs was able to detect virtually all clinical isolates tested in the study, one of them being reactive with PspA family 1 strains and the other with family 2 strains (Kolberg *et al.*, 2001). This reinforces the hypothesis that a PspA-based vaccine containing a single fragment from each major family should be effective against virtually all pneumococci.

Fig. 2. Western blot analysis of the reactivity of anti-PspA antibodies within clades and families. Polyclonal sera from mice immunized with the N-terminal regions of PspA clades 1 to 5 or the hybrid PspA1ABC-4B were tested for their reactivity with 35 pneumococcal extracts containing PspA from clades 1 to 5. Identification of the isolates is indicated on top of each blot; protein extracts of the strains from which the *pspA* fragments were cloned are indicated in bold and were used as positive controls; PspA clade 5-containing strains are underlined. Molecular mass markers (kDa) are indicated on the right.

To test this hypothesis, we examined the reactivity pattern of sera made against the hybrid PspA1ABC-4B. The results indicated that antisera to this hybrid extended to a certain degree the recognition capacity of antibodies raised only against PspA1ABC, showing a strong reaction with PspA clade 1-, 4- and 5-bearing pneumococcal isolates, whilst cross-reactivity with clade 2- and 3-containing strains was much lower (only four out of 16 strains of these two clades were reactive) (Fig. 2f). In general, these results showed that the fusion is indeed able to enhance the level of cross-reactivity, but that in order to achieve a broader effect, a longer fragment from family 2 should be included. In accordance with these results, our group has demonstrated that immunization with PspA hybrids induced antibodies with increased cross-reactivity, with the strongest effect obtained with the hybrid containing the longest PspA fragments (Darrieux *et al.*, 2007).

Nabors *et al.* (2000) previously assessed the degree of cross-reactivity of rabbit anti-PspA antibodies raised against PspAs from clades 1 to 5 against the same recombinant fragments. Analysis of PspA from family 1 showed that clades 1 and 2 had considerable cross-reactivity. However, in family 2, clade 3 PspA elicited antibodies with poor reactivity with clades 4 and 5. Furthermore, anti-PspA2 human antibodies were also analysed for reactivity against pneumococcal strains bearing different PspAs by ELISA. There was an increase in titres against all strains tested, but these results were influenced by prior natural exposure of the immunized subjects to pneumococci. However, analysis of sera raised by PspAs from clades 1 to 5 against several strains bearing PspAs from the same clade was not performed.

As important as defining which PspA clade(s) induces the most cross-reactive antibodies is determination of the

contribution of each protein domain to its immunogenicity; the two results combined could provide valuable information on the best composition for a PspA-based anti-pneumococcal vaccine.

It has been shown that immunization of mice with the A or B region induced high antibody levels against the whole N-terminal fragment of a homologous fragment (Roche *et al.*, 2003). In the present study, we proposed the opposite approach: identifying the level of recognition of each major domain by antibodies to the whole N-terminus.

Sera from mice immunized with PspA1ABC or the hybrid PspA1ABC-4B were analysed by ELISA against recombinant PspA clade 1 fragments containing regions A, AB or ABC. Anti-PspA1ABC had a significantly higher reactivity with the longer fragments (AB and ABC) compared with the smaller fragment (A) ($P=0.026$). No significant differences were observed between AB and ABC, indicating that the proline-rich region may have little contribution towards PspA1ABC immunogenicity in this model (Fig. 3a). The results also suggested that regions A and B were equally immunogenic. This result corroborates mapping studies using mAbs, which have shown that, from a pool of nine mAbs, five were reactive against the A region and four against the B region, indicating that these

two domains may be important for the immunogenicity of PspA (McDaniel *et al.*, 1994). This recognition pattern is probably due to PspA folding back on itself to generate a coiled-coil structure, which allows these different domains to be exposed on opposite sides of the same coiled-coil unit (Jedrzejewski *et al.*, 2000; Senkovich *et al.*, 2007). Moreover, a study with PspA fragments including different portions of the α -helical domain has demonstrated that, for family 2 strains, fragments including both ends of the α -helical region elicited the best protection (Roche *et al.*, 2003). This result suggests that the A and B regions are important not only for the immunogenicity of PspA, but also for protection against pneumococcal challenge.

In contrast to what was observed with sera from the PspA fragment, antibodies made against the hybrid PspA1ABC-4B did not show a significant difference in reaction with the A region compared with AB (Fig. 3b). This result may be due to differences in the hybrid structure, altering the accessibility of the regions to the immune system.

In conclusion, whilst it is important to determine which PspA fragments show the broadest cross-reactivity within the same family (or even the same clade, as is the case for the PspA clade 2 fragment), in order to extend reactivity to heterologous PspA families, it is important to have longer fragments for immunization, including the more conserved proline-rich region. This domain may also play a significant role in maintaining certain structural features necessary for the induction of cross-reactive antibodies. Furthermore, the use of a fusion protein bearing fragments of both families was able to increase the cross-reactivity of antisera, indicating that this could be a promising immunization strategy. Taken together, our results suggest that the best composition for a PspA-based anti-pneumococcal vaccine should include the whole N-terminal region of PspA clades 1 and 4, which could be expressed as a hybrid protein, as these are able to induce antibodies with a broad cross-reactivity. Moreover, future studies should also evaluate whether the protective ability of the antibodies correlates with the cross-reactivity data presented in this work.

Fig. 3. Serum reactivity with different PspA regions. Polyclonal sera from mice immunized with recombinant PspA1ABC (a) or PspA1ABC-4B (b) were analysed by ELISA against recombinant PspA fragments containing region A, AB or ABC of clade 1. The results are shown as log₂ of the titre. *Statistically significant difference ($P=0.026$).

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