

DNA vaccines expressing pneumococcal surface protein A (PspA) elicit protection levels comparable to recombinant protein

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Pneumococcal surface protein A (PspA) is a promising candidate for the development of cost-effective vaccines against *Streptococcus pneumoniae*. In the present study, BALB/c mice were immunized with DNA vaccine vectors expressing the N-terminal region of PspA. Animals immunized with a vector expressing secreted PspA developed higher levels of antibody than mice immunized with the vector expressing the antigen in the cytosol. However, both immunogens elicited similar levels of protection against intraperitoneal challenge. Furthermore, immunization with exactly the same fragment in the form of a recombinant protein, with aluminium hydroxide as an adjuvant, elicited even higher antibody levels, but this increased humoral response did not correlate with enhanced protection. These results show that DNA vaccines expressing PspA are able to elicit protection levels comparable to recombinant protein, even though total anti-PspA IgG response is considerably lower.

INTRODUCTION

Streptococcus pneumoniae is one of the most common aetiological agents of invasive diseases such as meningitis, septicaemia and pneumonia, especially in children under 2 years of age and in the elderly. Currently, two pneumococcal vaccines are available, a 23-valent polysaccharide and a 7-valent conjugate vaccine (Bogaert *et al.*, 2004). Although the 23-valent is poorly immunogenic and fails to induce protection in young children, the 7-valent has shown high efficacy against invasive infections in infants and the coverage is estimated as over 85% in the USA, 60–70% in Europe (Pelton *et al.*, 2003) and 58% in Brazil (Brandileone *et al.*, 2003). However, this latter vaccine elicits protection only against the seven included serotypes. To overcome these limitations, investigators have proposed the use of protective protein antigens conserved in all pneumococcal serotypes, such as pneumococcal surface protein A (PspA), pneumococcal surface antigen A and pneumolysin, as promising candidates for the development of cost-effective vaccines.

PspA acts in host–pathogen interactions, interfering with the activation and deposition of complement (Tu *et al.*, 1999; Ren *et al.*, 2004). Furthermore, it has been reported recently that PspA protects pneumococci from killing by apolactoferrin at mucosal sites (Shaper *et al.*, 2004). Many groups have shown that recombinant PspA (rPspA) can

elicit an antibody response that protects against lethal challenge in animal models, including passive protection (Briles *et al.*, 2000a, b). Induction of protective immunity using PspA through genetic immunization has also been described (McDaniel *et al.*, 1997; Borsage *et al.*, 2001; Miyaji *et al.*, 2002).

In the present study, DNA vaccine vectors expressing an N-terminal fragment of PspA were constructed with or without fusion to a secretory sequence. The immune response elicited by these vaccines was evaluated in terms of their protective efficacy compared with a recombinant protein administered with aluminium hydroxide as an adjuvant.

METHODS

Construction of DNA vaccine vectors. The non-secretion (pTG-*pspA3NS*) and the secretion (pSec-*pspA3NS*) vectors used for DNA immunization were based on pTARGET (Promega) and pSecTag2A (Invitrogen), respectively. In both vectors, expression of the gene is controlled by the human cytomegalovirus immediate-early promoter/enhancer. The pSecTag vector carries the secretion signal from the V-J2-C region of the mouse Ig kappa-chain, promoting efficient secretion of the recombinant protein. The DNA fragments encoding the N-terminal region of PspA3, without a signal sequence, as far as the proline-rich region, were amplified by PCR from pTG-*pspA3* (Miyaji *et al.*, 2002), using the primers 5'-TAGCTCGAGACCATGATCTTAGGGGCTGGTTT-3' and 5'-TAGTTATCTAGATTTTGGTGCAGGAGCTGG-3' for pTG-*pspA3NS* and 5'-GGTACCGGTAAGAGCAGAAGAAGAAGCCC-3' and 5'-CTCGAGTTATTTGGTGCAGGAGCTGG-3' for pSec-*pspA3NS*. The original sequence was amplified

Abbreviations: BHK, baby hamster kidney; rPspA, recombinant PspA.

from strain St 259/98 (*PspA* clade 3, serotype 14). Plasmid DNA was purified from *Escherichia coli* DH5 α by anion-exchange chromatography with QIAfilter Maxi kit from Qiagen.

Expression and purification of recombinant protein in *E. coli*.

Expression of rPspA3NS in *E. coli* was performed using the pAE vector (Ramos *et al.*, 2004), containing exactly the same insert as found in pTG-*pspA3NS*. The recombinant protein contains a 6 \times His tag at the N terminus to facilitate purification through Ni²⁺-charged column chromatography. Recombinant protein was purified from the soluble fraction of *E. coli* BL21 (SI) transformed with the vector, as described previously (Arêas *et al.*, 2004).

Antigen expression in transfected mammalian cells. To evaluate *in vitro* expression, baby hamster kidney (BHK) cells were transfected with the constructed vaccine vectors using Lipofectamine reagent (Invitrogen). Cells were harvested 24 h after transfection and transient expression was analysed by Western blotting, as previously described (Miyaji *et al.*, 2002), using anti-rPspA3NS antiserum developed in mice. Specificity of the antiserum had been tested previously using whole-cell extracts from several *S. pneumoniae* strains.

Immunization of mice and detection of anti-PspA antibodies.

The DNA vaccine vectors constructed were used to immunize female BALB/c mice (5–7 weeks old, from Instituto Butantan, São Paulo, Brazil). Groups of at least six mice were inoculated intramuscularly with 50 μ l of 10 μ M cardiotoxin (Laxotan, Valence, France) into each tibialis anterior muscle, 5 days before immunization with 50 μ g vaccine vector in PBS (100 μ l). Mice received a booster after 3 weeks with the same dose of plasmid DNA. For immunization with recombinant protein, mice were injected subcutaneously with 5 μ g rPspA3NS, using aluminium hydroxide (Production Division of Instituto Butantan) as the adjuvant, and also boosted 3 weeks after priming. Mice were bled from the retro-orbital plexus, 6 weeks after priming, for detection of serum anti-PspA antibodies by ELISA as described previously (Miyaji *et al.*, 2002), using rPspA3NS as the coating antigen. Differences between groups were analysed by Student's *t*-test. Isotyping of pooled sera was also performed by

ELISA using mouse monoclonal antibody isotyping reagents (Sigma). Reciprocal titres were considered as the last dilution of sera that registered an A_{492} of 0.1.

Intraperitoneal challenge. Immunized mice were challenged by intraperitoneal injection of 200 c.f.u. *S. pneumoniae* strain St 679/99 (*PspA* clade 3, serotype 6B) in 0.5 ml saline 6 weeks after priming. Animals were then observed for 2 weeks and inactive sick animals were euthanized. Differences in the overall survival rate between groups were analysed by Fisher's exact test.

RESULTS AND DISCUSSION

In order to evaluate expression of PspA by the constructed DNA vaccine vectors, mammalian cells were transfected and antigen expression was analysed by Western blotting. As expected, PspA3 was detected in cell lysates of both non-secretion (pTG-*pspA3NS*; Fig. 1, lane 2) and secretion (pSec-*pspA3NS*, lane 6) vectors, whereas secreted PspA3 was observed only in the supernatant of the cells transfected with pSec-*pspA3NS* (lane 6). A protein with a slightly higher molecular mass was detected in the cytosol of cells transfected with the secretion vector when compared with the

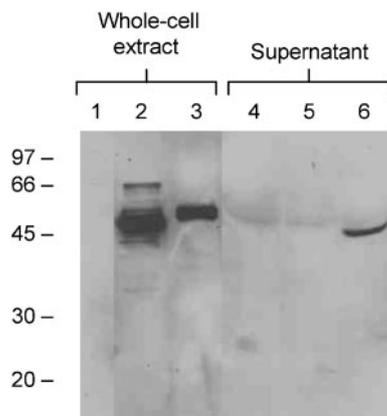


Fig. 1. Transient expression of PspA3 by DNA vaccine vectors in BHK cells. Total extracts (lanes 1–3) and supernatants (lanes 4–6) of BHK cells transfected or not with the vectors were analysed by Western blotting using anti-rPspA3NS antiserum. Lanes 1 and 4 indicate non-transformed cells; 2 and 5, cells transfected with pTG-*pspA3NS*; 3 and 6, cells transfected with pSec-*pspA3NS*. Migration of standard molecular mass markers is indicated.

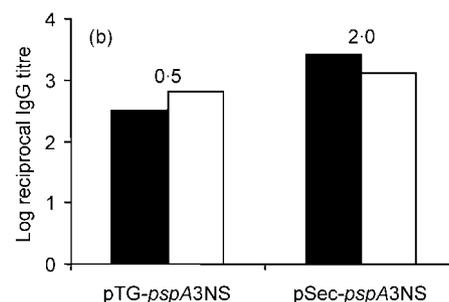
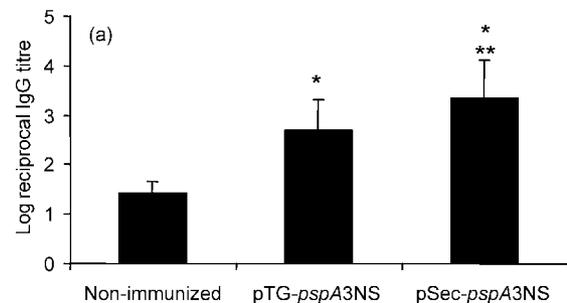


Fig. 2. Evaluation of the antibody response elicited by immunization with DNA vaccine vectors. Groups of at least 12 BALB/c mice were immunized intramuscularly with two doses of 50 μ g of the indicated vectors, sera were collected and total anti-PspA IgG of individual sera (a) or anti-PspA IgG1 (filled bars) and IgG2a (open bars) of pooled sera (b) was measured by ELISA in plates coated with rPspA3NS. Log of the reciprocal titre of anti-PspA IgG is shown. Numbers above columns are IgG1:IgG2a titre ratios. *, Statistically significantly different from non-immunized controls; **, statistically significantly different from pTG-*pspA3NS* ($P \leq 0.05$).

non-secretion vector. This difference is due to the presence of the V-J2-C secretion signal in the antigen expressed by pSec-*pspA3NS*, which is cleaved when the protein is exported to the culture supernatant.

BALB/c mice were then immunized with the constructed DNA vaccine vectors and the anti-PspA antibody response was analysed. As shown in Fig. 2(a), both vectors elicited a significant anti-PspA IgG response ($P \leq 0.0001$ for both non-immunized compared with pTG-*pspA3NS* and non-immunized compared with pSec-*pspA3NS*). Furthermore, mice immunized with pSec-*pspA3NS* elicited significantly higher levels of anti-PspA IgG than those observed for mice immunized with pTG-*pspA3NS* ($P = 0.03$), which indicates that expression of the antigen as a secreted protein is able to enhance the induced humoral response. Isotyping of antibodies demonstrated that mice immunized with both DNA vaccines showed a balanced IgG1 : IgG2a ratio (0.5 to 2.0), showing only a small difference due to the secretion of the antigen (Fig. 2b). Animals were then challenged with St 679/99 and survival was analysed. Both vectors elicited significant protection, at similar levels (Table 1), indicating that the lower IgG response elicited by the non-secretion vector is sufficient for the observed protection.

In order to compare genetic immunization and recombinant protein strategies, we next immunized mice twice with either pSec-*pspA3NS* intramuscularly or rPspA3NS subcutaneously (Fig. 3a). As expected, mice immunized with two doses of the recombinant protein showed significantly higher levels of anti-PspA IgG antibodies than mice immunized with pSec-*pspA3NS* ($P = 0.02$). Several studies have demonstrated that combining two or more vaccine modalities can enhance the immune response both qualitatively and quantitatively (reviewed by Doria-Rose & Haigwood, 2003). We have thus tested a prime–boost strategy, using DNA vaccine as priming and recombinant protein as booster, or vice versa. The groups that were immunized with the prime–boost strategies showed an enhancement of the antibody response, when compared with the mice that were immunized with two doses of pSec-*pspA3NS* vaccine vector, but the difference was not statistically significant. Isotyping

of antibodies demonstrated that mice immunized with DNA vaccines showed a more balanced IgG1 : IgG2a ratio (1.0), whereas animals immunized with recombinant protein induced a very high ratio (64) (Fig. 3b). Booster immunizations increased the anti-PspA levels but did not alter the isotypic nature of the response: DNA-vaccine-primed mice continued to show an antibody response with a more balanced ratio, whereas recombinant protein priming maintained a more Th2-biased humoral immune response, with higher IgG1 antibody production.

Mice were then challenged with St 679/99 and survival was analysed. All the immunized groups showed significant protection when compared with control groups (Table 1). Animals immunized with pSec-*pspA3NS* and those primed

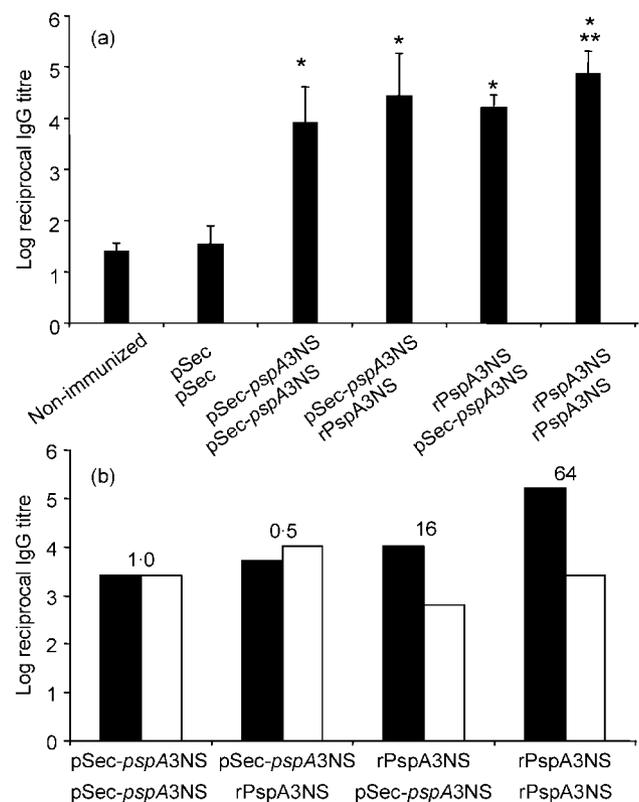


Fig. 3. Evaluation of the antibody response elicited by DNA vaccine and recombinant protein. Groups of six BALB/c mice were immunized with DNA vaccine or rPspA3NS, according to the indicated schedule of priming and booster doses. Sera were collected and total anti-PspA IgG of individual sera (a) or anti-PspA IgG1 (filled bars) and IgG2a (open bars) of pooled sera (b) was measured by ELISA on plates coated with rPspA3NS. Non-immunized and empty vector (pSec) controls are also shown. Log of the reciprocal titre of anti-PspA IgG is shown. Numbers above columns are IgG1 : IgG2a titre ratios. *, Statistically significantly different from non-immunized controls; **, statistically significantly different from pSec-*pspA3NS* ($P \leq 0.05$). Results are representative of two independent experiments.

Table 1. Survival after challenge with *S. pneumoniae* 679/99

Treatment	Alive/total	Survival (%)
Non-immunized	1/18	6
pSec + pSec	1/12	8
pTG- <i>pspA3NS</i> + pTG- <i>pspA3NS</i>	5/6	83*
pSec- <i>pspA3NS</i> + pSec- <i>pspA3NS</i>	15/18	83*
pSec- <i>pspA3NS</i> + rPspA3NS	7/10	70*
rPspA3NS + pSec- <i>pspA3NS</i>	6/12	50*
rPspA3NS + rPspA3NS	7/12	58*

*Statistically significantly different from animals immunized with the empty vector pSec ($P < 0.05$).

with DNA vaccine and boosted with recombinant protein showed a tendency towards increased survival when compared with animals primed with rPspA3NS, but the difference was not statistically significant. Thus, augmentation of the humoral response did not lead to higher survival. In our previous work we have used immunization with protein as a positive control for comparison with DNA vaccines expressing different non-secreted PspAs (Miyaji *et al.*, 2002, 2003). The proteins used were purified directly from *S. pneumoniae* through a choline chloride wash, resulting in contamination with other choline-binding proteins. PspA purified from *S. pneumoniae* consistently elicited higher protection levels when compared with the DNA vaccines. With the elimination of the pneumococcal contaminants and the use of exactly the same fragment, protection elicited by DNA vaccination was slightly higher (though not statistically significant) than for recombinant protein.

Anti-PspA antibodies are believed to overcome the anti-complement effect of PspA and to have opsonic activity that appeared to be mainly contributed by IgG2a, a Th1-characteristic immunoglobulin (Arulanandam *et al.*, 2001). Intramuscular immunization with DNA vaccines is known to induce immune responses dominated by Th1 cytokine production, induced in part by immunostimulatory CpG motifs within the bacteria-derived plasmid DNA (Chu *et al.*, 1997; Klinman *et al.*, 1997). Indeed, immunization with DNA vaccines expressing PspA elicited a humoral response with a much more balanced IgG1 : IgG2a ratio. Data on IgG isotyping (Fig. 3b) suggest that the higher antibody response elicited by recombinant protein is mostly due to IgG1, while IgG2a is induced at similar levels by both recombinant protein and DNA vaccination, with comparable protection. We thus conclude that DNA vaccines are able to elicit protection levels similar to those for recombinant protein, even though total IgG levels are considerably lower.

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