Serum Opacity Factor (SOF) of *Streptococcus pyogenes* Evokes Antibodies That Opsonize Homologous and Heterologous SOF-Positive Serotypes of Group A Streptococci

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Received 29 January 2003/Returned for modification 5 May 2003/Accepted 24 June 2003

Serum opacity factor (SOF) is a protein expressed by *Streptococcus pyogenes* that opacifies mammalian serum. SOF is also a virulence factor of *S. pyogenes*, but it has not been previously shown to elicit a protective immune response. Herein, we report that SOF evokes bactericidal antibodies against *S. pyogenes* in humans, rabbits, and mice. Rabbit antiserum against purified recombinant SOF2 opsonized SOF-positive M type 2, 4, and 28 S. *pyogenes* in human blood but had no effect on SOF-negative M type 5 *S. pyogenes*. Furthermore, affinity-purified human antibodies against SOF-positive M type 2 also opsonized SOF-positive streptococci. A combination of antisera against M2 and SOF2 proteins was dramatically more effective in killing streptococci than either antiserum alone, indicating that antibodies against SOF2 enhance the opsonic efficiency of M protein antibodies. Mice tolerated an intravenous injection of 100 μg of SOF without overt signs of toxicity, and immunization with SOF protected mice against challenge infections with M type 2 *S. pyogenes*. These data indicate that SOF evokes opsonic antibodies that may protect against infections by SOF-positive serotypes of group A streptococci and suggest that different serotypes of SOF have common epitopes that may be useful vaccine candidates to protect against group A streptococcal infections.

The group A streptococcus *Streptococcus pyogenes* causes a variety of diseases, ranging from mild and generally self-limiting infections of the pharynx and skin to more-severe and life-threatening infections, such as toxic shock syndrome and necrotizing fasciitis. The major sequelae of group A streptococcal infections are acute rheumatic fever and acute glomerulonephritis, which are thought to be due to autoimmune T- and B-cell responses induced by streptococcal products (2, 11–15, 20). Prior infections with group A streptococci may also lead to autoimmune neurological disorders (5, 34, 45).

Early efforts to develop a vaccine to prevent these diseases focused on M proteins because infections in humans were found to elicit an immune response to M proteins that was protective and long-lived (30). M proteins are the major virulence factor in group A streptococci and confer the abilities to multiply in nonimmune human blood and to attach to host cells (8, 13, 20). Structurally, M proteins are α-helical, coiled-coil proteins that radiate from the surface of the organism and that are composed of a variable N-terminal half and a highly conserved C-terminal half (20). The N-terminal 40 to 50 amino acids are hypervariable and elicit type-specific antisera. Both the conserved and variable domains of M proteins are targets of current vaccine efforts, and each approach has its own strengths and weaknesses.

The major strength of a vaccine based on the conserved domains of M proteins is that protection against both homologous and heterologous serotypes is provided (1, 4, 6, 7, 36–38). The major concern is that these conserved domains may stimulate T- and B-cell responses that target human tissues (12, 14, 15). Good and coworkers have, however, identified a peptide in the C repeats of M proteins that elicits bactericidal antibodies that do not cross-react with human tissues (1, 36, 37), but the level of bactericidal antibodies may not be adequate in some cases.

The major strengths of a vaccine based on the variable N termini of M proteins are that a strong bactericidal antibody response is evoked and that these antibodies are less likely to cross-react with human tissues (17, 23). The major problem is that protection is generally type specific, and there are more than 100 different M types produced by group A streptococci. This problem has been addressed by developing multivalent vaccines that target prevalent serotypes causing pharyngitis, invasive diseases, and rheumatic fever (23). Thus, a 26-valent vaccine targeted 84% of all group A streptococcal isolates and 74% of invasive isolates identified from 1998 to 2000 within the United States (23).

More-recent investigations have identified a number of other vaccine candidates, including the R28 protein (44), Spa (16, 32), C5a peptidase (25), the group A carbohydrate (41), Sfb1 (also termed protein F1) (22, 33, 43), FBP54 (27), SpeA (40), SpeB (26), SpeC (31), and lipoteichoic acid (LTA) (18). Some of these antigens elicit protection against only a limited number of serotypes, while other antigens, such as the group A carbohydrate, may require high concentrations of antibodies to be effective. Furthermore, the C5a peptidase, SpeA, SpeB, SpeC, Sfb1, and the R28 protein have not been shown to induce antibodies that opsonize group A streptococci. FBP54 evoked opsonic antibodies against two different serotypes, but its degree of coverage and efficacy of protection have not yet been thoroughly investigated (27). LTA induced antibodies that blocked colonization (18), but almost all gram-positive...
bacteria produce LTA. Therefore, a vaccine utilizing LTA would not be selective in the bacteria it targets. Because of these considerations, the M protein-based vaccine is considered to be very promising. However, not all types of M proteins evoke a protective antibody response (6), and there are serotypes for which a protective antigen (an antigen that evokes a protective immune response) has not yet been identified. Moreover, the current 26-valent vaccine targets serotypes primarily found in the United States, and these serotypes may not be representative of those causing infections in other areas such as Australia and Asia. Thus, there is a need to broaden the protective coverage of vaccines and to define the protective antigen in some serotypes. Herein, we report on the potential of the serum opacity factor (SOF) of group A streptococci to meet this need.

SOF is a >100-kDa, surface-bound and released protein of *S. pyogenes* that opacifies mammalian serum by interacting with high-density lipoproteins (42, 46, 47). It is composed of alternating variable and conserved domains and a highly conserved C-terminal domain with an LPASG anchoring motif (9, 28, 39). The C-terminal domain contains a tandemly repeated peptide that binds fibronectin and fibrinogen (9, 10, 28, 39). The opacification of serum can be inhibited by antisera against type-specific determinants of SOF, and this inhibition is the basis for the SOF typing scheme of group A streptococci (3). Interestingly, the type-specific determinants of SOF usually covary with those of M proteins in a given strain, and thus the M type can be predicted based on the SOF type (3). Inactivation of SOF decreased the virulence of M type 2 *S. pyogenes* in a mouse model, indicating that it is a virulence determinant (9). Because other virulence factors have been found to elicit protective immune responses, SOF was tested for its ability to induce protective antibodies. The results indicate that SOF evokes antibodies that protect against infections by SOF-positive group A streptococci.

**MATERIALS AND METHODS**

**Organisms and growth conditions.** The SOF-positive strains of *S. pyogenes* used in this study were the M type 2 strain T2MR, the M type 4 strain 52936, and the M type 28 strain 92448. The M type 5 strain Manfredo is SOF negative. The organisms used in this study were the M type 2 strain T2MR, the M type 4 strain 52936, and the M type 28 strain 92448. The M type 5 strain Manfredo is SOF negative. The growth conditions used for these experiments are described in Methods. The microorganisms were grown in Todd-Hewitt broth supplemented with 1.5% yeast extract to an optical density of 0.08 at 530 nm and diluted 1:10,000. Twenty microliters of this dilution was added to a tube containing 200 μl of anti-SOF2 serum or preimmune serum and 400 μl of heparinized human blood from a nonimmune donor. The blood was rotated for 3 h at 37°C, and the number of CFU was determined by plating dilutions on blood agar plates. The bactericidal assays were repeated on three separate occasions. In assays testing the combined effects of anti-M2(1-35) serum and anti-SOF2 serum, 100 μl of the serial 1:2 dilutions of anti-M2(1-35) was added to 100 μl of anti-SOF2 or normal rabbit serum (NRS). The mixtures were added to 400 μl of heparinized human blood and treated as described above. The percentage of streptococci killed in the bactericidal assays was calculated by the following formula: percent killing = [1 – (number of CFU in anti-SOF2 serum/number of CFU in preimmune serum)] × 100.

**The serum opacity reaction and its inhibition.** The ability of SOF in the culture supernatant of streptococci to opacity serum was tested by centrifugation of overnight cultures of the organisms, sterilization of the media by filtration, and addition of 100 μl of the filtrate to 1 ml of horse serum. After incubation at 37°C for 3 h, the absorbance at 405 nm was recorded. Assays for neutralization of the opacity reaction consisted of preincubating 100 μl of neutralizing serum and 100 μl of culture supernatant for 30 min at 37°C and then adding 1 ml of horse serum and recording the absorbance at 405 nm after 3 h and after an overnight incubation. In some cases, purified recombinant SOF (1 μg/ml) was used instead of culture supernatants in the inhibition experiments described above.

**Purification of human antibodies against SOF.** A donor whose serum inhibited the opacity reaction of SOF2 was selected. The donor’s serum was first chromatographed over a quaternary amine-ethyl-Sepharose column to remove other serum proteins that may bind to SOF. The α2M flowthrough containing the antibodies was then added to a column of SOF2-H(38-1047) or SOF2ΔFBD covalently linked to agarose. The columns were washed with buffer, and bound proteins were eluted with 0.05 M sodium acetate–0.1 M glycine, pH 5.0 and injected subcutaneously into New Zealand White rabbits. Booster injections of 500 μg in phosphate-buffered saline (PBS) were given at 4, 8, 10, and 15 weeks.

**Enzyme-linked immunosorbassays (ELISA).** Wells of a microtiter plate were coated with purified recombinant SOF2, SOF4, and SOF28 (10 μg/ml in 0.01 M sodium bicarbonate, pH 9.5). Control wells were coated with bovine serum albumin (BSA). After being coated, all wells were blocked with BSA (1 mg/ml in PBS). Serial 1:2 dilutions of a 1:1,000 dilution of rabbit anti-SOF2-H(38-1047) or preimmune serum were added to the wells and incubated for 30 min at 37°C. The wells were washed, and a 1:2,000 dilution of peroxidase-labeled goat anti-rabbit immunoglobulins (lg) was added. After 30 min, the wells were washed and the substrate tetramethylbenzidine was added. After color development, the absorbance at 650 nm was measured. The average value for wells coated with BSA served as a blank and was subtracted from all other values. All samples were tested in duplicate.

**Bactericidal assays.** Streptococci were grown in Todd-Hewitt broth supplemented with 1.5% yeast extract to an optical density of ~0.08 at 530 nm and diluted 1:10,000. Twenty microliters of this dilution was added to a tube containing 200 μl of anti-SOF2 serum or preimmune serum and 400 μl of heparinized human blood from a nonimmune donor. The blood was rotated for 3 h at 37°C, and the number of CFU was determined by plating dilutions on blood agar plates. The bactericidal assays were repeated on three separate occasions. In assays testing the combined effects of anti-M2(1-35) serum and anti-SOF2 serum, 100 μl of the serial 1:2 dilutions of anti-M2(1-35) was added to 100 μl of anti-SOF2 or normal rabbit serum (NRS). The mixtures were added to 400 μl of heparinized human blood and treated as described above. The percentage of streptococci killed in the bactericidal assays was calculated by the following formula: percent killing = [1 – (number of CFU in anti-SOF2 serum/number of CFU in preimmune serum)] × 100.

**FIG. 1. Model of SOF and recombinant peptides of SOF, indicating the locations of the functional domains of SOF and the recombinant peptides of SOF used in this study. The locations of the functional domains are based on the findings of Rakonjac et al. (39), Kreikemeyer et al. (28), and Courtney et al. (9).** Fn, fibronectin.
Antiserum against SOF2 cross-reacts with other types of SOF. Rabbit antiserum against SOF2-H(38-1047) did not inhibit the serum opacity reaction of SOF2 but did react strongly with SOF2 in ELISA as exhibited by a positive signal at a 1:128,000 dilution (Fig. 2). The anti-SOF2 serum also strongly cross-reacted with SOF4 and SOF28. It was anticipated that the anti-SOF2 serum would cross-react with both SOF4 and SOF28, because there is ~60% homology between SOF2 and SOF28 and ~53% homology between SOF2 and SOF4. The degree of cross-reactivity suggests that a significant proportion of the antibodies are directed against common epitopes.

Bactericidal activity of antiserum against SOF2. The ability of the rabbit antisera against SOF2 to opsonize M type 2, 4, and 28 S. pyogenes in nonimmune human blood was tested (Fig. 3). Rabbit antisera against SOF2-H(38-1047) not only opsonized and killed M type 2 S. pyogenes (65% killing) but also opsonized M type 4 and 28 S. pyogenes (72 and 71% killing, respectively). Two separate control experiments were performed to ensure that the antiserum did not aggregate the streptococci. In one experiment, an identical inoculum was added to preimmune serum and to anti-SOF2 serum, the mixtures were shaken, and the numbers of CFU were determined by plating. There was no difference in the numbers of CFU in the inocula, indicating that no aggregation occurred due to anti-SOF serum. In a second experiment, streptococci were added to freshly prepared human plasma containing either preimmune serum or anti-SOF2 serum. After 3 h of rotation, the numbers of CFU were determined. Again, no significant difference in the numbers of CFU between preimmune and immune serum was found, indicating that anti-SOF2 serum did not aggregate the streptococci. The results of the second experiment also demonstrate that neutrophils are needed to kill the streptococci and that antibodies and complement alone are not sufficient.

Next, it was of interest to determine if humans also produce opsonic antibodies against SOF2. A donor whose serum inhibited the serum opacity reaction of SOF2 was selected. The antibodies against SOF2 were purified from this serum by affinity chromatography utilizing either SOF2-H(38-1047) or SOF2AFBD as the matrix and tested in bactericidal assays using strain T2MR. In two separate experiments, antibodies eluted from SOF2AFBD killed 40 and 43% of streptococci in a bactericidal assay of whole human blood (Table 1). Antibodies eluted from SOF2-H(38-1047) killed 73% of the streptococci. These results indicate that SOF stimulates the production of bactericidal antibodies in humans.

The higher level of opsonization achieved with antibodies eluted from SOF2-H(38-1047) raised the possibility that antibodies against the FBD of SOF may also contribute to the opsonization of the bacteria. To evaluate this possibility, serum from the human donor was tested for antibodies that react with the FBD of SOF. Serum from the human donor did not react with the FBD of SOF (Table 2). Thus, it is unlikely that antibodies against the FBD contributed to the opsonization of T2MR by the affinity-purified antibodies. Gillen et al. (21) also found that human sera have little or no antibodies that react with the FBD of SOF. However, these investigators reported that rabbit antiserum against full-length SOF did not react with
the FBD of SOF. In contrast, we found that immunization of a rabbit with full-length SOF elicited antibodies that reacted with the FBD of SOF (Table 2).

**Table 2. Comparison of antibodies against SOF2 in rabbit and human immune serum**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Dilution</th>
<th>Substrate</th>
<th>( A_{50} ) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-SOF2-H(38-1047)</td>
<td>1:100</td>
<td>SOF2-H(38-1047)</td>
<td>&gt;2.5</td>
</tr>
<tr>
<td></td>
<td>1:1,000</td>
<td>SOF2-H(38-1047)</td>
<td>1.76 ± 0.197</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>FBD</td>
<td>1.63 ± 0.122</td>
</tr>
<tr>
<td></td>
<td>1:1,000</td>
<td>FBD</td>
<td>0.349 ± 0.074</td>
</tr>
<tr>
<td>Human donor (opsonizing)</td>
<td>1:100</td>
<td>SOF2-H(38-1047)</td>
<td>0.444 ± 0.056</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FBD</td>
<td>-0.009 ± 0.005</td>
</tr>
</tbody>
</table>

a. Rabbit anti-SOF2 serum and human serum (from the same donor that provided antibodies listed in Table 1) were diluted as indicated and reacted with microtiter wells coated with SOF2-H(38-1047) or FBD. The wells were washed and reacted with the appropriate peroxidase-conjugated second antisera as indicated in Materials and Methods. Wells were done in triplicate. Wells coated with BSA and treated as described above served as blanks, and the value for these was subtracted from test values. The \( A_{50} \) of wells coated with FBD were slightly lower than that for wells coated with BSA, resulting in a negative value for FBD.

**Table 1. Opsonization of M type 2 *S. pyogenes* by affinity-purified human antibodies against SOF2**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Affinity matrix</th>
<th>Inoculum (CFU)</th>
<th>CFU in:</th>
<th>% Killing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SOF2ΔFBD</td>
<td>72</td>
<td>Control buffer, Purified antibodies</td>
<td>1,000</td>
</tr>
<tr>
<td>2</td>
<td>SOF2ΔFBD</td>
<td>40</td>
<td>Control buffer, Purified antibodies</td>
<td>1,000</td>
</tr>
<tr>
<td>3</td>
<td>SOF2-H(38–1047)</td>
<td>85</td>
<td>Control buffer, Purified antibodies</td>
<td>1,000</td>
</tr>
</tbody>
</table>

a. Human antibodies against SOF2 were purified by SOF affinity chromatography, mixed with the indicated number of CFU of *S. pyogenes* strain T2MR, and added to human blood as described in Materials and Methods. The number of CFU after 3 h of rotation was determined by plating dilutions of the mixtures. Controls consisted of adding human IgG equivalent to the amount of affinity-purified SOF antibodies except for experiment 1, where Tris-saline buffer was used.

**Bactericidal effect of combining anti-M2 and anti-SOF2 sera.** To further evaluate the potential of SOF as a vaccine candidate, the ability of anti-SOF2 serum to enhance the opsonic effect of antiserum against the M protein was assessed. Serial 1:2 dilutions of rabbit antisera against a synthetic peptide copying the first 35 amino acids from the N terminus of the M type 2 protein from *S. pyogenes*, anti-sM2(1-35), were added to NRS or to rabbit anti-SOF2 serum. *S. pyogenes* strain T2MR and nonimmune human blood were added, and the mixtures were treated as described for the bactericidal assays. Anti-serum against SOF dramatically enhanced the ability of antiserum against the M2 protein to opsonize and kill group A streptococci (Fig. 4).

**Mouse toxicity and protection experiment.** The mouse toxicity and protection experiment was initially designed to determine if SOF was toxic to mice. Five mice were injected i.v. with 100 \( \mu \)g of SOF2-H(38-1047) and five mice were injected i.v. with 100 \( \mu \)g of SOF2-H(494-1047). SOF2-H(38-1047) encompasses the mature SOF2 protein and opacifies serum. SOF2-H(494-1047) does not opacify serum and served as a negative control. None of the mice exhibited any visible signs of illness, indicating that SOF2 is not overtly toxic to mice under these conditions. The mice were then used to determine if vaccination against SOF2 would protect against group A streptococcal infections. The mice were boosted by an i.p. injection of SOF2-H(494-1047) and challenged i.p. with \( 5 \times 10^7 \) CFU of M type 2 strain T2MR 11 days later. As a negative control, 15 nonimmunized mice were also challenged i.p. with T2MR. There was no difference in survival rate between mice immunized with SOF2-H(494-1047) and mice immunized with SOF2-H(38-1047); therefore, the two groups were combined. Thus, only 4 of the 10 mice immunized with SOF2 died, whereas, 14 of the 15 mice that were not immunized died (Fig. 5). These results suggest that immunization with SOF2 protects mice against infections by SOF-positive group A streptococci.

Next, we wanted to determine if the FBD of SOF was required to induce protection in mice. Ten mice were immunized with SOF2ΔFBD in CFA, and 9 mice were mock immunized with CFA. After a booster injection, blood was obtained from the tail veins of mice and tested for antibodies against SOF. The immunized mice developed significant levels of antibodies against SOF2ΔFBD, whereas the mock-immunized mice did not (Fig. 6). All of the mice were challenged i.p. with \( -1 \times 10^7 \) CFU of T2MR, and the number of surviving mice was monitored daily. None of the immunized mice died, whereas four of the nine mock-immunized mice died (Fig. 7). These data provide additional evidence that SOF induces a protective immune response and that the FBD of SOF is not required for this response.
DISCUSSION

In this report, SOF is shown to evoke a protective or bactericidal immune response by three independent experiments. First, immunization with SOF evoked opsonic antibodies in rabbits. Second, purified, human antibodies against SOF opsonized and killed S. pyogenes in human blood. Third, immunization of mice with SOF protected them against death from a challenge infection by SOF-positive streptococci.

Antibodies, in general, can provide protection against infections by several different mechanisms. Antibodies may bind to an adhesin on the surface of an organism and block adhesion of the bacteria to host cells, or antibodies may neutralize the function of a virulence factor. Alternatively, antibodies may opsonize bacteria. In the present case, the possibility of an antiadhesive effect can be excluded because the mice were challenged i.p., which effectively bypasses the adherence and colonization stage of an infection. We cannot rule out the possibility that neutralization of the opacity reaction of SOF may reduce virulence. However, our findings that rabbit antisera against SOF opsonized and killed SOF-positive S. pyogenes but did not neutralize the opacity reaction of SOF suggest that the protection afforded by immunization with SOF is most likely due to opsonic antibodies and not neutralizing antibodies. Others have also reported the lack of neutralizing antibodies in the serum of SOF-immunized animals (29).

Although our data suggest that protection from infection is due to opsonic antibodies that recognize SOF, other mechanisms may come into play under different conditions. For example, if mice were challenged intranasally, then antibodies that block adhesion could be protective. Recent experiments indicate that SOF may mediate the adhesion of group A streptococci to certain types of host cells (H. Courtney, unpublished data) and raise the possibility that antibodies against SOF may be able to prevent adhesion.

Antibodies against SOF2 not only opsonized the homologous M type 2 strain but also opsonized and killed M type 4 and 28 S. pyogenes. These data indicate that SOF contains common epitopes that can induce cross-reactive opsonic antibodies that recognize SOF on the surfaces of M type 2, 4, and 28 S. pyogenes. To our knowledge, this is the first instance in which a protective antigen in M type 4 S. pyogenes has been identified. It has been previously reported that the M type 4 protein of S. pyogenes did not confer resistance to phagocytosis (24) and that antiserum against the M type 4 protein was not opsonic (6). It is noteworthy that many of the serotypes of S. pyogenes that are poorly opsonized by antisera against M proteins are SOF positive (6, 23).

The common or shared epitope(s) of SOF that evokes cross-opsonic antibodies has not yet been identified. The FBD of SOF is an obvious candidate, especially since Schulze et al. (43) found that intranasal immunization of mice with the FBD of SfbI protected against an intranasal challenge of group A streptococci. Other domains of SfbI failed to stimulate a protective response. The primary protective effect was judged to be due to immunoglobulin A antibodies against the FBD of SfbI, which blocked adhesion of the streptococci to host cells (43). We have demonstrated that the FBD of SOF is not necessary for the induction of opsonic antibodies. Thus, epitopes outside of the FBD of SOF can induce protective
antibodies. Whether the FBD of SOF may also induce opsonic antibodies remains to be demonstrated.

SOF is also produced by *Staphylococcus epidermidis* and by group C streptococci (9, 19), which can cause infections of the respiratory tract and skin in humans. The sequence of SOF from a group C streptococcus, *Streptococcus dysgalactiae*, shares many domains with SOF from group A streptococci (9), and antisera against SOF from group A streptococci cross-reacts with SOF from group C streptococci (H. Courtney, unpublished data). Thus, the usefulness of SOF as a vaccine may extend to other pathogenic species of gram-positive bacteria besides *S. pyogenes*. Moreover, some of the SOF-producing bacteria cause infections in both humans and animals, suggesting that SOF may also be utilized to help prevent infections in animals.

In summary, we have shown that antisera against SOF can opsonize SOF-positive streptococci in human blood and protect mice against streptococcal infections. The finding that antisera against one type of SOF can opsonize both homologous and heterologous SOF-positive serotypes of group A streptococci suggests that different serotypes of SOF contain a shared epitope(s) that evokes opsonic antibodies. The potential impact of a vaccine that targets this epitope(s) could be significant when one considers that almost one-half of all clinical isolates and 45% of invasive strains of group A streptococci in the United States are SOF positive (35). A further indication of the potential effectiveness of SOF as a vaccine candidate is provided by the finding that antisera against SOF dramatically enhanced the opsonic efficiency of anti-M protein serum. It is envisioned that the identification of a common protective epitope(s) of SOF could lead to its incorporation in current vaccines to broaden their protective coverage and effectiveness.

**ACKNOWLEDGMENTS**

This study was supported by research funds from the U.S. Department of Veterans Affairs and from the U.S. Public Health Service, grant AI-10085 (J.B.D.). We express our appreciation for the technical assistance of Yi Li and Edna Chiang.

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