PROTECTIVE AND HEART-CROSSREACTIVE EPITOPES LOCATED WITHIN THE NH_2 TERMINUS OF TYPE 19 STREPTOCOCCAL M PROTEIN

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The M protein of group A streptococci is an α-helical coiled-coil molecule (1) that projects from the surface of these organisms and functions as the major virulence factor (2). Only antibodies directed against these structures are protective against subsequent infections (2). Vaccine development has been hampered by the finding that some M protein molecules contain tissue-crossreactive epitopes in addition to protective epitopes (3–8). Previous studies (9–11) have shown that protective M protein epitopes could be separated from tissue-crossreactive epitopes by using synthetic peptides copying limited regions of the NH_2 terminus of the native M protein molecules. For example, type 5 M protein evokes both protective and heart-crossreactive antibodies, and some of the heart-crossreactive antibodies also react with the heterologous types 6 and 19 M proteins (5). Synthetic peptides representing the NH_2-terminal 20 amino acids of type 5 (9) and type 6 (11) M proteins evoke type-specific, opsonic antibodies that are not heart crossreactive. These findings have suggested that the heart-crossreactive epitopes of M proteins might be avoided by using only the NH_2-terminal primary structures as vaccine components. To test the generality of this hypothesis, we decided to examine additional "rheumatogenic" serotypes of M protein.

In the present study, we investigated the protective and autoimmune epitopes of type 19 streptococcal M protein. We show that a purified pepsin extract of type 19 streptococci (pep M19) evokes not only opsonic antibodies, but also heart-crossreactive antibodies in rabbits. By using a combination of synthetic peptides and pep M19, we further show that autoimmune epitopes may be located within the NH_2-terminal regions of certain M protein molecules. Thus, the concept that protective epitopes can always be separated from autoimmune epitopes by focusing on the NH_2-terminal regions of the various M protein serotypes must be reevaluated.

Materials and Methods

Extraction and Purification of M Proteins. Type 19 M protein was extracted from intact type 19 group A streptococci by limited pepsin digestion at pH 5.8, and was purified by ammonium sulfate precipitation and ion-exchange chromatography as previously described (12).

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Pep M19 was further purified by electroelution of the 43-kD polypeptide from SDS-polyacrylamide slab gels. 1 mg of pep M19 was added to each of four gels and electrophoresed under reducing conditions. Each gel was briefly stained with Coomassie blue and destained overnight in an aqueous solution containing 10% methanol and 7.5% acetic acid. After washing the gels in distilled water, proteins with an apparent molecular weight of 43×10^3 were cut from the gels and placed in dialysis tubing (Spectrum Medical Industries, Inc., Los Angeles, CA) containing 0.003 M trizmabase, 0.02 M glycine, 20% methanol, pH 8.3 (transphor buffer). The proteins were electroeluted from the gels in a transphor electrophoresis unit (Hoeffer Scientific Instruments, San Francisco, CA) at 2°C with a current 0.9 mA for 4 h. The electroeluted proteins were dialyzed overnight against distilled H_2O at 4°C and then were lyophilized. Coomassie blue stain and residual salt were removed by applying the preparation to a Bio-Gel P6 molecular sieve column (Bio-Rad Laboratories, Richmond, CA) equilibrated with 2.5 M guanidine/HCl/0.05% Tris, pH 7.4. The column fractions representing the initial peak of the chromatogram (OD_{230}) were collected and pooled, dialyzed overnight in distilled water, and then lyophilized. The SDS was removed by resuspending the product in 1 ml of 1 M acetic acid, adding three drops of proprionic acid, and applying it to a Bio-Gel P2 molecular sieve column (Bio-Rad Laboratories, Richmond, CA) equilibrated with 0.1 M acetic acid. The initial peak of the chromatogram (OD_{230}) was collected, dialyzed overnight in distilled water, and lyophilized. The protein was judged to be homogeneous by SDS-PAGE.

Determination of the NH_2-Terminal Primary Structure of pep M19. The NH_2-terminal amino acid sequence of the purified pep M19 was determined by automated Edman degradation, as previously described (13, 14).

Chemical Synthesis of SM19(1-24)C. A peptide copying the NH_2-terminal 24-amino acid residues of pep M19 was chemically synthesized by an automated peptide synthesizer as reported (10, 14), and is designated SM19(1-24)C. Additionally, two overlapping subpeptides of SM19(1-24)C were synthesized and designated as SM19(1-14)C and SM19(11-24)C, respectively. The sequence of each synthetic peptide was confirmed by automated Edman degradation to the penultimate residues (14, 15).

Immunization Procedures. New Zealand White rabbits were immunized subcutaneously with 100 μg of pep M19 emulsified in CFA (12). Booster injections consisting of the same dose in 0.02 M phosphate/0.15 M NaCl, pH 7.4 (PBS) were given at 4 and 8 wk. Serum was obtained before the initial injection and at 2-wk intervals thereafter. Rabbits were immunized with 300 μg of SM19(1-24)C emulsified in CFA. Booster injections of the same dose in PBS were given and serum was obtained as described above.

M Protein Assays. Rabbit sera were tested for type-specific and crossreactive antibodies by an ELISA as previously reported (3, 16). The presence of opsonic antibodies was detected by in vitro opsonophagocytosis tests as described (12). Inhibition of opsonization was performed as previously described (12). Indirect bactericidal tests were performed as described (12).

Detection of Heart-crossreactive Antibodies Evoked by pep M19 and SM19(1-24)C. Heart-crossreactive antibodies evoked by pep M19 or SM19(1-24)C were detected by indirect immunofluorescence tests using purified sarcolemmal membranes of human heart tissue (3, 5). Inhibition of immunofluorescence was performed as described (5). The specificity of tissue-crossreactive M protein antibodies was determined using frozen sections (4 μm) of human skin, lymph node, lung, liver, and kidney.

Affinity Purification of SM19(1-24)C. Antibodies evoked in rabbits immunized with SM19(1-24)C were affinity purified over a column of SM19(1-24)C covalently linked to CH-Sepharose 4B (Pharmacia Inc., Uppsala, Sweden) by methods previously described (10). The peptide-specific antibodies were eluted from the column with 0.2 M glycine/0.2 M NaCl, pH 2.8 and were dialyzed overnight against PBS. The eluate was concentrated to the intial serum volume by membrane ultrafiltration (XM50 membrane; Amicon Corp., Lexington, MA).

Affinity Purification of Heart-crossreactive Antibodies. Heart-crossreactive antibodies evoked by SM19(1-24)C were adsorbed to and eluted from purified sarcolemmal membranes of human heart tissue as described previously (3-5). The eluted antibodies were dialyzed overnight against PBS and concentrated to the initial serum volume by membrane ultrafiltration (XM50 membrane; Amicon Corp.).

Immunoblot Analysis. SDS-PAGE of human myocardium was performed on continuous gradient gels ranging from 7.5 to 15% polyacrylamide (5). The electrophoresed proteins were transferred onto nitrocellulose strips which were incubated in 0.05 M Tris/0.15 M NaCl, pH 7.5, containing 1% BSA. The strips were incubated overnight with affinity-purified SM19(1-24)C immune serum diluted 1:100. Inhibition experiments were performed by adding 100 μg/ml of pep M19 or 100 nm/ml of SM19(1-24)C, SM19(1-14)C, or SM19(11-24)C to the antiserum before the addition of the nitrocellulose strips. After an overnight incubation the strips were extensively washed in 0.05 M Tris/0.15 M NaCl/0.05% Tween-20, pH 7.5. The strips were incubated with peroxidase-conjugated goat anti-rabbit IgG (Cappel Laboratories, Malvern, PA) diluted 1:2,000 for 2 h at room temperature. After repetitive washings, the strips were developed with horseradish peroxidase substrate (Bio-Rad Laboratories).

Results

Purification and NH_2-Terminal Sequence Analysis of pep M19. Pep M19 was purified from limited peptic digests of intact type 19 streptococci by ammonium sulfate precipitation, ion-exchange chromatography over DEAE cellulose (12), and electroelution from polyacrylamide gels. The purified protein migrated as a single polypeptide on SDS-polyacrylamide gel with an apparent molecular weight of 43×10^3 (Fig. 1). The NH_2-terminal amino acid sequence of the purified protein was determined by automated Edman degradation (Fig. 2). Analysis of the primary structure by the method of Chou and Fasman (17) revealed a high α-helical potential beginning at residue 11 and continuing throughout the remainder of the sequence. Because of the low α-helical potential of the first 10 residues and the proline at position 8, which is known to be a helix breaker, the first 10 residues are represented as a flexible structure (Fig. 2).

Opsonic and Heart-crossreactive Antibodies Evoked by pep M19. To determine the immunogenicity of pep M19, each of three rabbits was immunized with 100 μg of the purified protein emulsified in CFA. Immune sera from all three rabbits contained significant levels of pep M19 antibodies, as determined by ELISA, and opsonic anti-

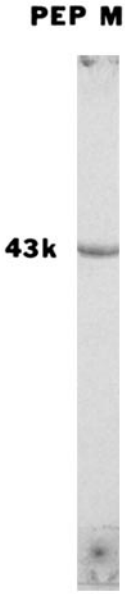

FIGURE 1. Coomassie blue stain of SDS-PAGE of the purified pepsin extracted type 19 streptococcal M protein revealing an apparent molecular weight of 43×10^3.

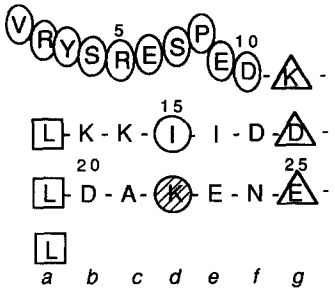

FIGURE 2. Primary structure of the NH_2 terminus of pep M19 showing the seven-residue periodicity of charged (amino acids in triangles at position g) and nonpolar residues (amino acids in boxes and circles at positions a and d, respectively), that predicts an α-helical three-dimensional structure. The first 10 residues (in ovals) are represented as a more flexible structure because of the low α-helical potential and the proline at position 8. The hatched circle indicates a charged residue at position d.

bodies against type 19 streptococci, as determined by in vitro opsonization tests (Table I). In addition, all three pep M19 antisera crossreacted with sarcolemmal membranes of human myocardium (Table I). Immunofluorescence tests of frozen sections of human skin, lymph nodes, lung, liver, and kidney were negative.

Because previous studies have shown that one serotype of M protein may contain epitopes that are shared with heterologous M antigens (5, 16), we assayed the pep M19 antisera for M protein-crossreactive antibodies by ELISA and opsonization tests. All three immune sera contained antibodies that crossreacted with pep M5 (with titers of 1,600; 1,600; and 800) and pep M6 (with titers of 3,200; 3,200; and 800), but not with pep M24 (all < 200). The crossreactive antibodies also opsonized the heterologous serotypes of streptococci with percent opsonization of type 5 streptococci ranging between 92 and 100 and type 6 streptococci between 76 and 92. Taken together, these data show that pep M19 evokes type-specific and crossreactive opsonic antibodies, as well as antibodies that crossreact with sarcolemmal membranes of human myocardium.

Immunogenicity of an NH_2-Terminal Synthetic Peptide of pep M19. In an effort to identify protective epitopes of pep M19 that were not heart crossreactive, we synthesized peptides copying the NH_2 terminus of pep M19 (Fig. 3). To determine the immunogenicity of SM19(1-24)C, rabbits were immunized with the unconjugated synthetic peptide emulsified in CFA. All three rabbits developed significant levels of antibodies against SM19(1-24)C and pep M19, as determined by ELISA (Table II).

TABLE I

Type-specific and Heart-crossreactive Antibodies Evoked in Rabbits Immunized with pep M19

Antiserum	ELISA titers against pep M19*	Opsonization of type 19 streptococci‡	Sarcolemmal immunofluorescence§
		%	
Preimmune pool	<200	0	0
8104	25,600	92	+ + + +
8105	6,400	92	+ +
8106	25,600	100	+ + +

* ELISA titers represent the reciprocal of the highest serum dilution resulting in an $OD_{450} \geq 0.1$

‡ Percent opsonization equals the percentage of neutrophils with associated streptococci.

§ On a scale of 0 to + + + + .

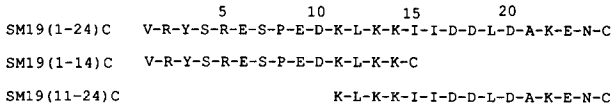

FIGURE 3. Primary structure of synthetic peptides copying the NH_2 terminus of pep M19.

TABLE II

Immunogenicity of SM19(1-24)C in Rabbits

Antiserum	ELISA titer against: SM19(1-24)C	ELISA titer against: pep M19	Opsonization of type 19 streptococci
			%
86126			
Preimmune	<200	<200	4
6 wk	12,800	12,800	100
86127			
Preimmune	<200	<200	8
6 wk	12,800	12,800	80
86128			
Preimmune	<200	<200	4
6 wk	12,800	12,800	88

All three antisera also opsonized type 19 streptococci. When screened for the presence of heart-crossreactive antibodies by indirect immunofluorescence tests, all three SM19(1-24)C antisera reacted with sarcolemmal membranes of human myocardium (Table III). Thus, the NH_2-terminal 24 residues of pep M19 contain type-specific opsonic epitopes as well as heart-crossreactive epitopes.

Immunoblot Analysis of SM19(1-24)C Heart-crossreactive Antibodies. To identify the myocardial proteins containing SM19(1-24)C crossreactive epitopes, immunoblot analyses were performed using SDS-extracted proteins from whole human myocardium that were electrophoresed and transferred to nitrocellulose strips (Fig. 4). The SM19(1-24)C antiserum reacted with a 60-kD myocardial protein (Fig. 4, lane *C*). Inhibition experiments showed that the heart-crossreactive antibodies were completely inhibited by SM19(1-24)C (lane *D*), partially inhibited by SM19(1-14)C (lane *E*), and totally inhibited by SM19(11-24)C (lane *F*) and by the native pep M19 (lane *G*).

TABLE III

Heart-crossreactive Antibodies Evoked by SM19(1-24)C

Immune rabbit serum	Sarcolemmal fluorescence
86126	
Preimmune	0
6 wk	+ + +
86127	
Preimmune	0
6 wk	+ +
86128	
Preimmune	0
6 wk	+

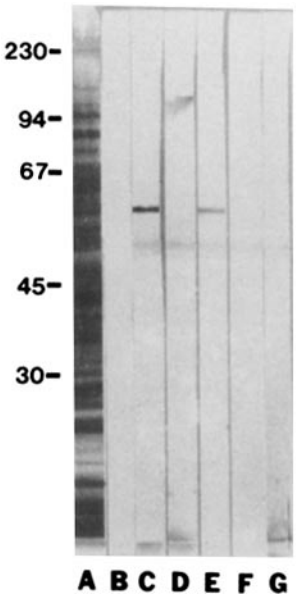

FIGURE 4. Type specificity of heart-crossreactive antibodies evoked by SM19(1-24)C as determined by immunoblot analysis. SDS-solubilized human myocardium was electrophoresed under reducing conditions on an SDS-polyacrylamide continuous gradient gel, ranging from 7.5 to 15%. Multiple bands were observed in the Coosmassie blue-stained gel (*A*). Immunblot analysis revealed that the preimmune serum did not react with human myocardial proteins (*B*), but the SM19(1-24)C immune serum that had been affinity purified over an SM19(1-24)C-Sepharose column reacted with a myocardial protein of an apparent molecular weight of 60 × 10^3 (*C*). The heart cross-reactive antibodies were inhibited by SM19(1-24)C (*D*), SM19(11-24)C (*F*), and pep M19 (*G*), but not SM19(1-14)C (*E*).

Crossreactve Opsonic Epitopes of SM19(1-24)C. The antisera against SM19(1-24)C were tested for the presence of crossreactive antibodies against heterologous serotypes of streptococci by ELISA and in vitro opsonization tests. Two of the three sera contained significant levels of antibodies against pep M5, but not pep M6 and pep M24, as determined by ELISA (Table IV). One of the antisera contained low levels of opsonic antibodies against type 5 streptococci, indicating the presence of cross-reactive epitopes exposed on the surface of the organism. To confirm the presence of type-specific and crossreactive opsonic epitopes on SM19(1-24)C, indirect bactericidal tests were performed (Table V). The SM19(1-24)C immune serum (rabbit No. 86127) promoted almost complete killing of both type 19 and type 5 streptococci during the 3-h rotation in whole blood. These results were similar to those obtained with antiserum raised against the native pep M19 antigen (Table V), indicating that

TABLE IV

Type-specific and Crossreactive Antibodies in SM19(1-24)C Immune Sera

Immune rabbit serum‡	ELISA titer against:*		Opsonization of:	
	pep M19	pep M5	Type 19 streptococci	Type 5 streptococci
			%	
Preimmune pool	<200	<200	2	0
86126	12,800	12,800	96	8
86127	12,800	3,200	96	34

* Additional serotypes tested that resulted in titers <200 were pep M6 and pep M24.

‡ Results are from assays performed using 10-wk antisera. pep M5 crossreactions were not observed before 8 wk after the initial injection. Rabbit 86128 died from an injury 7 wk after immunization.

TABLE V

Indirect Bactericidal Activity of SM19(1-24)C Antiserum Against Type 19 and 5 Streptococci

		Number of colonies of streptococci surviving 3 h rotation in test mixture					
		Type 19			Type 5		
Antiserum	Inoculum:	49	16	1	60	11	3
Normal rabbit serum		Laked	>2,000	>2,000	Laked	>2,000	400
Anti-SM19(1-24)C		1	0	0	0	0	0
Anti-pep M19		8	0	0	0	0	0

the synthetic peptide evoked significant levels of opsonic antibodies against the homogous type 19 serotype as well as type 5 streptococci.

To determine whether SM19(1-24)C shared heart-crossreactive epitopes with pep M5, we eluted SM19(1-24)C antibodies from sarcolemmal membranes (3, 5) and assayed for the presence of pep M5-crossreactive antibodies. The affinity-purified heart-crossreactive antibodies showed an ELISA titer of 1,600 against pep M5, indicating that SM19(1-24)C and pep M5 share at least one heart-crossreactive epitope. In an attempt to determine the primary structure of the shared epitope, ELISA inhibition experiments were performed by using SM19(1-24)C affinity-purified heart-crossreactive antibodies reacted with pep M5 and inhibited by synthetic peptides spanning the entire pep M5 molecule (18); the latter peptides were synthesized for previous studies (7, 8). None of the synthetic peptides inhibited the crossreactive antibodies (data not shown), suggesting that the heart-crossreactive epitope that is shared between pep M5 and SM19(1-24)C was not adequately represented by the series of synthetic type 5 M protein peptides.

Localization of Opsonic and Heart-crossreactive Epitopes of SM19(1-24)C. The NH_2 terminus of pep M19 was synthesized in an attempt to evoke protective, but not heart-crossreactive antibodies against type 19 streptococci. Having shown that SM19(1-24)C evoked antibodies of both types, we performed opsonization and immunofluorescence inhibition assays using the overlapping subpeptides in order to localize the type-specific and autoimmune epitopes (Table VI). Opsonization of type 19 streptococci and myocardial immunofluorescence were completely inhibited by pep M19, SM19(1-24)C, and the subpeptide SM19(11-24)C, but not by SM19(1-14)C (Table VI). These data indicate that the opsonic and heart-crossreactive epitopes of SM19 (1-24)C are contained within the same limited primary structure of SM19(11-24)C.

TABLE VI

Localization of Protective and Heart-crossreactive Eptiopes of SM19(1-24)C

Serum inhibited with:	Opsonization of type 19 streptococci	Saroclemmal fluorescence
	%	
Uninhibited	80	+ + + +
SM19(1-24)C	0	0
SM19(1-14)C	70	+ + +
SM19(11-24)C	4	0
pep M19	0	0

Discussion

In the present study we have shown that pep M19 is immunogenic in rabbits and evokes type-specific and crossreactive opsonic antibodies as well as heart-crossreactive antibodies. The NH_2-terminal primary structure of pep M19 was determined and a synthetic peptide copying the first 24-amino acid residues was synthesized. SM19(1-24)C was immunogenic in rabbits without a carrier protein and evoked significant levels of antibodies that reacted with the native pep M19 by ELISA and opsonic antibodies that reacted with the surface M protein of intact type 19 streptococci. Although our initial goal was to synthesize an immunogen that contained protective and not tissue-crossreactive epitopes of pep M19, all three SM19(1-24)C antisera crossreacted with sarcolemmal membranes of human myocardium. The antibodies specifically recognized a 60-kD myocardial protein, and the crossreactions were completely inhibited by pep M19, indicating that they were evoked by epitopes of SM19(1-24)C that were identical to those in the native antigen.

Although we cannot provide definitive evidence that the partial sequence of pep M19 represents the NH_2-terminus of the native molecule, it does have structural features in common with other M proteins. The low α-helical potential of the initial sequence, which predicts a more flexible structure is also found in types 1, 5, 6, and 24 M proteins (18–21). The location of a proline within the first 11 residues, which would function as a helix breaker, is a feature of types 1, 5, and 6 M proteins (18–20) and now also of pep M19. Thus, the evidence derived from the structural analysis of pep M19 suggests that this sequence represents the NH_2 terminus of the native molecule.

Previous studies in our laboratory (5) have shown that several M proteins contain multiple heart-crossreactive epitopes, some of which are shared by heterologous M types. Heart-crossreactive antibodies evoked by pep M5 also crossreacted with pep M6 and pep M19 (5). Some of the pep M5 heart-crossreactive antibodies were specific for sarcolemmal membrane proteins (8), while others reacted with the heavy chain of myosin (6, 7). Using synthetic peptides copying the primary structure of pep M5 (18), we localized the myosin-crossreactive epitopes to peptide 84–116 (7) and at least one of the sarcolemmal membrane–crossreactive epitopes to peptide 164–197 (8). The NH_2-terminal synthetic peptides of pep M5 and pep M6 were shown to contain only type-specific, opsonic epitopes and not tissue-crossreactive epitopes (9–11). The data presented here show for the first time that tissue-crossreactive determinants may be located within the NH_2 terminus of M protein molecules.

In the present study, SM19(1-24)C evoked heart-crossreactive antibodies that also reacted with pep M5. A comparison of the primary structures of each revealed that residues 12–17 of pep M19 (LKKIID) were identical to residues 168–173 of pep M5 (18) (LKKILD), except for a single isoleucine/leucine substitution in the fifth position of the hexapeptide. The inability of SM5(164–197), which we have previously shown contains at least one heart-crossreactive epitope (8), to inhibit the SM19(1-24)C antibodies suggests that the shared hexapeptide either is not part of the crossreactive epitope or that it is not presented in the appropriate conformation for antibody binding. A dodecapeptide copying amino acid residues 12–17 of pep M19 twice was synthesized, and this peptide did not inhibit the pep M5 crossreactive antibodies evoked by SM19(1-24)C (our unpublished data). These data suggest that SM19(1-24)C evokes

heart-crossreactive antibodies that react with pep M5 epitopes that are at least partially conformational and not adequately predicted by primary structure. In addition, immunoblot analyses have shown that SM5(164–197)C evokes antibodies that crossreact with a 40-kD myocardial protein (8), whereas SM19(1–24)C crossreacts with a 60-kD protein. These findings suggest that the two peptides contain different heart-crossreactive epitopes.

Previous studies have shown that the NH_2-terminal amino acid sequence of several M proteins is quite variable from one serotype to another (9–12, 22, 23), whereas the COOH-terminal sequences are highly conserved (22–24). The M protein–crossreactive and tissue-crossreactive epitopes of pep M5 are located toward the COOH terminus of the molecule (7, 8). However, in the present study we have shown that SM19(1–24)C contains potentially harmful tissue-crossreactive epitopes, indicating that this region of M protein molecules may not generally represent an ideal vaccine component. Nevertheless, synthetic peptides containing heart-crossreactive epitopes should serve as useful probes to identify myocardial proteins containing crossreactive epitopes. Such studies may provide information regarding the pathogenesis of acute rheumatic carditis and also eventually may lead to the development of safe and effective streptococcal vaccines.

Summary

M protein was purified to homogeneity from limited pepsin digests of intact type 19 streptococci (pep M19). The purified pep M19 when emulsified in CFA and injected into rabbits evoked type-specific and crossreactive opsonic antibodies, as well as heart-crossreactive antibodies. The NH_2-terminal primary structure of pep M19 was determined and a peptide copying the first 24 amino acids [SM19(1–24)C] was chemically synthesized. Rabbits that were immunized with the unconjugated peptide developed antibodies that recognized the native pep M19, as determined by ELISA, and opsonic antibodies against type 19 streptococci, as determined by in vitro opsonophagocytosis tests. The synthetic peptide also evoked antibodies that crossreacted with a 60-kD sarcolemmal membrane protein of human mycardium. By using overlapping synthetic subpeptides as immunoinhibitors, the opsonic and heart-crossreactive epitopes of SM19(1–24)C were localized to SM19(11–24)C. Our data confirm the presence of heart-crossreactive epitopes within the primary structure of pep M19 and show that these potentially harmful autoimmune epitopes may be located in the NH_2-terminal regions of certain M proteins. We conclude that continued efforts to identify the primary structures of protective and heart-crossreactive epitopes will be necessary to elucidate the pathogenesis of acute rheumatic heart disease and to develop safe and effective streptococcal vaccines.

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