New protective antigen of group A streptococci

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It is widely believed that the surface M protein of group A streptococci is the predominant surface protein of these organisms containing opsonic epitopes. In the present study, we identified a new surface protein, distinct from M protein, that evokes protective antibodies. A type 18 M-negative mutant was found to be both resistant to phagocytosis in human blood and virulent in mice. The wild-type strain, but not the M-negative mutant, was opsonized by antisera against purified recombinant M18 protein or a synthetic peptide copying the NH$_2$-terminus of M18. However, antisera raised against a crude pepsin extract of the M-negative mutant opsonized both strains, indicating the presence of a protective antigen in addition to type 18 M protein. This antiserum was used to identify and purify a 24-kDa protein fragment (Spa, streptococcal protective antigen) that evoked antibodies that opsonized the M18 parent and the M-negative mutant. The results of passive mouse protection tests confirmed the presence of protective epitopes within Spa. The deduced amino acid sequence of a 636-bp 5′ fragment of the *spa*18 gene showed no homology with sequences in GenBank. These studies reveal the presence of a new protective antigen of certain strains of group A streptococci that may prove to be an important component of vaccines to prevent streptococcal infections.

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Introduction

Group A streptococcal infections cause a wide array of clinical syndromes; these range from uncomplicated pharyngitis and pyoderma to serious, invasive infections and toxic shock syndrome. Protection against infection is largely mediated by antibodies against the surface M protein of the organisms. The M protein is an α-helical, coiled-coil molecule that extends from the surface with its hypervariable NH$_2$-terminus exposed to the outside and its conserved COOH-terminus buried in the cytoplasm (1). The NH$_2$-terminus contains type-specific epitopes evoking bactericidal antibodies that correlate with protection against the homologous serotype (2). The *emm* gene is located in a regulon controlled by the upstream positive regulator *mga* (3). Depending on the serotype, the regulon may contain 1, 2, or 3 *emm* and *emm*-like genes (4). In serotypes containing only 1 *emm* gene, deletion (5) or interruption (6) of the *emm* gene results in an avirulent organism that can no longer resist phagocytosis. In serotypes expressing several *emm*-like genes, each may partially contribute to phagocytosis resistance (7), but among the many defined surface proteins of group A streptococci, only antibodies against the M protein have been shown to be opsonic.

In this study, we identify a new surface antigen, streptococcal protective antigen (Spa) of group A streptococci, that evokes protective antibodies. A fully virulent M-negative mutant of type 18 streptococci was opsonized by rabbit antiserum raised against a crude pepsin extract of the M-negative mutant. This antiserum was used to identify and purify the new protective antigen, Spa. Anti-serum against the purified Spa protein was bactericidal against both the type 18 parent strain and, importantly, the M-negative mutant. Passive mouse protection tests using rabbit antisera raised against purified Spa confirmed that the new surface protein contained protective epitopes. In addition, the anti-Spa antibodies opsonized type 3 and type 28 streptococci, indicating that this new surface protein is a cross-protective antigen for certain strains of group A streptococci.

Methods

Bacterial strains. The parent type 18 streptococcal strain 87-282, has been the subject of previous studies in this laboratory (8) and others (9) and was obtained from Patrick Cleary (University of Minnesota, Minneapolis, Minnesota, USA). The M3 strain (3375) was provided by James Musser (Baylor College of Medicine, Houston, Texas, USA). The M28 strain (S2356) was from our laboratory collection.

Reagents. The preparation of rabbit antisera against the synthetic peptide SM18(1-30)C has been described previously (8). Antisera were also raised against recombinant M18 that was purified from periplasmic extracts of *Escherichia coli*, as previously described (8, 10). Rabbit antisera were similarly prepared against purified Spa (8). A peptide copying the NH$_2$-terminal 23 amino acid residues of Spa [S-Spa18(1-23)C] was commercially synthesized (Research Genetics Inc., Huntsville, Alabama, USA) and chemically coupled to KLH by methods described previously (11). Antisera against S-Spa18(1-23)C-KLH were raised in rabbits according to our standard, published protocol (11).

Insertion of the Ω-interposon into emm18 and transformation of type 18 streptococci. Insertional inactivation of *emm*18 was accomplished essentially as described previously for type 24 streptococci (6). Briefly, *emm*18 was ligated into pKK223-3 and then

Table 1

Growth of M18 and M18Ω streptococci in human blood

Organism		CFU surviving 3-hour rotation[A] (no. of generations)	
	Inoculum:	45	15
M18		12,550 (8.1)	4,800 (8.3)
	Inoculum:	55	20
M18Ω		9,940 (7.3)	4,210 (7.6)

[A]Streptococci were grown to early log-phase in Todd-Hewitt broth, and the inoculum indicated was added to 0.45 mL normal, heparinized human blood that did not contain M18 antibodies. The mixture was rotated end-over-end at 37°C for 3 hours. Surviving organisms were quantitated on pour plates of sheep blood agar using an aliquot of the mixture. The experiment was performed on 3 separate occasions, and the results shown are from a representative experiment. Although the absolute number of CFU varied somewhat from one experiment to another, growth in blood, as determined by the number of generations, was comparable.

cut with *Xho*I, which recognizes a single site between bases 136 and 141 of the *emm*18 gene. The ends were repaired with a Klenow fragment (12). pBR322-ΩKm2 was digested with *Sma*I, and the ΩKm2 fragment was purified from a 0.8% agarose gel and ligated into the cut *emm*18 gene (12). The resulting plasmid, pKKM18Ω, was electroporated into type 18 streptococci as described (6). One kanamycin-resistant colony, designated M18Ω, was selected for further study.

Southern blots. Streptococcal chromosomal DNA was digested with *Bsa*HI and electrophoresed in a 1% agarose gel. The DNA fragments were transferred to a nylon membrane (Sigma Chemical Co., St. Louis, Missouri, USA) and probed with digoxigenin-labeled ΩKm2 fragment or *emm*18 according to instructions provided by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, Indiana, USA).

Western blots. Western blots were performed using extracts of intact streptococci and purified proteins as described previously (13). In some experiments using purified recombinant Enn18 protein, the nitrocellulose membranes were first incubated in buffer containing 10% normal human serum to block nonspecific binding of immunoglobulins.

Cloning and sequencing enn18. The type 18 strain (87-282) used for these studies contains an M-like gene, *enn*18, downstream from the *emm*18 gene (4). Although Enn18 has been shown to be expressed only in low levels by this strain of group A streptococci (14), we cloned the *enn*18 gene and purified the recombinant protein from extracts of *E. coli*. The purified Enn18 was used in subsequent experiments to determine whether Spa antibodies cross-reacted with this additional surface protein. The *enn*18 gene was initially cloned by PCR using a forward primer that copied the 3′ end of the *emm*18 gene and a reverse primer that was specific for the SF3 subfamily of *emm*-like genes (4). The purified PCR product was ligated into pKK223-3, and the insert was sequenced by standard methods (12). The intact *enn*18 gene was later cloned using a reverse primer that copied the 5′ end of the *scp*A gene (15), and the purified PCR product was ligated into pQE-30 (QIAGEN Inc., Chatsworth, California, USA). The recombinant Enn18 protein was purified by affinity chromatography over a nickel column according to the instructions provided by QIAGEN Inc.

Opsonization and bactericidal assays. In vitro opsonization assays and bactericidal assays were performed using nonimmune whole human blood as described previously (16). Briefly, rotation mixtures contained 0.1 mL of test antiserum, a standard inoculum of streptococci, and 0.4 mL of heparinized (10 U/mL) human blood. For opsonization assays, the number of streptococcal CFUs per leukocyte in the mixture was approximately 10. The tubes were rotated end-over-end for 45 minutes at 37°C; smears were then made on glass slides and stained with

Wright's stain. Opsonization was quantitated by counting 50 neutrophils per slide and calculating the percentage with associated streptococci (percent opsonization). The bactericidal activity of test antisera was determined using a similar assay except that fewer streptococci were added to the mixture, which was rotated for 3 hours at 37°C. At the end of the rotation, 0.1-mL aliquots were added to sheep blood agar, and pour plates were made to quantitate viable bacteria.

Determination of LD$_{50}$. A comparison of the mouse virulence of the M18 parent strain and the M18Ω mutant was made after intraperitoneal challenge infections of Swiss white mice. Four groups of 6 mice each were challenged with 10-fold increasing inocula, ranging from 2.7×10^4 to 2.7×10^7 CFU of either M18 or M18Ω. Deaths were recorded for 7 days after challenge infections. The LD$_{50}$ was determined by the method of Reed and Muench (17).

Passive mouse protection tests. Eight-week-old female white Swiss mice were given 0.5 mL of rabbit antiserum via the intraperitoneal route (2). After 24 hours, they were challenged using the same route with the indicated number of streptococci that had been grown to early log-phase in Todd-Hewitt broth. Deaths were recorded twice daily for 7 days.

Extraction and purification of Spa from pepsin extracts of M18Ω. Extraction of peptide fragments from the surface of M18Ω was accomplished using dilute solutions of pepsin at suboptimal pH, as described previously for the extraction of pep M proteins (18). The extract was precipitated in 60% saturated ammonium sulfate, dialyzed extensively against distilled water, and then lyophilized. The mixture of surface proteins and peptides is referred to as crude pep M18Ω.

Spa was identified in the crude pepsin extract by opsonization inhibition assays. The crude pep M18Ω was separated by SDS-PAGE on a preparative 10% gel using reducing conditions. The entire gel was electroblotted to nitrocellulose paper, each end was cut vertically, and then these pieces were stained Coomassie brilliant blue to identify the protein bands. The center section of the nitrocellulose paper was cut into horizontal strips approximately 8–10 mm wide. Each strip was then used to absorb rabbit antiserum raised against the crude pep M18Ω for 2 hours at 37°C. Opsonization assays were performed using absorbed serum samples and unabsorbed serum, as described above. Once the opsonic inhibitory peptide had been identified
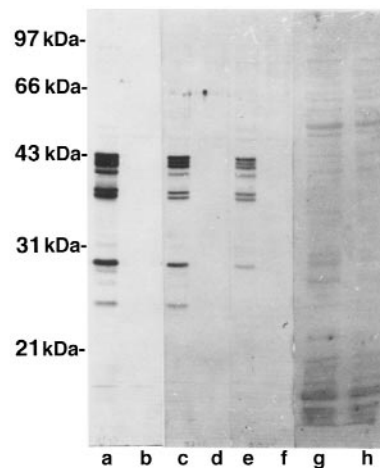
Figure 1

Western blot analysis of whole cell extracts of M18 (lanes **a**, **c**, and **e**) and M18Ω (lanes **b**, **d**, and **f**) reacted with rabbit antisera against rM18 (lanes **a** and **b**), SM18(1-30) (lanes **c** and **d**), and SM5(265-291) (lanes **e** and **f**). Coomassie blue–stained multiple proteins in extracts from both strains (M18, lane **g**; and M18Ω, lane **h**).

Table 2

Opsonization of M18 and M18Ω by rabbit antisera against recombinant M18, S-M18(1-30), and crude pep M18

Antiserum	Percent neutrophils with associated streptococci[A] (± SEM)	
	M18	M18Ω
Preimmune pool	0.7 ± 0.7	13.3 ± 1.3
Anti–crude pep M18	98.7 ± 0.7	94.0 ± 1.2
Anti-rM18	94.7 ± 0.7	15.3 ± 0.7
Anti–SM18(1-30)-KLH	95.3 ± 0.7	12.7 ± 0.7
Anti–SM5(1-15)-KLH	0.7 ± 0.7	15.3 ± 0.7

[A]Early log-phase cultures of streptococci were added to 0.1 mL of test serum and incubated for 15 minutes at ambient temperature. To this was added 0.4 mL of normal, heparinized human blood, and the entire mixture was rotated end-over-end for 45 minutes at 37°C. At the end of the rotation, a drop of each mixture was used to make thin smears on microscope slides. After staining the slides with Wright's stain, the percentage of neutrophils with associated streptococci (either ingested or attached) was estimated by counting at least 50 consecutive neutrophils. Experiments were repeated 3 times, and the data presented are the mean values ± SEM. Anti–SM18(1-30)-KLH, rabbit antiserum against a synthetic peptide that copied the NH_2-terminal 30 amino acids of M18 linked to KLH. Anti–crude pep M18, rabbit antiserum against a partially purified pepsin extract of whole type 18 streptococci. Anti–SM5(1-15)-KLH, rabbit antiserum against a synthetic peptide that copied the NH_2-terminal 15 amino acids of M5, which served as a control for the KLH antibodies in the SM18 antiserum.

in the crude pepsin extract, it was further purified by preparative gel electrophoresis (Prep Cell 491; Bio-Rad Laboratories Inc., Hercules, California, USA) by methods described previously (10).

Amino acid sequence analyses of Spa. The purified Spa protein was electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane and submitted to the Protein and Nucleic Acid Facility (Beckman Center, Stanford University Medical Center, Stanford, California, USA) for NH_2-terminal sequencing by Edman degradation. The sequence of an internal peptide of Spa was also determined in the Stanford University Facility. The intact protein on PVDF was digested with LysC protease (0.25 μmol/mL) at 37°C overnight. The resultant peptides were purified by HPLC on a Vydac C18 column. Selected fractions were assessed for purity by mass spectroscopy, and 1 peptide with a mass of 1.249 kDa was selected for NH_2-terminal sequencing.

PCR, cloning, and sequencing a fragment of the spa18 gene. A fragment of the *spa* gene was amplified by PCR using degenerate inosine-containing oligonucleotide primers. The forward set of primers derived from the NH_2-terminal sequence of Spa from amino acid residues 7–11 contained the sequence GAR GTI GCI GAY CC. The reverse primers, from the NH_2-terminal sequence of the internal peptide, contained the sequences RTG IGA YTC RCT RTC and RTG RCT YTC IGA RTC. PCR was performed as described previously (10) using chromosomal DNA from type 18 streptococci as the template. The forward primer in combination with the second reverse primer listed above resulted in a single PCR product of 336 bp, which was ligated into pCR2.1-TOPO (Invitrogen Corp., San Diego, California, USA). DNA sequencing was performed by automated techniques in the University of Tennessee Molecular Resources Center using primers from the 5′ and 3′ flanking ends of the plasmid. The identity of the *spa* sequence was confirmed by comparing the translated DNA sequence to the amino acid sequence of Spa that was not used to construct the degenerate PCR primers.

An additional sequence of *spa*18 was obtained by methods reported previously by Evans et al. (19). The 336-bp PCR product obtained above was purified, biotinylated, and then bound to streptavidin-coated magnetic beads. Chromosomal DNA from type 18 streptococci was partially digested with *Sau*3A and

then ligated to synthetic oligonucleotide pairs (19) containing each half of the *Sau*3A sequence. The ligation mixture and bound PCR target were melted and mixed to capture *spa*-containing chromosomal sequences. The magnetic beads were washed, and then PCRs were performed using an internal *spa* primer and each of the 4 extension primers. PCR products were cloned into pCR2.1-TOPO, which was used to transform *E. coli.* Plasmids from positive transformants were screened for inserts and sequenced by automated techniques. One ~600-bp PCR product yielded an overlapping sequence plus 300 bases of a unique downstream sequence.

Results

Characterization of the M-negative mutant (M18Ω). The new streptococcal protective antigen was discovered during studies of an M-negative mutant of type 18 streptococci. The M-negative M18 strain was constructed by interrupting the *emm*18 gene with an Ω element. Southern blot analyses of chromosomal DNA from M18 and M18Ω strains were probed with ΩKm2 and *emm*18 fragments and PCR analyses using primers from the *emm* gene and the Ω element; these probes revealed that there was a single copy of the Ω element inserted ~140 bp beyond the start codon of the *emm*18 gene. In addition, PCR analyses using primers from the *emm*18 gene revealed that there was not another copy of the *emm*18 gene in the M18Ω strain (data not shown).

To confirm that the *emm*18 gene was not expressed in the M18Ω strain, immunoblots were performed using extracts of whole bacteria and rabbit antisera evoked by purified recombinant M18 (rM18), a synthetic peptide of M18, SM18(1-30), and synthetic peptide SM5(265-
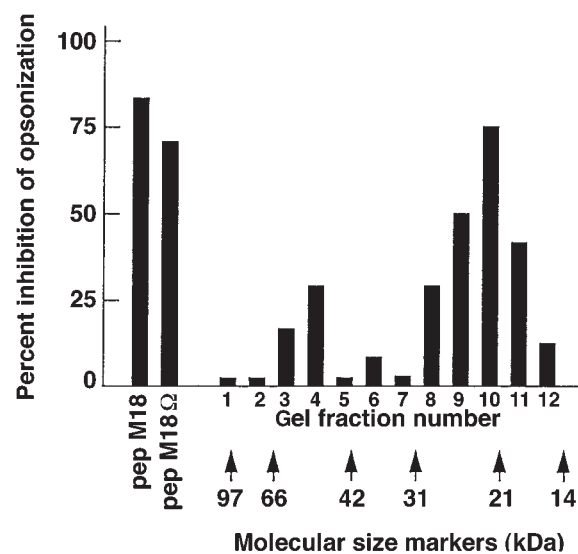
Figure 2

Identification of Spa in a crude pepsin extract of M18Ω. The extract was separated by preparative PAGE, and the entire gel was transferred to nitrocellulose paper. Each bar, labeled **1**–**12**, indicates the gel fraction that was used to absorb opsonic antibodies from a rabbit antiserum raised against a crude pepsin extract of M18Ω (see Methods). The unfractionated pepsin-extracted proteins from M18 and M18Ω (labeled pep M18 and pep M18Ω, respectively) at a concentration of 1 mg/mL inhibited opsonization by 70–80%. Absorption of the antiserum with gel fraction number **10**, which contained a protein of approximately 24 kDa, resulted in a similar level of inhibition of opsonization.

Table 3

Opsonization of type 18 streptococci by rabbit antisera against purified Spa and a synthetic peptide of Spa

Antiserum	Percent neutrophils with associated streptococci[A] (± SEM)	
	M18	M18Ω
Preimmune pool	0.7 ± 0.7	13.3 ± 1.3
Anti-Spa	97.3 ± 0.7	98.7 ± 0.7
Anti–S-Spa(1-23)C	72.7 ± 1.8	94.7 ± 1.3
Anti-rM18	94.7 ± 0.7	15.3 ± 1.3

[A]Details of the assay are provided in the legend to Table 2. Experiments were performed 3 times, and the data presented are the mean values ± SEM. Anti–S-Spa (1-23)C, rabbit antiserum against a synthetic peptide that copied the NH$_2$-terminal 23 amino acids of Spa linked to KLH.

291), which copies the C-repeat domain of type 5 M protein that is common to all M proteins (1) (Figure 1). Rabbit antiserum against intact, recombinant type 18 M protein (anti-rM18) reacted with a triplet of proteins extracted from the parent strain, the largest of which had a molecular mass of ~43 kDa (Figure 1, lane a). The immunoreactive proteins with lower molecular mass are presumably degradation products of M18 that were present in the crude extracts of whole bacteria. There was no reaction of rM18 antiserum with the extract from the M18Ω mutant (Figure 1, lane b). Anti–SM18(1-30) reacted only with proteins in extracts from the parent strain (Figure 1, lane c), and the pattern was identical to that observed with antiserum against rM18. Interestingly, antiserum against the C-repeat peptide of M protein, SM5(265-291), reacted only with an extract from the parent strain (Figure 1, lane e) and not with an extract from the mutant (Figure, lane f). This suggests that the mutant did not express another M protein or M-like protein containing these shared repeating epitopes. These data, taken together with the results of the Southern blot analyses and PCR, indicate that the Ω element was inserted in the *emm*18 gene and that the M18 protein was not expressed by M18Ω.

Growth in blood and mouse virulence of M18 and M18Ω.. The virulence of the M-negative mutant of type 18 streptococci was first assessed by its ability to grow in nonimmune human blood (Table 1). The parent strain of M18 grew to just over 8 generations after a 3-hour rotation in blood. The M18Ω mutant grew to approximately 7.5 generations in the same assay. The most stringent laboratory assay for virulence is intraperitoneal challenge infections of nonimmune mice. The LD$_{50}$ of M18 and M18Ω were calculated after intraperitoneal injections of 10-fold increasing doses of each organism. The LD$_{50}$ of the parent M18 strain was 0.73×10^5, and the LD$_{50}$ of the M18Ω was 1.26×10^5. Of the 24 mice challenged with each organism, 8 challenged with M18 died, and 7 challenged with M18Ω died. Organisms recovered from the spleens of mice that succumbed to challenge infections with M18Ω were kanamycin resistant and M18 negative, indicating that there was no reversion to the parent phenotype in vivo. These results demonstrated that the expression of M18 was not required for virulence of type 18 streptococci.

Opsonization of M18 and M18Ω. Interruption of *emm*18 expression could have resulted in a virulent mutant that

did not express a protective antigen, or alternatively, one that expressed a second protective antigen on its surface. In vitro opsonization experiments were performed to determine whether M18Ω expressed a surface antigen that contained opsonic epitopes (Table 2). Only the M18 parent strain was opsonized by antisera against SM18(1-30) or recombinant M18. The association of M18Ω with polymorphonuclear neutrophils remained at baseline levels in the presence of both of these antisera. Antiserum raised in rabbits against a crude pepsin extract of the M18Ω strain, however, opsonized both the parent and the M-negative mutant (Table 2). This antiserum was used in subsequent experiments to identify and purify the putative new protective antigen of type 18 streptococci.

Identification and purification of Spa. To identify the surface antigen of M18Ω containing opsonic epitopes, we performed opsonization inhibition experiments using fractions of the crude pepsin extract of M18Ω that had been separated by SDS-PAGE and then transferred to nitrocellulose paper. One section of the nitrocellulose paper was reacted with pep M18Ω antiserum to identify the immunoreactive bands, and another section was cut into horizontal strips that were used to absorb opsonic antibodies in the pep M18Ω antiserum. The pep M18Ω extract contained a protein with a molecular mass of 24 kDa (Spa) that absorbed the majority of the opsonic antibodies in the pep M18Ω antiserum (Figure 2).

Spa was purified from the crude pepsin extract of M18Ω by ammonium sulfate precipitation and preparative gel electrophoresis. The purified Spa migrated as a single band, as assessed by SDS-PAGE, with a molecular mass of 24 kDa (Figure 3b). Edman degradation of the purified protein revealed the sequence of the first 23
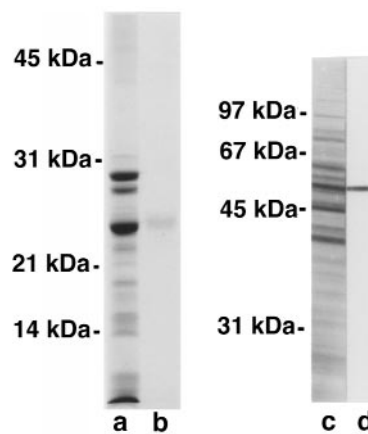
Figure 3

PAGE of the purified fragment of Spa (lanes **a** and **b**) and Western blot analysis of the native protein extracted by phage lysin (lanes **c** and **d**). The crude pep M18Ω (lane **a**) and the purified Spa (lane **b**) were electrophoresed in a 10% polyacrylamide gel under reducing conditions and stained with Coomassie blue. The purified Spa migrated with a molecular mass of 24 kDa. Western blot analysis was performed to identify the native Spa that was released from the cell wall of M18Ω by group C streptococcal phage-associated lysin (19). The crude lysin extract contained multiple proteins that stained with Coomassie blue (lane **c**). Antiserum against the synthetic peptide of Spa reacted with a single protein in the extract with a molecular mass of 50 kDa (lane **d**).

Table 4

Bactericidal activity of rabbit antisera against rM18 and purified Spa against M18 and M18Ω

	CFU surviving a 3-hour rotation[A]	
Antiserum	M18	M18Ω
Inoculum:	16	19
Normal rabbit serum	2,300	3,780
Anti-rM18	80	3,860
Anti-Spa	150	0
Anti-rM18 + anti-Spa	0	ND

[A]Indirect bactericidal assays were performed by adding a small inoculum of the wild-type (M18) or the M-negative mutant (M18Ω) to 0.4 mL of nonimmune human blood that contained 0.1 mL of test rabbit antiserum. The mixtures were rotated end-over-end for 3 hours at 37˚C, and the organisms surviving were quantitated on pour plates of sheep blood agar. The experiment was performed 3 times with similar results. The data presented are from a representative experiment. Anti-rM18 + anti-Spa, a 50:50 mixture of the 2 antisera added to the test mixture in a total volume of 0.1 mL. ND, not done.

amino acids to be DSVSGLEVADPSDSKKLIELGLA. In addition, an internal peptide purified from a LysC digest of the intact Spa ontained the NH₂-terminal sequence YRLDSESHLK.

Bactericidal activity of Spa antibodies. To directly assess the presence of bactericidal epitopes in Spa, rabbits were immunized with 100 μg of the purified protein in CFA at 0, 4, and 8 weeks. Antiserum obtained 10 weeks after the first injection contained antibodies that opsonized the M18 parent and M18Ω (Table 3). None of the Spa antisera cross-reacted with purified recombinant M18 or purified recombinant Enn18, as determined by ELISA (data not shown). The results of the opsonization assays were confirmed by indirect bactericidal tests (Table 4). The Spa antiserum was bactericidal against both M18 and M18Ω. Antiserum against rM18 was bactericidal against only the parent M18 strain and not against M18Ω (Table 4). A mixture of Spa and rM18 antisera resulted in complete killing of the M18 parent strain, suggesting that the 2 antisera have an additive bactericidal effect (Table 4).

Passive mouse protection tests with anti-Spa and anti-rM18. Passive mouse protection tests were performed to demonstrate the presence of protective epitopes within

Spa. In both experiments, groups of 10 mice each were pretreated with either anti-Spa, anti-rM18, or an equal mixture of the 2 antisera, and then later challenged with M18 or M18Ω (Table 5). Of the mice challenged with the parent M18 strain, only 3 out of 20 control animals survived. Those pretreated with anti-rM18 or anti-Spa had higher rates of survival, but these were not statistically different from the control group. Interestingly, the mice that received a mixture of anti-Spa and anti-rM18 were significantly protected from challenge infections with the parent M18 strain (Table 5). Of the mice challenged with M18Ω, those that were pretreated with anti-Spa alone were significantly protected from death when compared with the control animals receiving normal rabbit serum (Table 5). Taken together, these results directly demonstrate the presence of protective epitopes within Spa.

Identification of opsonic epitopes within the NH₂-terminus of Spa. To confirm the presence of opsonic epitopes within the covalent structure of Spa, a peptide [S-Spa18(1-23)C] copying the NH₂-terminal 23 amino acids of the purified 24-kDa fragment was chemically synthesized. The peptide was covalently linked to KLH (11), and 3 rabbits were immunized with 100-μg doses using the same schedule described above. Serum from all 3 rabbits opsonized both the parent and M18Ω strains of group A streptococci (Table 3), confirming the presence of opsonic epitopes within this limited region of Spa. The antiserum against the synthetic peptide of Spa was also used to identify the native protein in phage lysin extracts of M18Ω (20). The S-Spa18(1-23)C antiserum reacted in the lysin extract with a single protein with a molecular mass of 50 kDa (Figure 3d), suggesting that the pepsin-derived Spa was a fragment of the larger native protein.

Opsonization of heterologous serotypes of group A streptococci by Spa antiserum. To determine whether Spa evoked opsonic antibodies against serotypes of group A streptococci other than type 18, opsonization assays were performed with antisera against purified Spa or against the pep M18Ω and a series of selected streptococci (Table 6). Both antisera opsonized type 3 and type 28 streptococci, in addition to type 18 organisms. Serotypes tested that were not opsonized by either antiserum were M1, M2, M5, M6, M13, M14, M19, and M24, all of which

Table 5

Passive protection of mice against challenge infections with M18 or M18Ω by rabbit antisera against rM18 and Spa

	No. mice surviving/No. mice challenged with[A]						
	M18				M18Ω		
Antiserum	Exp. no. 1	Exp. no. 2	Total		Exp. no. 1	Exp. no. 2	Total
Inoculum	1.2×10^7	2.5×10^7			1.8×10^7	4.0×10^7	
Normal rabbit serum	2/10	1/10	3/20		5/10	1/10	6/20
Anti-rM18	4/10	3/10	7/20 (NS)		ND	ND	
Anti-Spa	3/10	2/10	5/20 (NS)		9/10	8/10	17/20 ($P = 0.001$)
Anti-rM18 + anti-Spa	7/10	9/10	16/20 ($P = 0.0001$)		ND	ND	

[A]Passive mouse protection tests were performed according to the original protocol described by Lancefield (2). Briefly, white Swiss mice were injected via the intraperitoneal route with 0.5 mL of rabbit serum and then challenged 24 hours later with virulent streptococci from early log-phase cultures. Deaths were recorded twice daily for a total of 7 days. Each experiment was performed using an identical protocol, with the exception that in experiment no. 2, the pooled antisera against rM18 and Spa were concentrated to 50% of the original volume and each animal received 0.5 mL. Statistical analyses were performed using a χ^2 test on MultiStat software (Biosoft, Cambridge, United Kingdom).

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                      10           20           30           40           50           60           70
                       *            *            *            *            *            *            *
                  GAA GTG GCG GAC CCC TCT GAT AGT AAG AAA CTT ATT GAA TTA GGT TTG GCT AAA TAC CTT AAT GAT AAA TTA CCC TTT
Asp Ser Val Ser Gly Leu Glu Val Ala Asp Pro Ser Asp Ser Lys Lys Leu Ile Glu Leu Gly Leu Ala Lys Tyr Leu Asn Asp Lys Leu Pro Phe

     80           90          100          110          120          130          140          150          160          170
      *            *            *            *            *            *            *            *            *            *
AAA ACT AAA GAA GAT TCA GAG ATT TTA TCT GAG TTA CGT GAT GTA TTA AAA AAT GCT AAT CCA GAA ACA TTA AAA AGT TTA CTT AAT GGT ATG GAT
Lys Thr Lys Glu Asp Ser Glu Ile Leu Ser Glu Leu Arg Asp Val Leu Lys Asn Ala Asn Pro Glu Thr Leu Lys Ser Leu Leu Asn Gly Met Asp

    180          190          200          210          220          230          240          250          260          270
      *            *            *            *            *            *            *            *            *            *
CAA GGA CAT ATA TCA TTT TCT GAT AGA AAT AAT CGC TAC AAC CGT TTA TCT CAA TAT ATA AAT AGT TTT AGA AAA GAT GAT GAT GAC TAC CTA CAT
Gln Gly His Ile Ser Phe Ser Asp Arg Asn Asn Arg Tyr Asn Arg Leu Ser Gln Tyr Ile Asn Ser Phe Arg Lys Asp Asp Asp Asp Tyr Leu His

    280          290          300          310          320          330          340          350          360
      *            *            *            *            *            *            *            *            *
AAT GGA TAT TCT TTA GGA AGT TTA GTG ATT GAA GCA ATT AAA TAC CGT TTA GAT AGT GAG TCA CAT CTA AAG GAA GAA TTA CTT AAA CAG ACT GCA
Asn Gly Tyr Ser Leu Gly Ser Leu Val Ile Glu Ala Ile Lys Tyr Arg Leu Asp Ser Glu Ser His Leu Lys Glu Glu Leu Leu Lys Gln Thr Ala
                                                                             ------------------------------------------
    370          380          390          400          410          420          430          440          450          460
      *            *            *            *            *            *            *            *            *            *
GAA CTT GAG CAA CGT AAG AAT GCA GAA GTT GAT TTA AAA TCT GAA AAA AAG AGA CTT GAA GCG CAA ATA GAA AAA GTA GGA TAT GAT ATT GCT AAT
Glu Leu Glu Gln Arg Lys Asn Ala Glu Val Asp Leu Lys Ser Glu Lys Lys Arg Leu Glu Ala Gln Ile Glu Lys Val Gly Tyr Asp Ile Ala Asn
-----------------------------------------------------------------------------------------------------------------------
    470          480          490          500          510          520          530          540          550
      *            *            *            *            *            *            *            *            *
AAA CAG CAA GAA TTA GAA AAA GCG CGT TCA GAT CAA AAA GAG TTA AGT GAA TCT ATT CAA AAA TTA ACG TCA CGA TTT AAA AAA GAA AGT GAT GAT
Lys Gln Gln Glu Leu Glu Lys Ala Arg Ser Asp Gln Lys Glu Leu Ser Glu Ser Ile Gln Lys Leu Thr Ser Arg Phe Lys Lys Glu Ser Asp Asp
-----------------------------------------------------------------------------------------------------------------------
    560          570          580          590          600          610          620          630
      *            *            *            *            *            *            *            *
GCT AAA CAA AAA GAA CTA GAT GAA GCT AAG GCG GCA AAT AAA TCT CTT TCA GAG TCA GCA ACA AAA ACA TTA GCT AGA
Ala Lys Gln Lys Glu Leu Asp Glu Ala Lys Ala Ala Asn Lys Ser Leu Ser Glu Ser Ala Thr Lys Thr Leu Ala Arg
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Figure 4

Partial DNA sequence of the *spa* gene and the deduced amino acid sequence. The solid underline indicates the amino acid sequence of purified Spa determined by Edman degradation. The unmarked DNA sequence (bases 1–336) was determined from the PCR product obtained using chromosomal DNA and degenerate oligonucleotide primers based on the NH2-terminal and internal amino acid sequences. The dashed line indicates the sequence derived from an overlapping DNA fragment that was captured from a *Sau*3A digest of chromosomal M18 DNA using the original 336-bp PCR fragment. The sequence has been submitted to GenBank and has the accession no. AF086813.

were from our laboratory collection. The anti–S-Spa18(1-23)C antisera did not opsonize types 3 or 28 streptococci, indicating that this limited region of Spa does not contain cross-opsonic epitopes.

Cloning and sequencing a fragment of the spa18 gene. A fragment of the *spa*18 gene was cloned using PCR and degenerate primers based on the NH2-terminal peptide sequence and the sequence of an internal peptide purified from a LysC digest of Spa. The PCR product was 336 bp in length, and the translated amino acid sequence contained residues 12–23 of the sequence derived from the Spa protein (Figure 4), confirming that the DNA was a fragment of *spa*18. An additional sequence of *spa*18 was obtained from an overlapping *Sau*3A fragment of chromosomal DNA that was captured using the original PCR fragment (Figure 4). A search of the current entries in GenBank and the type 1 streptococcal genome sequence database (http://dna1.chem.ou.edu/strep.html) revealed that the 636-bp *spa*18 gene fragment did not share sequence homology with any known proteins, suggesting that Spa is a new protective antigen of group A streptococci.

Discussion

Previous studies have shown that the surface M proteins of group A streptococci play a dual role in the pathogenesis of infection. M proteins are a major determinant of virulence and confer upon group A streptococci the ability to resist phagocytosis and killing by neutrophils (2). In addition, M proteins elicit bactericidal antibodies thought to protect the host against subsequent infection by the same serotype (2). The antibodies with the greatest bactericidal activity are directed against the NH2-terminal, hypervariable epitopes of M proteins (21–24). Among the many defined surface proteins of group A streptococci, only antibodies against the M protein have been shown to be opsonic. In the present study, however, we have identified a new protective antigen, distinct from M protein, that evokes bactericidal antibodies.

The identification of Spa was facilitated by the fact that the M-negative mutant of type 18 streptococci was fully virulent when compared with the parent strain. This finding is somewhat contradictory to the recent results of Moses et al. (14), who showed reduced virulence of an M-negative mutant of the same M18 strain (87-282). They concluded that the hyaluronate capsule and the surface M protein played significant but unequal roles in resistance to phagocytosis and in mouse virulence. The identification of Spa in this strain raises the possibility that it too may play a role in virulence. This has not yet been tested directly, but experiments are now underway to create a Spa-negative/M-negative mutant that will be assessed for mouse virulence and its ability to grow in human blood.

The concept that group A streptococci may express more than 1 surface protein that contains protective epitopes is not new, but to date there has been insufficient data to prove the hypothesis. As early as 1939, Hirst and Lancefield (25) reported cross-protective antisera raised against crude extracts of different serotypes of group A streptococci. Wiley and Wilson (26) later reported the occurrence of 2 M antigens on the surface of certain strains of type 14 organisms. Other similar studies reported cross-protective immune responses evoked by various antigen preparations (27, 28). We now recognize that some M proteins contain cross-protective and type-specific epitopes (29). However, none of these earlier studies provided definitive evidence that any of the organisms actually expressed 2 distinct surface proteins containing protective epitopes.

A more recent study of cross-protective immunity by Chappell and Stuart (30) may be directly related to the protective epitopes of Spa. In these experiments, mice that were parentally immunized with an M-negative mutant of type 28 streptococci were protected against intravenous challenge infections with the type 28 parent and types 3 and 18 streptococci. In addition to the apparent non-M protein–mediated protective immune responses, the investigators also observed that the type 28 M-negative mutant was as virulent in mice as the M-positive type 3 and type 18 strains (30). Our finding that rabbit antisera against Spa opsonized all 3 of these serotypes suggests that the cross-protective immune responses observed by these investigators may have indeed been evoked by a Spa-like protein on the surface of type 28 streptococci.

In the present studies, we have shown that opsonic epitopes reside on both the M18 protein and Spa18. Both proteins evoked antibodies in rabbits that were bactericidal against the parent M18 strain in human blood. Although a greater number of mice survived when treated with anti-M18 or anti-Spa18 than when treated with preimmune serum, neither antiserum alone provided statistically significant protection against challenge infections of mice with the parent strain. Only the mice that received a mixture of the 2 antisera were protected to a statistically significant degree. Although these results suggest that both antigens may be required to evoke a fully protective immune response, we have not ruled out the possibility that our findings simply reflect the absolute quantity of antibodies against rM18 or Spa in each of the immune rabbit sera. Studies are currently in progress to quantitate the relative role of each antibody population in bactericidal killing.

The identification of Spa raises many questions that remain to be answered. Experiments to determine the complete sequence of the *spa* gene and its position in the streptococcal chromosome are currently in progress, as are experiments to determine the frequency of the gene and its expression by other serotypes of group A streptococci. Other important questions relate not only to the prevalence of *spa* within different strains of type 18 streptococci but also to the regulation of its expression. Type 18 streptococci have been identified as an important serotype in the resurgence of acute rheumatic fever in Utah (31). In addition, types 3 and 18, and to a lesser extent type 28, streptococci have all been implicated in the recent resurgence of serious streptococcal diseases (32). Differential expression of *spa* could potentially result in new subtypes of the organism with different virulence characteristics that may be partly defined by the immune status of the host and the population as a whole. The ability of an individual M serotype to alter the protective antigens expressed on its surface could potentially impact the changing epidemiology of group A streptococcal infections.

The discovery of a new protective antigen of group A streptococci has obvious implications for the development of vaccines that would prevent these infections. Our current approach is to use limited NH$_2$-terminal fragments of M proteins that are linked in tandem to evoke protective immune responses against each

Table 6

Opsonization of heterologous serotypes of group A streptococci by rabbit antisera against purified Spa and a crude pepsin extract of M18Ω

Serotype (strain)	Percent neutrophils with associated streptococci[A] (± SEM) in the presence of		
	Normal rabbit serum	Anti–crude pep M18Ω	Anti-Spa
M18 (282)	0.6 ± 0.7	91.3 ± 0.7	96.7 ± 0.7
M3 (3375)	1.3 ± 0.7	59.3 ± 1.3	26.0 ± 1.2
M28 (S2356)	1.3 ± 0.7	17.3 ± 1.3	25.3 ± 1.8

[A]Details of the assay are provided in the legend to Table 2. Serotypes that were tested but were not opsonized by either antiserum were M1, M2, M5, M6, M13, M14, M19, and M24. Experiments were repeated 3 times, and the data presented are the mean values ± SEM.

serotype represented in the vaccine (10, 23, 24). This has the advantage of limiting the total amount of protein contained in the vaccine. However, because each NH$_2$-terminal M protein fragment is type specific, this will likely necessitate the development of relatively complex vaccines to prevent the majority of streptococcal infections in a given population or geographic region. If Spa and Spa-like proteins are found to elicit significant cross-protection, this could facilitate the development of broadly protective vaccines that are considerably less complex than those presently in development.

To our knowledge, this is the first study to show that group A streptococci may express 2 distinct surface proteins that contain opsonic epitopes. Whether Spa is involved directly in the pathogenesis of streptococcal infections is not yet known. We believe that continued studies of Spa and related proteins that may be expressed by other serotypes of group A streptococci will provide new insights into the pathogenesis of these infections and the protective immune responses that they elicit.

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