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Intranasal Immunization with C5a Peptidase Prevents Nasopharyngeal Colonization of Mice by the Group A *Streptococcus*

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Early inflammatory events are initiated by phased production of C5a and interleukin-8 in tissue. Most serotypes of group A streptococci express a surface-bound peptidase (SCPA) which specifically cleaves mouse and human C5a chemotaxins. This study investigates the impact of SCPA on colonization of the nasopharyngeal mucosa of mice and evaluates its potential to induce protective immunity. Two strains, serotypes M6 and M49, which contain insertion and deletion mutations in the SCPA gene (*scpA*) and represent the two major subdivisions of group A streptococci, were characterized and compared in a mouse intranasal infection model. In this model, SCPA mutants were more rapidly cleared from the nasopharynges of inoculated mice compared with wild-type strains. A 2,908-bp fragment of *scpA49* gene, obtained by PCR, was ligated to the expression vector pGEX-4T-1 and expressed in *Escherichia coli*. The affinity-purified ΔSCPA49 protein proved to be highly immunogenic in mice and rabbits. Although the purified ΔSCPA49 immunogen lacked enzymatic activity, it induced high titers of rabbit antibodies which were able to neutralize peptidase activity associated with M1, M6, M12, and M49 streptococci in vitro. This result confirmed that antipeptidase antibodies lack serotype specificity. Intranasal immunization of mice with the deleted form of the SCPA49 protein stimulated significant levels of specific salivary secretory immunoglobulin A (IgA) and serum IgG antibodies and reduced the potential of wild-type M1, M2, M6, M11, and M49 streptococci to colonize. These experiments suggest a new approach to vaccine development for prevention of streptococcal pharyngitis.

Group A streptococcal pharyngitis is responsible for 10 to 30% of all office calls to a general medical practice. More recently, this organism has emerged again to become a frequent cause of systemic infections associated with toxic shock and aggressive soft tissue wound infections. Virulence is orchestrated by a variety of surface proteins and secreted toxins which together present a barrier to phagocytic defenses or have a profound pathophysiological impact on the host. For more than 40 years, the antiphagocytic M proteins have been a primary focus of epidemiologists, molecular biologists, and those involved in vaccine development (12). M proteins block the deposition of opsonin C3b onto the bacterial surface, function as adhesins for epithelial cells, and bind a variety of human plasma proteins. Antibody directed at M protein is opsonic (12), can be protective in animal models (1, 4), but can also induce a tissue cross-reactive immune response (11).

Several years ago our laboratory discovered that the surface of virulent strains of group A streptococci also displays a highly specific peptidase (SCPA), an endopeptidase which cleaves the leukocyte binding site of the complement-derived chemotaxin C5a (26). The peptidase was previously shown to cleave C5a at His^{67}. Moreover, it is highly specific for this complement component and is unable to cleave native C5 or several other proteins known to contain internal histidines (9). Because this peptidase is strategically bound to the bacterial surface, near the source of C5a, generated by interaction of streptococci with the alternative complement pathway, we proposed that SCPA delays infiltration of phagocytes, retarding clearance of bacteria from mucosal surfaces. Circumstantial evidence, coregulation of SCPA and M protein expression, and existence of similar enzymes on the surface of other human pathogenic streptococcal species support its role in virulence (23). More recent studies confirmed that SCPA retards infiltration of granulocytes to sites of infection created by injection of streptococci below the dermis of mice (18). Elimination of SCPA by mutation enhances granulocyte infiltration, clearance of streptococci, and trafficking of streptococci to lymph nodes, even though they produce M protein and resist phagocytosis in vitro.

The development of a safe and effective group A streptococcal vaccine is necessary and important for prevention of serious group A streptococcal infection. The *scpA* gene is ubiquitous among group A streptococci. More than 160 strains of group A streptococci, representing 40 serotypes, were found to express SCPA (5, 7). The group B streptococcal C5a peptidase gene (*scpB*), which is 97 to 98% similar to *scpA* (6), was found in more than 30 strains, representing all serotypes of group B streptococci (8). In the present study, an intranasal infection model was used to compare the capacities of strains devoid of SCPA to colonize the mouse nasopharynx. The protective immunogenicity of a recombinant, subunit SCPA49 protein, produced from a 2.9-kb fragment of *scpA* which originated from serotype M49 strain, was tested by using the same model. Other investigators have used this model to demonstrate that M protein is required for virulence (10) and that antibody against M protein can prevent lethal infection (1, 4). Our results show that SCPA is critical for group A streptococcal colonization of the mouse nasopharynx and that the recombinant ΔSCPA49 protein can induce serotype-independent immunity to group A streptococcal infection.

MATERIALS AND METHODS

Bacterial strains. Streptococcal strains CS101, CS210, and CS463 are spontaneous streptomycin-resistant derivatives of serum opacity-positive (OF^+), class II, serotype M49, M2, and M11 strains, respectively. MJ3-15 is strain CS101 with an internal in-frame deletion in the *scpA49* gene (18). Streptococcal strains

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90-131 and UAB200 are spontaneous streptomycin-resistant derivatives of OF$^-$, class I, serotype M1 and M6 human isolates of group A streptococci, respectively. Streptococci were cultured in Todd-Hewitt broth supplemented with 2% neopeptone or 1% yeast extract (THY) or on sheep blood agar. In some experiments, streptococci were grown in the culture medium containing streptomycin (200 μg/ml) or erythromycin (1 μg/ml). *Escherichia coli* ER1821 (New England Biolabs, Inc., Beverly, Mass.) was used as the recipient for the thermosensitive suicide vector, plasmid pG$^+$host5. pG$^+$host5 was obtained from Appligene, Inc., Pleasanton, Calif. *E. coli* ER1821 containing plasmid pG$^+$host5 was grown in Luria-Bertani broth containing erythromycin (300 μg/ml) at 39°C.

Construction of the *scpA* insertion mutants. The *scpA6* insertion mutant AK1.4 was constructed as described previously (18). Recombinant plasmid DNA, pG::scpA1.2, contains an internal *Bgl*II-*Hin*dIII fragment of the *scpA* gene. This plasmid was electroporated into UAB200 recipient cells, and transformants were selected on THY agar plates containing erythromycin at 30°C. A chromosomal integrant of pG::scpA1.2, strain AK1.4, which resulted from recombination between the plasmid insert and the chromosomal *scpA6* was selected by growth on agar medium containing erythromycin at 39°C. Insertion into *scpA6* was confirmed by Southern blotting using *scpA* as the probe and by PCR using an M13 universal primer (5′ GTAAAACGACG GCCAGT 3′), specific for the plasmid, and an *scpA* For835 primer (5′ AAGGACGACAC ATTGCGTA 3′), specific for the chromosomal *scpA* of group A streptococci.

Construction, expression, and purification of ΔSCPA. A 2.9-kb fragment of *scpA49* (from bp 1033 to 3941) was amplified by PCR using an *scpA* forward primer containing a *Bam*HI recognition sequence (5′ CCCCCCGGATCCACC AAAACCCCACAAACTC 3′) and an *scpA* reverse primer (5′ GAGTGGCCC TCCAATAGC 3′) (5). Sequences which code for the signal peptide and membrane anchor regions of the SCPA protein were deleted from the resulting PCR product. PCR products were digested with *Bam*HI and ligated to the thrombin recognition site of the glutathione *S*-transferase gene on the pGEX-4T-1 high-expression vector from Pharmacia Inc. (Piscataway, N.J.). The recombinant plasmid was transformed into *E. coli* DH5α. The ΔSCPA fusion protein from one transformant, *E. coli*(pJC6), was purified by affinity chromatography on a glutathione-Sepharose 4B column. Following digestion with thrombin, thrombin was removed by chromatography on a benzamidine-Sepharose 6B column. Methods of expression and purification are described by the manufacturer. This affinity-purified, truncated ΔSCPA protein lacked peptidase activity when tested by the polymorphonuclear leukocyte (PMN) adherence assay.

Western blot techniques. Mutanolysin extracts from streptococci were prepared as described previously (18). Briefly, 100 ml of a streptococcal overnight culture was pelleted and washed twice in ice-cold 0.2 M sodium acetate (pH 5.2). The pellet was suspended in 1 ml of TE-sucrose buffer (1 mM Tris, 1 mM EDTA, 20% sucrose) with 40 μl of mutanolysin. After rotation at 37°C for 2 h, the mixture was centrifuged for 5 min at 1,500 × *g*. Phenylmethylsulfonyl fluoride (100 mM) was added to the resulting supernatant. Western blotting was performed as previously described (18). Anti-SCPA antibody was prepared by immunization of rabbits with affinity-purified recombinant ΔSCPA protein.

PMN adherence and neutralization assays. SCPA activity was measured by using a PMN adherence assay. Recombinant human C5a (rhC5a; C5788; Sigma, St. Louis, Mo.) was incubated at 37°C for 45 min with whole bacterial cells. Residual rhC5a was measured by its ability to activate PMNs, which become adherent to bovine serum albumin (BSA)-coated wells of a microtiter plate (3). PMNs were isolated from fresh human blood by density gradient centrifugation in Ficoll-Hypaque (18). Neutralization of SCPA activity by rabbit anti-SCPA serum was assayed by using the PMN adherence assay. Approximately 10^7 heat-killed bacteria in 0.5% BSA–phosphate-buffered saline (PBS) were rotated with 1.4 ml of rabbit anti-ΔSCPA49 serum or normal rabbit serum for 1 h at 37°C. The bacteria were then resuspended in 40 μl of 0.5% BSA–PBS buffer and incubated with rhC5a for 45 min before residual chemotaxin was measured by the PMN adherence assay.

Phagocytosis assay. In vitro human blood phagocytosis assays were performed as described previously (19). Briefly, log-phase cultures of the group A streptococci were diluted in THY to 10^3 to 10^4 CFU/ml. One-tenth milliliter of diluted cultures and 0.9 ml of human blood were mixed and rotated at 37°C for 3 h. Initial viable counts and counts after 3 h of rotation were determined by plating diluted samples on blood agar.

Mouse intranasal infection model. Sixteen-hour cultures of challenge streptococcal strains (1×10^8 to 9×10^8 CFU), grown in Todd-Hewitt broth containing 20% normal rabbit serum and resuspended in 10 μl of PBS, were administered intranasally to 25-g female CD1 (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) or BALB/c (Sasco, Omaha, Neb.) mice. Viable counts were determined by plating dilutions of cultures on blood agar plates. Throat swabs were taken daily from anesthetized mice for 6 to 10 days after inoculation and streaked onto blood agar plates containing 200 μg of streptomycin per ml. After overnight incubation at 37°C, beta-hemolytic colonies on plates were counted. All challenge strains were marked by streptomycin resistance to distinguish them from beta-hemolytic bacteria which may persist in the normal flora. Throat swabs were cultured on blood agar containing streptomycin. The presence of one beta-hemolytic colony was taken to indicate a positive culture.

Immunization and challenge protocol. Four-week-old, outbred, CD1 female mice were immunized by administration of 20 μg of affinity-purified ΔSCPA49 in 10 μl of PBS into each nostril. Mice were immunized three times on alternating days and boosted again 3 weeks after the third immunization. After 2 weeks of rest, mice were again boosted (1). Control mice received only PBS. Prior to infection, all mice which were immunized with ΔSCPA protein were determined by enzyme-linked immunosorbent assay (ELISA) to have high titers of antibodies against ΔSCPA antigen in their sera and saliva. Group A streptococcal strains CS101 (2.0×10^8 CFU), CS210 (3.6×10^8 CFU), CS463 (7.8×10^8 CFU), 90-131 (3.4×10^8 CFU), and UAB200 (9.6×10^8 CFU) were used to intranasally challenge the mice 7 days after the last vaccine booster. Animal studies were performed according to National Institutes of Health guidelines.

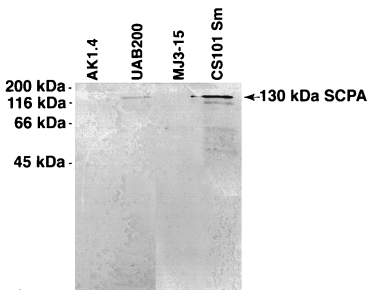

FIG. 1. Western blot analysis of SCPA proteins expressed by wild-type and mutant streptococci. Blots were exposed to 1:1,000 dilution of anti-ΔSCPA49 antibody. Bound antibody was detected as previously described (18).

Sample collection and ELISA. Blood and saliva samples were collected from anesthetized mice after immunization. All sera were tested for the presence of SCPA49 antibodies by ELISA as previously described (20). Purified SCPA49 protein was bound to microtiter wells by addition of 500 ng of purified protein in 0.05 M bicarbonate buffer (pH 9.6). After overnight incubation at 4°C, the wells were washed and then blocked with 0.5% BSA–PBS for 1 h. Salivation was stimulated in mice by injection of 100 μl of a 0.1% pilocarpine (Sigma) solution subcutaneously. Saliva samples were collected and spun at 14,000 rpm for 5 min in an Eppendorf microcentrifuge. The supernatants were tested for the presence of secretory immunoglobulin A (sIgA) against ΔSCPA49 protein by ELISA. ELISA titers represent the highest dilution of individual serum and saliva which had an optical density at 405 nm (OD_{405}) of ≥0.1.

Statistical analyses. The χ^2 test was used to analyze the data from animals experiments. A P value of <0.05 was considered significant.

RESULTS

Characterization of SCPA$^-$ mutants. The overall goal of this study was to compare the potentials of wild-type and isogenic SCPA$^-$ mutant streptococci to colonize the nasopharynges of mice. Strains UAB200 and AK1.4 represent the parent and SCPA$^-$ mutant, respectively, of an M6 strain. Strains CS101 and MJ3-15 are the parent and SCPA$^-$ mutant, respectively, of an M49 strain. Mutanolysin extracts of cell surface proteins from parent and mutant cultures were analyzed by Western blotting using SCPA-specific serum. Mutants were confirmed to lack SCPA (Fig. 1). Extracts of SCPA$^-$ mutants AK1.4 (Fig. 1, lane 1) and MJ3-15 (Fig. 1, lane 3) did not react with anti-SCPA serum. SCPA proteins of the expected sizes were observed in extracts from the wild-type strains CS101 and UAB200. Failure of mutant strains AK1.4 and MJ3-15 to produce C5a peptidase activity was verified by comparing their capacities to destroy rhC5a. Exposure of isolated PMNs to rhC5a induces them to become adherent to BSA-coated microtiter wells. Incubation with streptococci or purified SCPA specifically cleaves rhC5a and alters its potential to activate PMNs (18). PMNs that respond to residual rhC5a and bind to BSA-coated wells were stained and then measured spectrophotometrically. Incubation of rhC5a with parent cultures, UAB200 and CS101, destroyed rhC5a, which inhibited PMN adherence by 58.8 and 54.5%, respectively. In contrast SCPA$^-$ mutants AK1.4 and MJ3-15 did not alter rhC5a or adherence of PMNs to BSA-coated wells (Table 1). This experiment confirms the Western blot results and demonstrates that SCPA$^-$ cultures lack other proteases which might degrade rhC5a.

TABLE 1. Phagocytosis assay and PMN adherence assay of wild-type and mutant strains

Strain	Description	CFU/ml		Fold increase in CFU/ml	% Inhibition of C5a-induced PMN adherence[a]
		0 h	3 h		
UAB200	$M6^+$ $SCPA^+$	1.8×10^3	7.2×10^4	40	58.8
AK1.4	$M6^+$ $SCPA^-$	1.2×10^3	4.5×10^4	37.5	0
CS101	$M49^+$ $SCPA^+$	1.0×10^4	4.9×10^5	49	54.5
MJ3-15	$M49^+$ $SCPA^-$	1.5×10^4	2.1×10^5	14	0

[a] Percent inhibition = [(OD_{570} of PMNs activated by C5a alone − OD_{570} of PMNs activated by C5a preincubated with bacteria)/OD_{570} of PMNs activated by C5a alone] × 100.

Although M protein expression is not expected to be influenced by mutations in *scpA*, assays were performed to assess whether $SCPA^-$ mutant streptococci still expressed M protein and had the ability to resist phagocytosis. Growth of streptococci in fresh human blood during 3 h of incubation is indicative of antiphagocytic M protein on the cell surface (19). As expected, levels of parent streptococcal strains UAB200 and CS101 increased 40- and 49-fold, respectively (Table 1). Levels of the M^+ $SCPA^-$ cultures, strains AK1.4 and MJ3-15, increased 37.5- and 14-fold, respectively, confirming that *scpA* mutations had little effect on M protein expression or resistance to phagocytosis in whole human blood. The somewhat poorer growth of both mutant strains in rotated blood was reproducible and unexpected. The growth rates of mutant and parent cultures in human plasma were indistinguishable. It is possible that inactivation of SCPA allows C5a to accumulate in rotated blood, which in turn activates PMNs. Activated PMNs are more phagocytic and better able to kill M^+ streptococci. Surface protein extracts contain M6 and M49 antigen when analyzed by Western blotting using anti-M49 and anti-M6 antisera (unpublished data), confirming that mutations in SCPA did not alter M protein expression.

The C5a peptidase is required for nasopharyngeal colonization. CD1 outbred mice were inoculated intranasally to evaluate the relative capacity of wild-type and $SCPA^-$ streptococci to colonize the nasopharynx. Throat swabs were taken daily for 1 to 10 days from anesthetized mice and streaked onto blood agar plates containing antibiotics selective for the strain in question. BALB/c mice were used for the M6 strain UAB200 infections in order to conform with earlier studies using this strain (1). The inoculum size which results in colonization of approximately 50% of the mice for up to 5 days was first determined (unpublished data). Significant differences between M^+ $SCPA^+$ and M^+ $SCPA^-$ type 49 streptococci were observed on days 3 to 9 after inoculation (Table 2). By day 4, 50% (9 of 18) of mice infected with strain CS101 still produced positive throat cultures, whereas only 11% (2 of 18) of mice infected with MJ3-15 retained streptococci in their throats. Differences in the number of colonies on the blood agar plates were also consistent with more rapid clearance of M^+ $SCPA^-$ streptococci. We found that 59% (31 of 54) of positive cultures from mice infected with wild-type streptococci contained more than 100 CFU, whereas only 14% (2 of 14) of positive cultures from animals infected with $SCPA^-$ streptococci contained more than 100 CFU (statistically significant to $P < 0.001$). Moreover, 22% (4 of 18) of the mice died from infection with M^+ $SCPA^+$ streptococci. None of the mice died from infection with M^+ $SCPA^-$ streptococci. This difference is also statistically significant ($P < 0.05$). Comparison of $SCPA49^+$ and $SCPA49^-$ variants of strain CS101 was repeated two more times with similar results (data not shown).

Because the spectrums of the diseases caused by OF^+ and OF^- strains differ significantly, the impact of SCPA on colonization by an OF^- serotype, M6, was also investigated (Fig. 2). Again the $SCPA6^-$ strain AK1.4 was cleared more rapidly than the parent strain UAB200. Four days after infection, all mice had completely cleared $SCPA^-$ streptococci from the throat, whereas 30% of mice infected with wild-type streptococci remained culture positive. Greater than 98% of all positive cultures had more than one colony on the blood agar plate. In this experiment, all mice were free of streptococci by day 5 postinoculation.

Construction and production of recombinant ΔSCPA49 immunogen. An enzymatically inactive form of SCPA49 protein was constructed for use in immunization studies. A 2,908-bp *scpA49* fragment with additional *Bam*HI recognition sequences was obtained by PCR and ligated into plasmid pGEX-4T-1, which had been digested with *Bam*HI and *Sma*I. In this construct, plasmid pJC6, the *scpA49* sequence was fused in frame to the glutathione *S*-transferase gene. The streptococcal insert did not include the *scpA* signal or cell wall anchor sequences (Fig. 3). Vaccine preparations of purified ΔSCPA49 protein were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting to confirm purity. Several protein bands in the purified ΔSCPA49 preparation reacted with polyclonal rabbit antiserum directed against recombinant ΔSCPA49 protein (Fig. 4). The size of the major band was approximately 100 kDa, the estimated size of the deletion form of SCPA49. Smaller bands were assumed to be degradation products of SCPA, a common feature of overexpressed proteins in *E. coli*. The antiserum did not react with any protein isolated from *E. coli* DH5α(pGEX-4T-1) without a streptococcal insert. The procedure described in Materials and Methods routinely yielded 2 to 3 mg of highly pure ΔSCPA49 protein from 1 liter of culture. The purified ΔSCPA49 protein was found to lack C5a peptidase activity when assayed by the PMN adherence assay (data not shown).

Immunogenicity of ΔSCPA49. Rabbits were immunized with purified ΔSCPA49 to evaluate the immunogenicity of the subunit ΔSCPA49 vaccine. The rabbits developed high levels of antibodies against ΔSCPA49 protein as determined by ELISA (data not shown). Although the purified ΔSCPA49 immunogen lacked functional activity, hyperimmune rabbit antiserum could neutralize the peptidase activity of purified wild-type SCPA49 enzyme in vitro. Moreover, undiluted rabbit antiserum against ΔSCPA49 protein was able to neutralize C5a peptidase activity associated with different serotypes. C5a peptidase activity associated with intact M1, M6, and M12 streptococci was inhibited by this antiserum, confirming that antibody against ΔSCPA49 protein lacks serotype specificity (Fig. 5).

Systemic and mucosal antibody responses in mice. Serum and saliva samples were obtained from 10 immunized and 10 control mice to assess the immunogenicity of ΔSCPA49 pro-

TABLE 2. Colonization of wild-type M49 streptococci and the SCPA$^-$ mutant in throats of infected mice

Strain	Mouse no.	CFU/ml on indicated day postinoculation									
		1	2	3	4	5	6	7	8	9	10
CS101 (SCPA$^+$)	1	>200	>200	133	0	0	0	0	0	0	0
	2	>200	2	4	0	0	3	>200	>200	Dead	
	3	>200	>200	>200	133	152	86	16	0	2	0
	4	5	71	6	156	41	28	103	>200	>200	Dead
	5	5	3	0	0	0	0	0	0	0	0
	6	>200	0	0	0	0	0	0	0	0	0
	7	14	0	0	17	35	3	0	0	0	0
	8	>200	117	16	4	0	0	0	0	0	0
	9	82	0	0	0	0	0	0	0	0	0
	10	10	48	5	3	0	0	0	0	0	0
	11	94	>200	>200	>200	2	0	0	0	0	0
	12	12	>200	>200	>200	15	96	>200	>200	Dead	
	13	90	>200	>200	Dead						
	14	>200	90	>200	141	146	>200	>200	>200	>200	>200
	15	>200	>200	96	>200	58	164	125	>200	>200	6
	16	>200	27	9	0	0	0	0	0	0	0
	17	167	31	184	0	0	0	0	0	0	0
	18	>200	>200	18	0	0	0	0	0	0	0
No. positive		18	14	14	9	7	7	6	5	4	2
MJ3-15 (SCPA$^-$)	1	1	1	0	0	0	0	0	0	0	0
	2	>200	0	0	0	0	0	0	0	0	0
	3	53	20	20	0	0	0	0	0	0	0
	4	39	10	0	0	0	0	0	0	0	0
	5	>200	177	3	0	0	0	0	0	0	0
	6	9	30	0	0	0	0	0	0	0	0
	7	145	>200	3	0	0	0	0	0	0	0
	8	101	4	0	0	0	0	0	0	0	0
	9	50	136	0	0	0	0	0	0	0	0
	10	3	101	>200	>200	0	0	0	0	0	0
	11	>200	33	15	2	82	6	23	0	0	0
	12	>200	143	0	0	0	0	0	0	0	0
	13	5	0	0	0	0	0	0	0	0	0
	14	129	143	6	0	0	0	0	0	0	0
	15	28	>200	4	0	0	0	0	0	0	0
	16	172	0	0	0	0	0	0	0	0	0
	17	0	0	0	0	0	0	0	0	0	0
	18	11	0	19	0	14	0	0	0	0	0
No. positive		16	12	8[a]	2[b]	2[a]	1[a]	1[a]	0[b]	0[a]	0

[a] Significant difference at $P < 0.05$ by χ^2 analysis.
[b] Significant difference at $P < 0.01$ by χ^2 analysis.

tein when administered via the intranasal route without adjuvants. Mice which were immunized with purified ΔSCPA49 protein developed high titers of SCPA-specific IgG in their sera compared to control mice immunized with PBS. Titers of serum IgG directed against ΔSCPA49 ranged from 1:10,240 to 1:20,480. In contrast, the SCPA49-specific IgG titer for control mice was not detectable in sera. Mice immunized with purified ΔSCPA49 protein also showed a significant increase in SCPA49-specific salivary sIgA relative to control mice. Specific sIgA titers in saliva of immunized mice were greater than 1:16. In contrast, sIgA directed against SCPA49 in the saliva of control mice was not detectable. The concentrations of IgG and sIgA in serum diluted 1/2,560 and saliva diluted 1/2, respectively, are shown in Fig. 6. These results demonstrate that purified ΔSCPA49 protein is an effective immunogen for the induction of specific systemic and secretory antibody responses in mice when administered intranasally. Assays were performed with homologous antigen in microtiter wells. Whether these antibodies have equal affinities for antigen from other serotypes is under investigation.

Immunization with the C5a peptidase enhances clearance of streptococci from the nasopharynx. Both hyperimmune rabbit and human sera (20) which contain high levels of anti-SCPA antibody can neutralize SCPA activity in vitro. The fact that SCPA significantly facilitates colonization of the oral mucosa suggests that immunization of mice with purified ΔSCPA49 could reduce the capacity of streptococci to colonize the nasopharynx. Mice were immunized intranasally with affinity-purified, genetically inactivated SCPA to test this possibility. The truncated protein, ΔSCPA49, was administered intranasally without adjuvants or carriers. Pharyngeal colonization of vaccinated mice by wild-type M$^+$ SCPA$^+$ streptococci differed significantly from that of mice immunized with PBS in three independent experiments using mice vaccinated with two different preparations of purified ΔSCPA49 protein (representative data are shown in Fig. 7). Only 1 of 13 mice immunized with ΔSCPA49 protein was culture positive for streptococci 10 days after inoculation (Fig. 7). In contrast, 30 to 58% of the nonvaccinated controls remained culture positive for 6 days, and some were still positive 10 days after infection. The num-

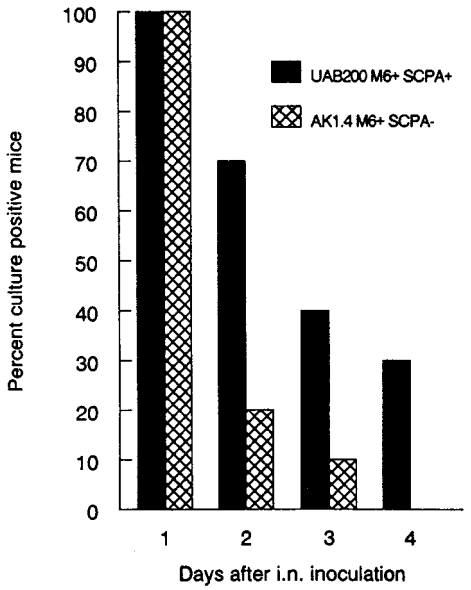

FIG. 2. Comparison of the abilities of SCPA$^-$ mutant and wild-type group A streptococci to colonize mice following intranasal (i.n.) infection. BALB/c mice (10 in each experimental group) were inoculated with 2×10^7 CFU of M6 streptococci. Throat swabs were cultured each day on blood agar plates containing streptomycin. Mice were considered positive if plates contained one beta-hemolytic colony. Data were analyzed statistically by the χ^2 test.

bers of beta-hemolytic, streptomycin-resistant colonies on blood agar plates also showed a significant difference between ΔSCPA49-vaccinated and control mice. Different sets of immunized mice cleared serotype M49 streptococci significantly more rapidly from the nasopharynx than nonimmunized controls.

Cross-protection against serotype M2, M11, M1, and M6 group A streptococcal colonization. An effective vaccine must prevent infection by more than 80 different serotypes of group A streptococci. The fact that rabbit serum directed against ΔSCPA49 protein from serotype M49 streptococci neutralizes peptidase activity associated with several serotypes suggested that intranasal immunization with a single-subunit vaccine might reduce or eliminate pharyngeal colonization by those serotypes. To explore this possibility, four groups of 20 mice were immunized by intranasal inoculation with affinity-purified ΔSCPA49 protein as described above. Control mice received

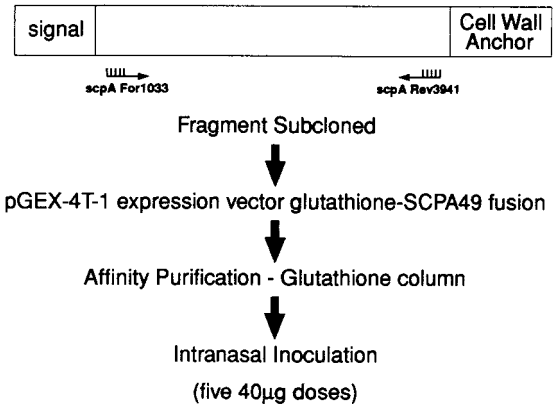

FIG. 3. Construction of ΔSCPA49 vaccine and immunization protocol.

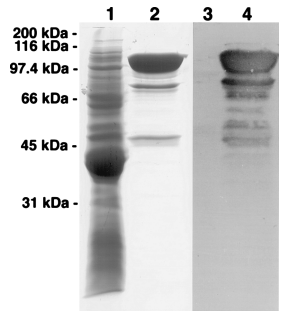

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis of purified ΔSCPA49 protein. Lanes 1 and 3 contain sonicates of *E. coli* DH5α(pGEX-4T-1) without an *scpA49* insert. Lanes 2 and 4 contain affinity-purified ΔSCPA49. Lanes 1 and 2 are stained with Coomassie blue, and lanes 3 and 4 were exposed to anti-SCPA49 which was detected with goat anti-rabbit IgG-alkaline phosphatase.

PBS. Prior to being challenged with streptococci, serum and saliva samples from randomly chosen, immunized and control mice were assayed for anti-SCPA antibody. All immunized mice tested had developed a strong serum and measurable salivary antibody response (data not shown). Pharyngeal colonization of mice immunized with ΔSCPA49 protein by strains of all four serotypes was reduced relative to that of nonimmunized controls. Differences were most significant on days 3 and 5 after inoculation (Table 3). Statistically significant differences were observed between immunized and control mice inoculated with serotype M2, M11, and M1 strains. However, the OF$^+$ M2 and M11 strains were more efficiently eliminated by immunized mice than were the OF$^-$ M1 and M6 strains. M1 streptococcal colonization of immunized mice was significantly reduced relative to that of control mice. Only 10.5% of the immunized mice were culture positive by day 5 postinfection. In contrast, 37% of the control mice were culture positive with this strain. Although immunized mice appeared to clear M6 streptococci more rapidly, the differences were not statistically significant. As in previous experiments, there were significantly fewer beta-hemolytic streptococcal colonies on blood agar plates in samples taken from vaccinated mice than in those taken from control animals (data not shown).

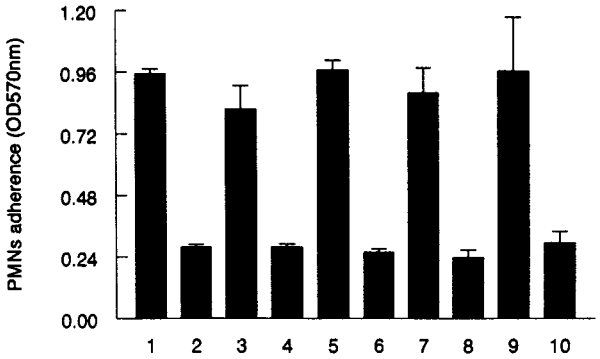

FIG. 5. Rabbit antibody neutralizes SCPA activity associated with different serotypes. Lane 1 is a positive control and contained rhC5a which was not preincubated before exposure to PMNs. Whole, intact bacteria, preincubated with normal rabbit serum (bar 2, M1 90-131; bar 4, M6 UAB200; bar 6, M12 CS24; bar 8, M49 CS101) or preincubated with rabbit anti-SCPA49 serum (bar 3, M1 90-131; bar 5, M6 UAB200; bar 7, M12 CS24; bar 9, M49 CS101), were incubated with 20 μl of 5 μM rhC5a for 45 min. Residual rhC5a was assayed by its capacity to activate PMNs to adhere to BSA-coated microtiter plate wells. Adherent PMNs were stained with crystal violet.

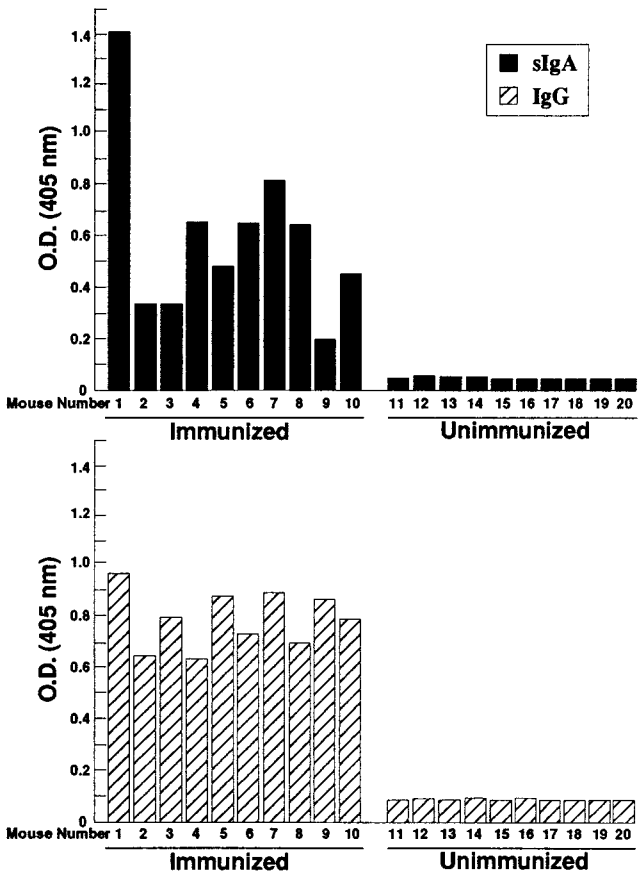

FIG. 6. Serum IgG and sIgA responses after intranasal immunization of mice with the purified ΔSCPA49 protein. Serum and saliva levels of SCPA49-specific IgG were determined by indirect ELISA. Serum from each mouse was diluted to 1:2,560 in PBS; saliva was diluted 1:2 in PBS.

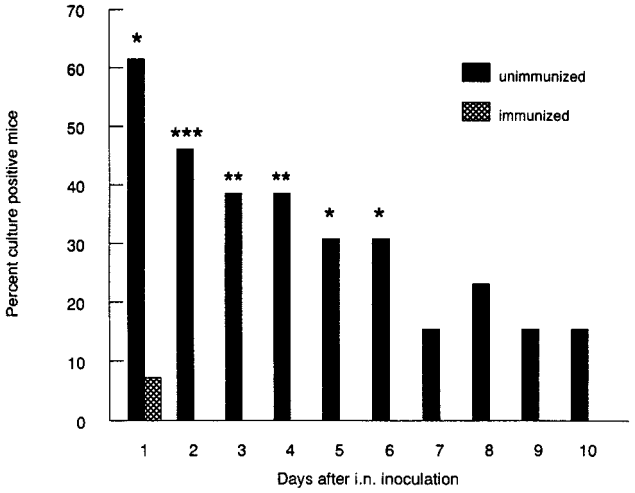

FIG. 7. Comparison of the abilities of serotype M49 streptococci to colonize immunized and nonimmunized CD1 female mice. Each experimental group contained 13 mice which were infected intranasally (i.n.) with 2.0×10^8 CFU. The data were analyzed statistically by the χ^2 test. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

DISCUSSION

The early inflammatory response can be initiated by the accumulation of the complement chemotaxin C5a in tissue. Neutrophil chemoattractants are generated in two stages: activation of the complement pathways produces C5a, which attracts neutrophils that in turn produce interleukin-8, a second chemokine which further amplifies the inflammatory response (17). We previously proposed that group A streptococci modulate early inflammatory events by eliminating C5a at the surface of streptococci where it is generated (18, 26). This provides an opportunity for streptococci to become locally established by delaying the inflammatory response. Mutations were introduced into *scpA* by gene replacement or plasmid insertion to test this model. Mutant streptococci were constructed from two serotypes, M49 and M6, which represent OF^+ and OF^- strains, the two primary lineages of group A streptococci, respectively. OF^+ serotypes, such as the M49 strain CS101, are associated with skin infections and glomerulonephritis, whereas OF^- serotypes, such as the M6 strain UAB200, are more often associated with pharyngitis, scarlet fever, and rheumatic fever (2). OF^+ and OF^- strains are also distinguished by differences in their *vir* gene clusters. The M6 strain contains only *emm6* and *scpA6* genes, whereas the M49 cluster contains *fcrA49*, *emm49*, *enn49*, *scpA49*, and other unlinked genes (14, 15). The strains used in this study, UAB200 and CS101, differ in other ways which may influence their capacity to colonize the nasopharynx. The M6 protein alone is sufficient for strain UAB200 to resist phagocytosis in whole blood, whereas CS101 requires other factors in addition to the M49 protein to resist phagocytosis (unpublished data and reference 22). In stationary-phase cultures of strain CS101, most SCPA protein is associated with the bacterial surface. In contrast, a large fraction of SCPA produced by strain UAB200 is present in the culture supernatants of stationary-phase cultures (unpublished data).

Mouse C5a is proteolytically destroyed by SCPA. Therefore, we used two different mouse models to test its impact on virulence (18). We showed that mutations in *scpA* reduce clearance and trafficking of streptococci from subdermal sites of infection to lymph nodes and increase the influx of PMNs and other mononuclear phagocytes to the site of infection (18). The experiments reported here investigated the role of SCPA in colonization of the oral mucosa. Intranasal inoculation of mice (1, 4) and rats (16) with group A streptococci has been used by several investigators to evaluate virulence and the protective potential of antibody induced by M protein. Although mice lack platine tonsils, and their oral lymphoid tissues are poorly developed, this in vivo model has confirmed many in vitro predictions. In most studies, streptococci were first multiply passaged in mice to select for variants which are more mouse virulent. Mouse-passaged strains become highly encapsulated (25) and may express a spectrum of M-like pro-

TABLE 3. Immune protectivity is serotype independent

Serotype	Day 3 after inoculation				Day 5 after inoculation			
	Nonimmune		Immune		Nonimmune		Immune	
	+[a]/total	%	+/total	%	+/total	%	+/total	%
M2	10/19	52.6	2/19[b]	10.5	3/19	15.8	1/19	5.2
M11	17/20	85	11/20[b]	55	8/20	40	2/20[b]	10
M1	16/19	84.2	11/19	57.9	7/19	37	2/19[b]	10.5
M6	14/20	70	12/19	63.2	8/20	40	4/19	21.1

[a] +, culture-positive mice.
[b] Differences between immunized and nonimmunized mice are statistically significant ($P < 0.05$). *P* values were calculated by χ^2 analysis.

teins different from that of the original culture (24). Therefore, initial experiments used cultures which were not mouse passaged to avoid these potential confounding variables. Colonies on blood agar plates from mouse throat cultures appeared highly mucoid, in agreement with the findings of Wessels et al. (25), again suggesting that the hyaluronic acid capsule also plays a critical role in persistence by group A streptococci in mice. The presence or absence of capsule is not known to influence cell-associated SCPA activity. Again as predicted by our model, both SCPA49$^-$ and SCPA6$^-$ streptococci were cleared more rapidly from the nasopharynx than were their wild-type parent strains.

The importance of M protein for initial colonization was first questioned by studies in rats (16). Our data also provide reason to doubt the importance of M protein and resistance to phagocytosis in colonization. Both SCPA$^-$ cultures and their parents produce M protein and resist phagocytosis in vitro, yet the mutants are more readily cleared from both subdermal (18) and nasopharyngeal sites of infection. Moreover, blocking M protein production by mutation in these strains did not significantly reduce their capacity to colonize mice (unpublished data). In vitro resistance to phagocytosis appears to be less important in these animal models. One possible explanation for this contradiction is that tissue phagocytes, i.e., macrophages and Langerhans cells, may be more important for clearance of streptococci locally than PMNs, and M protein is unable to prevent uptake of streptococci by these phagocytes. The fact that peritoneal macrophages can engulf and destroy M$^+$ streptococci is consistent with this explanation (13). The relative roles of SCPA and M protein are presently under investigation.

The findings presented above prompted us to explore the possibility that antibody against SCPA could neutralize peptidase activity, preserve and possibly amplify the C5a mediated inflammatory response, and prevent colonization of the nasopharyngeal mucosa by group A streptococci. A truncated peptide which lacked the signal sequence and membrane anchor domains of SCPA proved to be highly immunogenic and induced strong salivary sIgA and serum IgG responses when administered intranasally to mice. Measurements of anti-SCPA49 sIgA in saliva revealed titers greater than 1:16. These measurements underestimate the actual concentration of specific antibody because mice were induced to salivate, and therefore immunoglobulins present in normal mucosal secretions were diluted. Presence of SCPA-specific sIgA in saliva is correlated only with protection. These experiments do not demonstrate that protection is dependent on the secretory antibody response. Protection may not be exclusively associated with the capacity to neutralize SCPA activity. Our data cannot be compared to those of Bronze et al. (4) and Bessen and Fischetti (1) because they used adjuvant to augment the immune response. Moreover, Bronze et al. (4) concentrated the immunoglobulin fraction in saliva before specific antibody was quantitated. The high serum titers were somewhat surprising but may have been induced by antigen inhaled into the lung.

Intranasal immunization of mice dramatically reduced the capacity of the homologous M49 strain to colonize the nasopharynx beyond 2 days after intranasal inoculation. The M49 strain was used to challenge three different sets of mice, vaccinated with two different preparations of affinity-purified ΔSCPA49. Protection was observed in each case. Although the degree of protection is comparable to that reported by Bessen and Fischetti (1), the results cannot be compared directly. These investigators immunized with synthetic peptide which corresponded to a segment of the C repeats of M protein and used the B subunit of cholera toxin as a carrier. Furthermore, their mice consumed streptomycin as a means to select for the challenge strain. Experiments reported by Bronze et al. are also not comparable because they used mouse-passaged cultures which are highly virulent for mice relative to the strains used in this study. This permitted these investigators to use death as the endpoint in their studies (4).

Clearance of streptococci from immunized animals appeared to be more rapid from mice with the highest antibody titers, although this conclusion is tentative because antibody titers were assessed only on a random sample of immunized mice (unpublished observation). We are also unable to conclude from our data whether secretory or serum antibodies are a more important deterrent to colonization. We assume that neutralization of peptidase activity on the streptococcal surface permits a more vigorous recruitment of granulocytic phagocytes to the mucosal surface and activation of phagocytes by C5a. It is also possible, however, that anti-SCPA antibody enhances activation of the complement pathway, which in turn amplifies a more effective inflammatory response.

Serum from rabbits vaccinated with this protein contained antibody which was able to neutralize the peptidase activity associated with several serotypes, confirming early experiments which showed that human sera with unknown specificity could neutralize SCPA activity (20). Lack of serotype specificity is expected because C5a peptidase genes from serotype M12 (5) and M49 (21) group A streptococci and group B streptococci are 97 to 98% identical in sequence (6). The potential of hyperimmune rabbit serum to neutralize peptidase activity associated with multiple serotypes prompted us to test whether immunization of mice with a single antigen would prevent colonization by several serotypes. Four other sets of mice were immunized with ΔSCPA49, and each was challenged with a different serotype of group A streptococci. Significant protection from colonization by M2, M11, and M1 cultures was observed. Protection against M6 streptococci was also apparent, but the differences between immunized and nonimmunized mice were smaller and less statistically significant. We attribute the poorer protection against the M6 strain to strain variation and the fact that this strain is less able to persist in nonimmunized mice. None of the strains used in these experiments were particularly mouse virulent; therefore, large inocula were required to colonize nonimmunized mice, a variable which may dampen the impact of antibody in mucosal secretions. The amino acid sequence of SCPA49 protein from an OF$^+$ serotype is 3% different from that of SCPA12 from an OF$^-$ serotype. It is possible that this small difference accounts for the lesser cross-protection against heterologous strains.

The foregoing experiments confirm that the streptococcal C5a peptidase is critical for efficient colonization of the nasopharynx in mice. Immunization experiments suggest that in the absence of neutralizing antibody, granulocytes initially ignore SCPA$^+$ streptococci, creating a period of time for the bacteria to become locally established. Alternatively, if the host has antibody in salivary secretions which can neutralize the peptidase, then phagocytes are recruited which clear the inoculum before mucosal tissues become colonized. Streptococci are cleared if they lack SCPA activity, whether eliminated by mutation or neutralized by antibody in serum and mucosal secretions. Experiments described here encourage us to further investigate SCPA as a vaccine candidate. More than 40 serotypes of group A streptococci are known to produce antigenically similar C5a peptidases. Sequence similarity between peptidases suggests that intranasal administration of SCPA may induce immunity across group A streptococcal serotypes and to other human beta-hemolytic streptococcal pathogens.

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