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Intranasal immunization with serum opacity factor (SOF) of *Streptococcus pyogenes* fails to protect mice against lethal mucosal challenge with a heterologous strain

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Abstract

Streptococcus pyogenes is a human pathogen causing invasive and non-invasive diseases, as well as severe sequels, such as rheumatic fever. Several bacterial factors have been proposed as candidate vaccine antigens. Among them, the serum opacity factor (SOF), which was able to confer protective immunity against an intraperitoneal challenge after vaccination by the parenteral route. In an attempt to develop more efficient vaccines, we combined SOF with an additional well-known protective antigen, namely, the fibronectin-binding protein I (SfbI). Intranasal immunization of mice with SOF and SfbI stimulates strong systemic and mucosal immune responses against both antigens. Animals vaccinated with SfbI, alone or in combination with SOF, were also efficiently protected against a lethal challenge with a SOF/SfbI-positive virulent *S. pyogenes* strain (80% survival). In contrast, those vaccinated with SOF alone were not protected against a mucosal challenge (100% lethality), which mimics more closely natural infections. These results highlight the importance of developing adequate experimental animal models to evaluate vaccine efficiency, according to the selected antigen.

Keywords: *Streptococcus pyogenes*; Mucosal delivery; Vaccine; SOF; SfbI

1. Introduction

Streptococcus pyogenes exclusively colonizes humans leading to either a variety of localized infections of the skin and mucosa (e.g. impetigo, pharyngitis), or life-threatening invasive diseases (e.g. sepsis, necrotizing fasciitis and toxic shock-like syndrome) [1,2]. *S. pyogenes* infections can also result in severe non-infectious sequels, such as acute post-streptococcal glomerulonephritis, rheumatic fever and rheumatic heart disease [3]. These diseases are particularly wide-spread in certain populations, such as the Australian Aborigines [4–6].

So far, most of the research addressing the development of *S. pyogenes* vaccines has been concentrated on a handful of proteins, which are mainly involved in the pathogenesis process [7]. Among them, surface exposed molecules, such as the M protein [8,9], which has anti-phagocytic and adhesive properties, or the fibronectin binding proteins, which play a major role in bacterial attachment to and invasion of epithelial cells [10–16]. The C5a peptidase, a surface-bound peptidase that cleaves C5a, and the extracellular cysteine protease, which cleaves fibronectin and converts IL-1^{ß} precursor to biologically active IL-1^{ß} [17–19]. More recently, it was shown that parenteral immunization of mice with the streptococcal serum opacity factor (SOF; Fig. 1) stimulates a protective immune response against a systemic challenge [20]. The SOF is a large extracellular and surface-exposed protein, which opacifies mammalian serum and shows fibronectin-binding capacity [21,22]. Interestingly, up to 96% of the sera from aboriginal patients tested positive for antibodies against SOF, suggesting that it is expressed and recognized during natural infections [23].

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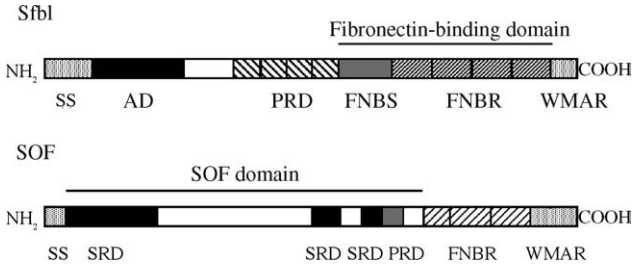

Fig. 1. Schematic structure of the SfbI and SOF proteins used in this work. SS: signal sequence, AD: aromatic domain, PRD: proline-rich domain, FNBS: fibronectin-binding spacer, FNBR: fibronectin-binding repeats, WMAR: wall–membrane anchor region, and SRD: serine-rich domain.

In an attempt to develop more efficacious vaccine formulations against this *S. pyogenes*, we decided to combine the SOF with the well-characterized protective antigen SfbI [24–28]. To this end, we evaluated the usefulness of SOF as candidate vaccine antigen, alone or in combination with the SfbI protein, using a respiratory murine experimental infection model mimicking more closely natural infections. Animals vaccinated with SfbI or SfbI + SOF were efficiently protected against challenge. In contrast, despite the stimulation of strong humoral and cellular immune responses, SOF-vaccinated animals were not protected against a lethal mucosal challenge with a virulent SOF-positive strain.

2. Materials and methods

2.1. Immunization, sample collection and challenge

The recombinant His-tagged fusion proteins were generated by cloning a 615 bp *Bam*HI/*Sal*I fragment encompassing positions 1240–1854 of the *sfbI* gene and a 910 bp *Bam*HI/*Sal*I fragment encompassing positions 109–2838 of the *sof* gene into the pQE30 vector, thereby generating the recombinant plasmids pSTH12 and HT1 [16,29]. Overexpression and purification by affinity chromatography of the His-tagged fusion proteins using Ni-NTA columns (PIERCE, Rockford) was performed under native conditions; according to the producer recommendations. Groups ($n = 5$) of BALB/c mice (Harlan Winkelmann, Borchen, Germany) were immunized by intranasal route with the recombinant polypeptides co-administered with 10 μg of cholera toxin B subunit (CTB; Sigma Chemie GmbH, Deisenhofen, Germany) as adjuvant or with CTB alone on days 0, 7 and 14, according to established protocols [30]. Serum samples were collected on days 0, 7, 14, and 25 and stored at −20 °C prior to determination of SfbI- and SOF-specific antibodies. Lung lavages were obtained by flushing the organs with several aliquots of PBS supplemented with 50 mM EDTA, 0.1% bovine serum albumin (BSA), and 10 mM PMSF with a final volume of 1 ml. Lavages were then centrifuged to remove debris (10 min at 3000 × *g*), and supernatant fluids were stored at −20 °C. Vaccinated animals ($n = 10$) were intranasally challenged with 10^8 colony-forming units (CFU) of the heterologous virulent *S. pyogenes* strain NS192 (blood isolate from the Australian Northern Territories) on day 35 and mortality was recorded daily [24,27].

2.2. Evaluation of antibody responses

Sera were tested for the presence of antigen-specific Ig by ELISA. Studies were performed using microtiter plates (Nunc Roskilde, Denmark) coated with 50 μl/well of the corresponding antigen (2 μg/ml) in coating buffer (bicarbonate, pH 9.6). After overnight incubation at 4 °C, plates were blocked with 1% BSA in phosphate buffered saline (PBS, pH 7.4) for 1 h at 37 °C. Serial 2-fold dilutions of sera from immunized mice in PBS/1% BSA were added (100 μl/well), and plates were incubated for 2 h at 37 °C. After four washes, secondary biotinylated antibodies were added followed by a 1 h incubation at 37 °C. After another six washes, peroxidase conjugated streptavidin (Pharmingen) was added (50 μl/well), and plates were further incubated for 45 min at room temperature. After six final washes, ABTS [2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)] diluted in 0.1 M citrate–phosphate buffer containing 0.01% H_2O_2 was added, and plates were further incubated for 30–60 min at room temperature. The end-point titers were expressed as the reciprocal $\log_2$ of the last dilution giving an A_{405} of 0.1 U above the A_{405} of the negative controls.

The IgG isotypes present in serum samples were determined by ELISA as previously described [31], using as secondary antibodies biotin-conjugated rat anti-mouse IgG1, IgG2a, IgG2b or IgG3 (Southern Biotechnology Associates, Birmingham, UK).

The amount of total and antigen-specific IgA and IgG present in lung washes was determined by ELISA, as previously described [24,31]. To compensate for variations in the efficiency of recovery of antibodies between animals, the results were normalized and expressed as percentage of antigen-specific IgA and IgG with respect to the total amount of IgA and IgG present in the sample, respectively. To establish the IgG standard curve, plates coated with goat anti-mouse IgG (Sigma Chemie) as capture antibody were incubated with serial dilutions of purified mouse IgG (Dianova, Hamburg, Germany). As secondary antibody, biotinylated goat anti-mouse IgG (Sigma Chemie) was used, plates were developed as described above.

2.3. T-cell proliferation assays

To investigate T-cell proliferation, spleen cell suspensions were adjusted to 5×10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml of penicillin, 50 μg/ml of streptomycin and 1 mM L-glutamine (GIBCO BRL, Karlsruhe, Germany). Cells were seeded (100 μl/well) in a flat-bottomed 96-well microtiter plates (Nunc), which were incubated at 37 °C for 4 days in the presence of 5, 10 and 20 μg/well of the relevant antigen in triplicates. During the final 18 h of culture, 1 μCi of [^{3}H]thymidine