Sortase A Confers Protection against *Streptococcus pneumoniae* in Mice

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*Streptococcus pneumoniae* sortase A (SrtA) is a transpeptidase that is highly conserved among pneumococcal strains, whose involvement in adhesion/colonization has been reported. We found that intraperitoneal immunization with recombinant SrtA conferred to mice protection against *S. pneumoniae* intraperitoneal challenge and that the passive transfer of immune serum before intraperitoneal challenge was also protective. Moreover, by using the intranasal challenge model, we observed a significant reduction of bacteremia when mice were intraperitoneally immunized with SrtA, while a moderate decrease of lung infection was achieved by intranasal immunization, even though no influence on nasopharynx colonization was seen. Taken together, our results suggest that SrtA is a good candidate for inclusion in a multicomponent, protein-based, pneumococcal vaccine.

*S. pneumoniae* colonizes the nasopharynx of humans and represents a leading cause of severe diseases, such as otitis media, pneumonia, and meningitis. *S. pneumoniae* is one of the major causes of bacterial pneumonia in developing countries (19). It is estimated that each year, nearly 1 million children worldwide die because of pneumococcal diseases (10). In countries (19). It is estimated that each year, nearly 1 million children worldwide die because of pneumococcal diseases (10). Besides children, groups at high risk of pneumococcal infection are immunocompromised subjects and the elderly, for whom a high case fatality rate is also observed. The last decades have seen an increase in investigations of protein antigens, and several protein candidates have been proposed for a vaccine for *S. pneumoniae* (2) to overcome the problems inherent to the currently available polysaccharide-based vaccines. In fact, the 23-valent polysaccharide pneumococcal vaccine is not effective in children under 2 years of age, whose immune systems are unable to mount a T-independent response to polysaccharides. On the other hand, the 7-valent polysaccharide conjugate vaccine, although efficacious, induces serotype replacement (5, 20). Moreover, while more than 90 *S. pneumoniae* serotypes are presently known, both polysaccharide pneumococcal vaccines and polysaccharide conjugate vaccines are effective only against the serotypes included in the vaccine. Efforts to identify new *S. pneumoniae* factors that play a role in colonization and pathogenesis may contribute to the indication of possible targets of either new therapeutic agents or vaccines.

Sortase A (SrtA) is a membrane-anchored transpeptidase expressed by gram-positive bacteria (12). The role of SrtA in the processing of sorting signals at the LPXTG motif to anchor surface proteins to the cell wall envelope was first described for *Staphylococcus aureus* (21), in which an isogenic SrtA mutation resulted in a strongly reduced ability to infect animals (13, 23). SrtA has been shown to participate in the colonization and/or pathogenesis of several *Streptococcus* species (1, 6, 8, 22, 24). *S. pneumoniae* SrtA has been described as playing a role in adhesion to human pharyngeal cells in vitro (7), in nasopharyngeal colonization in chinchilla (3), and in pneumonia, bacteremia, and nasopharyngeal colonization in murine models (15). Although SrtA seems to be dispensable in pilus biogenesis, its possible role in repressing pilus islet expression has been very recently proposed (9). SrtA has been found to be widely expressed among *S. pneumoniae* isolates and highly conserved, with a DNA identity of 99 to 100% (15). Although all of these findings suggest that pneumococcal SrtA might be useful as a protein vaccine, to the best of our knowledge no data have been provided so far on the protective efficacy afforded by SrtA immunization in animal models. Thus, we investigated the protective role of SrtA in murine models of *S. pneumoniae* infection.

**MATERIALS AND METHODS**

**Protein expression and purification.** The gene portion corresponding to the amino acid sequence from positions 30 to 247 of the SrtA of pneumococcal strain D39 (218 amino acids; calculated molecular mass, 24.81 kDa) was cloned into the pET151/D-TOPO vector (Invitrogen). Recombinant SrtA was then expressed in *Escherichia coli* with a six-histidine tag and purified from bacterial lysate by affinity chromatography on His-Trap high-performance columns (GE Healthcare), equilibrated, and eluted by following the manufacturer’s instructions. Finally, the SrtA, obtained in soluble form, was dialyzed against saline. The protein purity was higher than 90%, as evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by gel scanning densitometry.

**Bacterial strains and culture.** The following *S. pneumoniae* strains were used: TIGR4 (serotype 4), D39 (serotype 2), and 35B-SME15 (serotype 35B). Bacteria were grown for 24 h at 37°C under a 5% CO2 atmosphere on tryptic soy agar (Difco) plates containing colistine (10 mg/liter), oxolinic acid (5 mg/liter), and 5% defibrinated sheep blood. Bacteria were then harvested and used to inoculate liquid cultures in tryptic soy broth (Difco). Liquid cultures were carried out statically at 37°C under a 5% CO2 atmosphere until they reached an A600 of 0.25. Bacteria were then harvested by centrifugation at a relative centrifugal force of 3,500 for 20 min at 4°C and either used for challenge or frozen at −80°C in tryptic soy broth containing 20% glycerol and 20% fetal bovine serum. The
CFU/ml of each bacterial preparation were determined by plating culture aliquots at serial dilutions and counting CFU after 24 h of culture, which was carried out as described above.

**Animal treatments and evaluation of infection and mortality.** Animal experiments were done in compliance with the current Italian law and approved by the internal Animal Ethics Committee of Novartis Vaccines and Diagnostics.

Female, 6-week-old, specific-pathogen-free BALB/c mice (Charles River) received three intraperitoneal administrations, 2 weeks apart, of 20 μg of SrtA along with Freund’s adjuvant. Controls received the same course of saline plus adjuvant. In other experiments, mice were immunized intranasally with the same schedule. For intranasal immunization, animals were anesthetized by intraperitoneal injection of xylazine and ketamine (0.1 and 0.01 mg/kg of body weight, respectively) and then received into their nostrils 10 μl of saline containing 20 μg of SrtA along with 2 μg of LTK63, the nontoxic mutant of heat-labile E. coli enterotoxin, as a mucosal adjuvant (16). Two weeks after the completion of the immunization cycle, samples of sera were obtained for evaluation of the antibody response.

Three weeks after the last immunization, the animals were challenged intraperitoneally with one of the following *S. pneumoniae* strains: TIGR4, 1.4 × 10³ CFU/mouse; D39, 10⁴ CFU/mouse; or 35B-SME15, 7 × 10⁴ CFU/mouse. For the intraperitoneal challenge, frozen bacteria were thawed and brought to the desired concentration in saline.

Bacteremia was evaluated by using blood samples taken 24 h postchallenge and plated on blood agar plates at serial dilutions made in saline, starting with a 1:5 dilution. After 24 h of culture, carried out as described above, CFU were counted and the CFU/ml of blood were calculated. Mortality was recorded for 10 days postchallenge, twice per day for the first 4 days and then once per day. For the passive-protection experiment, each mouse received 0.3 ml of anti-SrtA rabbit serum intraperitoneally 15 min before TIGR4 intraperitoneal challenge. Control animals received 0.3 ml of normal rabbit serum.

For the intranasal challenge, animals anesthetized by xylazine and ketamine as described above received, via the nostrils, 50 μl of bacterial suspension containing 10¹ CFU of freshly harvested TIGR4. Two days after the intranasal challenge, the animals were sacrificed and samples of blood, nasal wash, and lung wash were taken. Nasal wash samples were obtained by flushing 0.5 ml saline through the nostrils. Lung wash samples were obtained in a final volume of 1 ml by two cycles of injection/extraction of 0.5 ml saline into the lungs. Samples were then plated at serial dilutions, starting from a 1:5 dilution (blood) or from no dilution (nasal and lung wash samples), for CFU counts as described above and for cytological analyses (nasal and lung wash samples) as detailed below.

The limit of detection of bacteria in blood was 125 CFU/ml, while that in nasal and lung wash samples was 25 CFU/ml.

**Cytological analysis.** Aliquots of nasal and lung wash samples (undiluted and diluted 1:5 in saline, respectively), taken as described above 2 days after TIGR4 challenge from mice previously immunized intranasally, were subjected to cytological analysis. Samples were cytospun centrifuged and deposited onto a microscope slide by using a Shandon Cytopsin 4 (Thermo Electron Corp.) and then subjected to May-Grünwald-Giemsa staining. Macrophages and polymorphonucleates (PMN) were counted for each mouse as the means of counts in three different microscope fields at 400× magnification, and the mean for each group was calculated.

**Antibody titer evaluation.** Quantification of immunoglobulin G (IgG) or IgA was made by an enzyme-linked immunosorbent assay of mouse sera. Single sera were analyzed.

Serial dilutions of sera were dispensed in Maxisorp 96-well plates (Nalge Nunc International) coated with recombinant SrtA at 0.2 μg/well. Antibody binding was detected by alkaline phosphatase-conjugated anti-mouse IgG or IgA (Southern Biotechnology Associates), followed by the substrate p-nitrophenyl-phosphate (Sigma). Absorbance was measured at 405 nm. The antibody titer was expressed as the reciprocal of the serum dilution, giving an AD50 of 1, and reported as the mean log₁₀ titer ± the standard deviation.

**Statistical analysis.** Antibody titer, bacteremia, and mortality course data were analyzed by a one-tailed Mann-Whitney U test. Cytological analysis data were analyzed by a two-tailed Mann-Whitney U test. P values of ≤0.05 were considered significant.

### RESULTS AND DISCUSSION

**SrtA is immunogenic in mice.** The specific IgG response was quantified with sera of mice immunized intraperitoneally with SrtA (*n* = 46). A good IgG response was detected for all immunized animals, with a mean log₁₀ titer of 4.87 ± 0.38, corresponding to a serum dilution of about 1:75,000. The IgG titer quantified for immunized mice was significantly higher (*P* < 0.0001) than the background value of 2.06 ± 0.42 detected in control sera (*n* = 40). The specific IgG response was also measured for the serum from mice immunized intranasally with SrtA (*n* = 8). The IgG mean log₁₀ titer result was 3.23 ± 0.81, more than 40 times lower than that obtained by intraperitoneal immunization, and with higher variability; it included data from two nonresponders. In the control sera (*n* = 8), an IgG mean log₁₀ titer of 2.18 ± 0.08 was detected (**P** = 0.052; **P** = 0.0003 when including data from the two nonresponders). In the same sera, the specific IgA mean log₁₀ titer was 1.41 ± 0.34, while the control group gave an IgA mean log₁₀ titer of 0.93 ± 0.12 (**P** = 0.002; **P** = 0.001 when excluding data from the two nonresponders). Indeed, mucosal immunization is expected to elicit a measurable amount of IgA isotype, even though the amount can be very low, as in this case.

**Immunization with SrtA protects mice against intraperitoneal challenge.** Results of protection afforded by SrtA against intraperitoneal challenge are shown in Fig. 1 and 2. After challenge with the TIGR4 strain, 15 out of 16 control mice were bacteremic, and only 6 mice were alive at 10 days. In contrast, bacteremia was detectable only in 4 out of 16 mice immunized with SrtA, with a geometric mean about 2 logs lower than that of the control group (Fig. 1A) (3.3 × 10⁴ versus 4.1 × 10³ CFU/ml, **P** = 0.00005). Survival of SrtA-immunized mice was significantly increased (**P** = 0.023), with 11 out of 16 mice alive at 10 days (Fig. 1B).

When challenge was carried out with the strain D39, all control mice (*n* = 8) were bacteremic and died within 3 days postchallenge, while in the SrtA-immunized group (*n* = 7), a 40-fold reduction of bacteremia was achieved (Fig. 1C) (2.7 × 10³ versus 1.1 × 10² CFU/ml, **P** = 0.047) and survival time was significantly increased (Fig. 1D) (**P** = 0.005).

By using the strain 35B-SME15 for the challenge, an ~15-fold reduction of bacteremia was achieved for immunized mice (*n* = 16) compared with controls (*n* = 24) (Fig. 1E) (2.5 × 10⁴ versus 3.6 × 10⁵ CFU/ml, **P** = 0.010), and a trend of increased survival was observed, although it was not significant (Fig. 1F) (**P** = 0.121). It must be pointed out that in our experimental model of infection, the 35B-SME15 strain efficiently infects mice, as judged on the basis of bacteremia quantified 24 h postchallenge; on the other hand, it requires a relatively high doses to cause mortality, and it has a high rate of variability, as observed in the experiments done to define the challenge dose with this strain (data not shown).

To investigate the involvement of antibodies in the protection conferred by SrtA, an experiment of passive protection against strain TIGR4 was carried out. Significant reduction of bacteremia was achieved, with the group receiving the anti-SrtA serum (*n* = 24) having >20-fold-reduced CFU/ml compared with that in samples from the control group (*n* = 24) that had received the same amount of normal rabbit serum (Fig. 2A) (2.1 × 10² versus 4.6 × 10³ CFU/ml, **P** = 0.012), and a corresponding increase in survival time was observed (Fig. 2B) (**P** = 0, 016).

**Immunization with SrtA protects mice against intranasal challenge at either the systemic or the mucosal level, depending on the immunization route.** The intraperitoneal and intranasal immunization routes conferred protection against intrana-
sal challenge at different levels. Intraperitoneal immunization resulted in an ~50-fold decrease of bacteremia (Fig. 3A) ($2.2 \times 10^7$ versus $1.3 \times 10^5$ CFU/ml, $P = 0.014$; $n = 8$ for both the immunized and the control groups), but did not influence nasopharyngeal carriage or lung infection (Fig. 3B and C) ($1.4 \times 10^5$ versus $1.3 \times 10^5$ and $2.7 \times 10^3$ versus $4.3 \times 10^3$ CFU/ml, respectively). Conversely, intranasal immunization did not affect bacteremia or nasopharyngeal carriage (Fig. 3D and E) ($2.3 \times 10^5$ versus $2.8 \times 10^5$ and $1.0 \times 10^5$ versus $1.4 \times 10^5$ CFU/ml, respectively; $n = 8$ for both the immunized and the control groups), while it was able to decrease lung infection by about 1 log (Fig. 3F) ($1.4 \times 10^4$ versus $1.5 \times 10^5$ CFU/ml, $P = 0.032$).

The results of the cytological analysis of nasal and lung wash samples, taken after challenge from mice immunized intranasally, are summarized in Table 1. In the nasal washes, no difference in macrophage or PMN counts was observed between immunized and control groups, while in the lung washes, the immunized group showed a trend toward reduction of macrophage numbers ($P = 0.094$) and a very marked decrease of PMN ($P = 0.0003$).

The finding that numbers of inflammatory cells were decreased in the lungs of immunized mice reinforces the observation that in the same site, the number of detectable bacteria were decreased, suggesting that mice immunized intranasally with SrtA cleared bacteria from the lower respiratory tract with faster kinetics than those of controls. These results suggest that at least partial protection against pneumonia can be afforded by mucosal immunization with SrtA. In contrast, immunization with SrtA did not afford protection against nasopharynx colonization. The relatively low antibody titers measured after intranasal immunization might indicate that an optimal immune response was not achieved. Therefore, additional work to enhance protection, including the optimization of adjuvants and of the immunization schedule and routes, could lead to an improved efficacy. On the other hand, our results could suggest a scarce relevance of SrtA to colonization in the model we used. A role of SrtA in colonization has been described previously (15); however, in that study, different bacterial and mouse strains were used, and more importantly, the results were based on the evaluation of the colonization ability of pneumococcal SrtA deletion mutants. Our results do not exclude the possible relevance of SrtA to colonization, but they suggest that immunity against SrtA might be scarcely or not effective at this stage. In other words, in the model we used, SrtA might be scarcely or not accessible to the immune system effectors during colonization, while it might become more accessible during lung infection and invasion.
Conclusions. SrtA has been reported to be highly conserved among S. pneumoniae strains (15) and to be involved in adhesion/colonization (3, 7, 15). Results reported here clearly show that SrtA is immunogenic in mice and is able to confer protection in the mouse model of intraperitoneal challenge against three different S. pneumoniae strains (TIGR4, D39, 35B-SME15) by active immunization with the recombinant protein. Moreover, the passive transfer of immune serum conferred protection against intraperitoneal challenge with strain TIGR4, indicating the role of antibodies in the protective mechanism. Finally, we observed at least partial protection against intranasal challenge with strain TIGR4 in terms of bacteremia or lung infection, depending on whether the route of immunization was intraperitoneal or intranasal, respectively.

The body of evidence presently available supports the notion that it is unlikely that a single protein antigen can afford protection against all S. pneumoniae serotypes, even though it can confer either partial protection against a broad range of pneumococcal strains or high protection against a subset of strains. Conceivably, an effective vaccine should be multicomponent (2, 4, 14), as is the case for some vaccines on the market (e.g., acellular pertussis vaccines) (17) and others under development (e.g., protein-based vaccines against group B meningococci or against group B streptococci) (11, 18).

Our results indicate that SrtA is a promising pneumococcal antigen that could be considered for inclusion in a multicomponent protein vaccine against S. pneumoniae.

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All authors declare potential conflicting interests; all authors are Novartis employees, with the exception of Giacomo Rossi, who has a contract with Novartis.

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