

MULTIPLE, HEART-CROSS-REACTIVE EPITOPES OF STREPTOCOCCAL M PROTEINS

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Group A streptococci contain certain antigens that are immunologically cross-reactive with human tissues, especially the myocardium (1-8). Some of the cross-reactive antigens have been identified as components of the protoplast membrane (3, 8), while others have been localized to the cell wall (2, 5, 6). The precise location of these host tissue-cross-reactive antigens within the streptococcus is of considerable interest to investigators attempting to develop vaccines composed of purified M proteins, the protective surface antigens of group A streptococci. Because M protein vaccines are intended to protect the host against acute rheumatic fever, a nonsuppurative sequela of streptococcal pharyngitis, the fear has been that host tissue-cross-reactive antigens contained within the vaccine may theoretically cause, rather than prevent, the disease.

Indeed, recent studies in our laboratory (9) have shown that type 5 M protein contains within its covalent structure at least one heart cross-reactive epitope. The purified heart-cross-reactive antibodies opsonized types 5 and 19 streptococci, indicating that they were directed against protective M protein epitopes. These results indicate the need to precisely identify the heart-cross-reactive antigenic determinants on M protein molecules of potentially rheumatogenic serotypes of group A streptococci so that these regions may be excluded from vaccine preparations.

In the present study, we show that highly purified M proteins from three different serotypes of rheumatogenic streptococci contain antigens that cross-react with sarcolemmal membrane proteins of human myocardium. The number and distribution of these epitopes on each purified M protein were determined by using a combination of immunofluorescence inhibition tests and enzyme-linked immunosorbent assays. The affinity-purified, heart-reactive antibodies opsonized all three serotypes of streptococci, indicating that they were directed against protective M protein epitopes that were exposed on the surface of virulent organisms. Immunoblot analyses were used to identify the human sarcolemmal membrane proteins containing the cross-reactive antigens and to demonstrate their M protein serotype specificity.

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Materials and Methods

Extraction and Purification of M Proteins. M proteins were purified from limited peptic extracts of types 5, 6, 19, and 24 group A streptococci (10–13). The purified M proteins (pep M)¹ were judged to be homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (10).

Immunization of Animals. Three New Zealand White rabbits were immunized with a single injection of 300 µg of pep M5 emulsified in complete Freund's adjuvant (10). Serum was obtained before the first injection and at 2-wk intervals thereafter. Only the immune sera from those rabbits whose preimmune serum did not contain heart-reactive antibodies (see below) were used in these studies.

M Protein Antibody Assays. Rabbit antisera were tested for the presence of type-specific and cross-reactive M protein antibodies by an enzyme-linked immunosorbent assay (ELISA), as previously described (9, 16). ELISA inhibition tests were performed by using increasing concentrations of either homologous or heterologous M protein as soluble inhibitors of antibodies directed against a solid phase M antigen (15).

Type-specific and cross-reactive opsonic antibodies were detected by *in vitro* opsonophagocytic tests as described (10). The test mixtures consisted of 0.05 ml of a standard suspension of streptococci, 0.1 ml of test serum, and 0.4 ml fresh human blood supplemented with 10 U/ml heparin. The tubes were rotated for 45 min at 37°C and the percentage of neutrophils with associated streptococci (percent phagocytosis) was estimated by microscopic counts of stained smears (10).

Affinity Purification of M Protein Antibodies. Type-specific and cross-reactive M protein antibodies were purified by affinity chromatography with columns of pep M5, pep M6, or pep M19, covalently linked to Sepharose 4B (Pharmacia, Inc., Uppsala, Sweden), as previously described (9). One ml of pep M5 immune rabbit serum was loaded onto the column and incubated at ambient temperature for 30 min. Serum proteins were eluted with phosphate-buffered saline (PBS) until ultraviolet absorption at 230 nm reached the baseline level. M protein-specific antibodies were eluted with 0.2 M glycine/0.2 M NaCl, pH 2.8 (glycine-HCl), immediately dialyzed against 0.02 M phosphate/0.15 M NaCl, pH 7.4 (PBS), and then concentrated to the original volume by membrane ultrafiltration (YM30 membrane; Amicon Corp., Scientific Systems Div., Lexington, MA).

Specific heart-reactive antibodies were affinity purified from pep M5 rabbit antisera by using sarcolemmal membrane preparations (8, 9). 500-µg sarcolemmal membranes were washed by centrifugation with 3 ml PBS, followed by 3 ml glycine-HCl, and finally with PBS. 3 ml of immune rabbit serum were added to the washed membranes; the mixture was rotated end-over-end at 37°C for 45 min and then centrifuged at 500 g for 15 min. The absorbed serum was retained and the membranes were washed three times with 3 ml PBS. Specific heart-reactive antibodies were eluted by incubating the membranes in 3 ml glycine-HCl with constant mixing for 10 min. After centrifugation, the supernatant was sterilized by membrane filtration (0.45 pore size; Millipore Corp., Bedford, MA), immediately dialyzed against PBS, and concentrated by membrane ultrafiltration (see above) to a volume of 1 ml.

Immunofluorescence Tests. Immune rabbit sera and affinity-purified antibodies were tested for the presence of heart-cross-reactive antibodies by indirect immunofluorescence tests using purified sarcolemmal membrane sheaths or frozen sections (4 µm) of human heart, as previously described (9). Immunofluorescence inhibition assays were performed by preincubating immune sera (diluted 1:4 with PBS) or affinity-purified M protein antibodies with either pep M5, pep M6, pep M19, pep M24, or pep M6 plus pep M19 (500 µg/ml) for 15 min at ambient temperature. A drop of the mixture was applied to frozen sections of heart tissue or sarcolemmal membranes fixed to glass microscope slides, incubated for 30 min at ambient temperature, and then washed three times in PBS. The slides were similarly treated with a 1:40 dilution of fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) and then mounted with 1% Gelvatol, pH

¹ *Abbreviations used in this paper:* BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; pep M, purified pepsin extracts of types 5, 6, 19, and 24 streptococci; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

7.0 (Monsanto, Springfield, MA) and a coverslip. The tissue was examined under a fluorescence microscope (E. Leitz, Inc., Rockleigh, NJ).

SDS-PAGE and Transblot Analyses. SDS-PAGE of sarcolemmal membrane proteins was performed as previously described (9, 10) on continuous gradient gels ranging from 10 to 20%. Electrophoresed proteins were transferred to nitrocellulose paper (9) which was cut into strips and incubated with affinity-purified M protein antibodies diluted 1:100 in 0.05 M Tris/0.15 M NaCl, pH 7.4 with 1% bovine serum albumin (Tris-BSA). Inhibition experiments were performed by adding pep M protein (20 μ g/ml) to the antibody preparation before adding the nitrocellulose strips. After incubating at 37°C for 2 h, the strips were washed extensively with 0.05 M Tris/0.15 M NaCl, pH 7.4, then incubated with peroxidase-conjugated goat anti-rabbit IgG (Cappel Laboratories), diluted 1:2000 in Tris-BSA, for 2 h at 37°C, and again washed extensively. The nitrocellulose paper was incubated with horseradish peroxidase substrate (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions.

Results

Our previous studies (9) showed that of nine rabbits immunized with 100 μ g pep M5, only one developed heart-cross-reactive antibodies. To determine whether the immunity to tissue-cross-reactive epitopes could be obtained more consistently with a higher dose of M protein, we immunized three rabbits with a single 300 μ g dose of pep M5 emulsified in complete Freund's adjuvant. All three rabbits developed type-specific antibodies against the immunizing antigen, as measured by ELISA and by opsonophagocytic tests of homologous type 5 streptococci (Table I). When tested for the presence of heart-cross-reactive antibodies, all three immune sera were strongly positive, as determined by indirect immunofluorescence tests with purified sarcolemmal membrane sheaths (Table I). To determine the pattern of antibody deposition, the antisera were reacted with frozen sections of fresh human myocardium (Fig. 1). The pattern of fluorescent antibody staining was sarcolemmal in distribution and identical for each of the three antisera.

Serotype Distribution of M Protein Heart-cross-reactive Epitopes. Because previous studies (9, 15) in our laboratories have shown that pep M5 shares epitopes with type 6 and type 19 M proteins, we tested each of the heart-cross-reactive pep M5 antisera for the presence of cross-reactive antibodies against purified

TABLE I
*Type-specific and Heart-reactive Antibodies Raised in Rabbits
Immunized with Pep M5*

Antiserum	ELISA titer against Pep M5	Percent phagocytosis of type 5 streptococci	Sarcolemmal immuno- fluorescence
8329 preimmune	<200	2	0
10 wk	25,600	88	++++
8331 preimmune	<200	2	0
10 wk	25,600	80	++++
8332 preimmune	<200	6	0
10 wk	12,800	80	++++

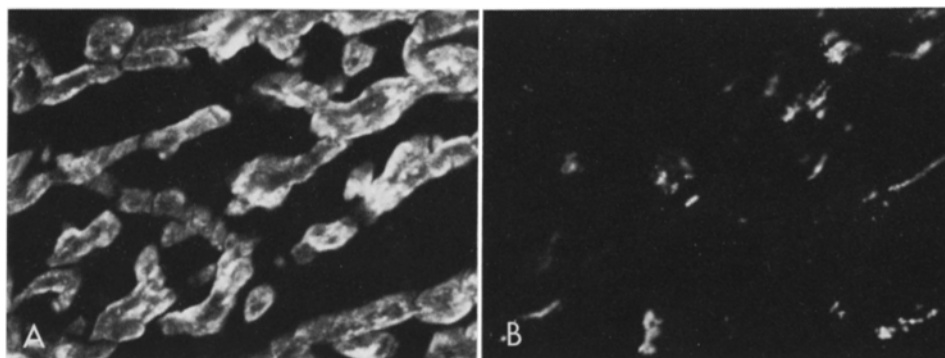


FIGURE 1. Immunofluorescence staining of human myocardium by pep M5 rabbit antiserum (8332). (A) The immune serum bound to the sarcolemma in a somewhat patchy distribution, while the preimmune serum (B) was negative (the bright staining areas were yellow rather than apple green, and are presumed to represent autofluorescence).

TABLE II
*Type-specific and Cross-reactive Antibodies Raised in Rabbits
Immunized with Pep M5*

Rabbit antiserum	ELISA titer against:			
	Pep M5	Pep M6	Pep M19	Pep M24
8329	25,600	1,600	6,400	<200
8331	25,600	3,200	6,400	<200
8332	12,800	1,600	3,200	<200
Preimmune pool	<200	<200	<200	<200

pep M6, pep M19, and pep M24 (Table II). All of the antisera showed significant cross-reactions with pep M6 and pep M19, but not with pep M24. A sample of pooled preimmune sera was negative against all of the M proteins tested.

Because all of the antisera raised against pep M5 were cross-reactive with human heart tissue and also with pep M6 and pep M19, it was of interest to determine the serotype distribution of the heart-cross-reactive epitopes. One of the antisera (8332) was chosen for further study in immunofluorescence inhibition assays (Table III). M protein-specific antibodies were affinity purified with pep M5, pep M6, or pep M19 covalently linked to Sepharose 4B. The concentration of the purified antibodies was adjusted to result in a 3+ immunofluorescence reaction with sarcolemmal membranes. Purified pep M proteins were used as soluble inhibitors of type-specific or cross-reactive, affinity-purified antibodies in immunofluorescence inhibition assays.

All of the affinity-purified antibodies reacted strongly with sarcolemmal membranes by indirect immunofluorescence (Table III), indicating that pep M6 and pep M19 share at least one heart-cross-reactive determinant with pep M5. The binding of pep M5-specific antibodies to heart tissue was totally inhibited by pep M5, but only partially inhibited by pep M6 and pep M19, even at concentrations of 500 $\mu\text{g}/\text{ml}$. When equal amounts of pep M6 and pep M19 were used together as soluble inhibitors at a total concentration of 500 $\mu\text{g}/\text{ml}$, the pep M5 heart-reactive antibodies were totally inhibited. These results suggested that pep M5

TABLE III
*Type Specificity of Pep M5 Heart-reactive Antibodies Determined by
 Immunofluorescence Inhibition of Affinity-purified Antiserum*

Antigen used for affinity purification	Antigen used as inhibitor	Sarcolemmal immunofluorescence
Pep M5	None	+++
	Pep M5	0
	Pep M6	+
	Pep M19	+
	Pep M6 + Pep M19	0
	Pep M24	+++
Pep M6	None	+++
	Pep M5	0
	Pep M6	0
	Pep M19	+
	Pep M24	+++
Pep M19	None	+++
	Pep M5	0
	Pep M6	+
	Pep M19	0
	Pep M24	+++

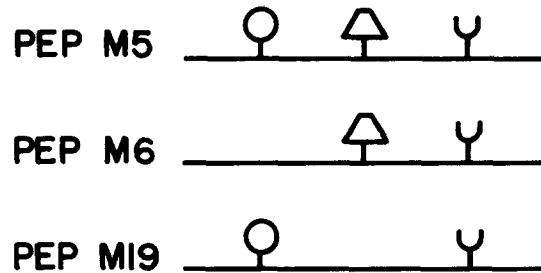


FIGURE 2. Schematic representative of M protein heart-cross-reactive epitopes based on data obtained from the immunofluorescence inhibition assays (see Table III).

contains more than one heart-cross-reactive epitope and that they are shared in some combination by pep M6 and pep M19.

To further define the location of the heart-cross-reactive epitopes on pep M6 and pep M19, the pep M5 antibodies were affinity purified on columns of pep M5, pep M6, or pep M19. Sarcolemmal immunofluorescence produced by antibodies affinity purified with pep M6 was totally inhibited by pep M5, the immunogen, and pep M6, the antigen used for purification, but only partially inhibited by pep M19 (Table III). Immunofluorescence by the pep M19-purified antibodies was likewise partially, but not totally, inhibited by pep M6. The minimum number of pep M5 heart-cross-reactive epitopes indicated by these results is three (Fig. 2). One of these epitopes is shared by pep M6 and pep M19, resulting in partial inhibition of the pep M5-purified, heart-reactive antibodies. Pep M6 and pep M19 each contain one of the other cross-reactive antigenic

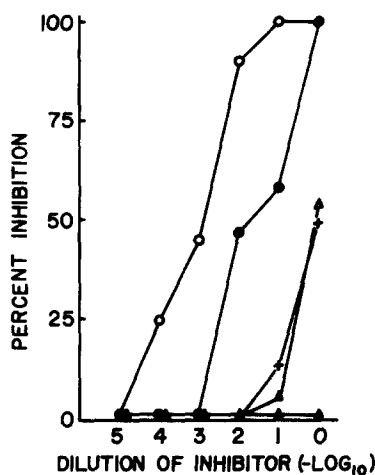


FIGURE 3. Type specificity of pep M5 heart-cross-reactive epitopes determined by ELISA inhibition. Specific heart-reactive antibodies were eluted from sarcolemmal membranes and inhibition of binding to pep M5 was measured with increasing concentrations of pep M5 (○), pep M6 (+), pep M19 (△), pep M24 (▲), or a combination of pep M6 and pep M19 (●). The highest concentration of soluble inhibitor was 40 $\mu\text{g}/\text{ml}$.

determinants, resulting in complete inhibition of pep M5 antibodies when the heterologous M proteins are used in combination.

To confirm the proposed distribution of heart-cross-reactive epitopes among the M proteins, ELISA inhibition assays were performed by using affinity-purified, heart-reactive antibodies eluted from sarcolemmal membranes. Pep M5, pep M6, and pep M19 were used as soluble inhibitors of antibodies directed against immobilized pep M5 (Fig. 3). Increasing concentrations of pep M5 totally inhibited the binding of purified, heart-reactive antibodies to immobilized pep M5. Identical concentrations (10 $\mu\text{g}/\text{tube}$) of pep M6 or pep M19 resulted in only $\approx 50\%$ inhibition, while pep M6 and pep M19, combined at a total concentration of 10 $\mu\text{g}/\text{tube}$, resulted in 100% inhibition (Fig. 3). These results confirm the presence of heart-cross-reactive epitopes on pep M6 and pep M19 that are shared with pep M5, but only partially shared with each other (Fig. 2).

Opsionization of Homologous and Heterologous Streptococci by Pep M5 Heart-reactive Antibodies. To determine whether the heart-cross-reactive epitopes of types 5, 6, and 19 M proteins were protective determinants, the heart-reactive antibodies were affinity purified by absorption and elution from sarcolemmal membranes and then used in opsonization tests (Table IV). The immune serum opsonized types 5, 6, and 19, but not type 24, streptococci. Absorption with sarcolemmal membranes partially reduced opsonization of types 5 and 6 organisms and totally abolished opsonization of type 19 streptococci (Table IV). The heart tissue-specific antibodies opsonized all three serotypes of streptococci, indicating that at least one of the heart-cross-reactive epitopes on each M protein was exposed on the surface of the organism and was available for antibody binding.

Type Specificity of the Sarcolemmal Membrane Antigens that Cross-react With Streptococcal M Proteins. Immunoblot analyses were used to identify the sarcolemmal membrane proteins that cross-react with types 5, 6, and 19 M proteins

TABLE IV
Opsonization of Types 5, 6, and 19 Streptococci by Heart-reactive Antibodies Eluted from Human Heart Tissue

Antiserum	Percent of polymorphonuclear cells with associated streptococci:			
	Type 5	Type 6	Type 19	Type 24
8332 (10 wk)	80	76	57	0
8332 absorbed with sarcolemmal membranes	66	14	4	0
8332 eluted from sarcolemmal membranes	86	84	96	0
8332 preimmune	6	0	0	0

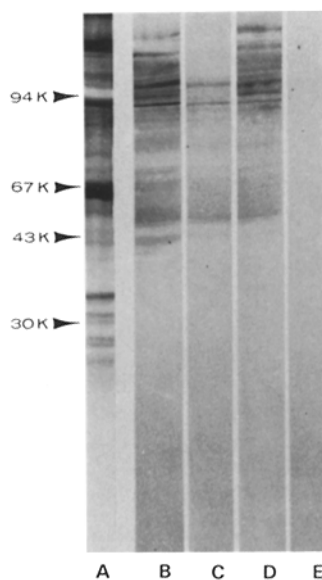


FIGURE 4. Serotype specificity of M protein-cross-reactive sarcolemmal membrane proteins determined by immunoblot inhibition assays. Sarcolemmal membrane proteins were electrophoresed in an SDS-polyacrylamide gel (A) and then transferred to nitrocellulose paper for immunoblot analyses (B-E). Pep M5-specific antibodies reacted with multiple proteins (B). Pep M6 inhibited antibody binding to lower and higher molecular weight proteins (C) and pep M19 inhibited antibody binding to lower molecular weight proteins only (D). Pep M5 totally inhibited antibody binding to the electrophoresed proteins (E).

(Fig. 4). Multiple sarcolemmal membrane proteins were discerned after staining SDS-polyacrylamide gels that were electrophoresed under reducing conditions (Fig. 4 A). The membrane proteins were transferred to nitrocellulose paper and reacted with antibodies that were affinity purified over a pep M5 column. The serotype specificity of the membrane antigens was determined by using pep M5, M6, and M19 as soluble inhibitors of antibody binding. The pep M5-specific antibodies reacted with multiple membrane proteins, ranging from 40,000 to 200,000 mol wt (Fig. 4 B). Pep M6 inhibited antibody binding to several proteins of higher and lower molecular weight (Fig. 4 C), and pep M19 inhibited antibody binding to lower molecular weight proteins (Fig. 4 D). Pep M5 totally inhibited antibody to the sarcolemmal membrane proteins (Fig. 4 E), indicating that all of

the antibodies used in the assay were specific for pep M5. The preimmune rabbit serum was not reactive with any of the sarcolemmal membrane proteins (data not shown).

Discussion

In the present study, we have shown that three serologically different M proteins from potentially rheumatogenic group A streptococci contain epitopes within their covalent structures that are cross-reactive with sarcolemmal membrane proteins of human myocardium. By using affinity-purified antibodies in immunofluorescence and ELISA inhibition tests, we are able to schematically map the locations of the heart-cross-reactive antigens on pep M5, pep M6, and pep M19. Although the minimum number of cross-reactive epitopes within the pep M5 molecule that satisfy our results is three, the possibility that more epitopes are present cannot be excluded by our data. The affinity-purified, heart-cross-reactive antibodies opsonized types 5, 6, and 19 streptococci, indicating that at least one of the tissue-cross-reactive epitopes on each native M protein is a protective antigenic determinant.

Previous studies have shown that tissue-cross-reactive antigens of group A streptococci are located in various subcellular components of the organism, including the cell wall (2, 5, 6) and protoplast membrane (3, 8). This was of concern to early investigators attempting to develop streptococcal vaccines, because of the fear that partially purified M protein preparations might be contaminated with these cross-reactive antigens. Only recently (9) did we provide evidence that type 5 M protein contains at least one epitope that is shared with human heart tissue. By immunizing with a higher dose of pep M5, we have now shown the presence of multiple cross-reactive epitopes shared among types 5, 6, and 19, but not 24, M proteins. Therefore, we can no longer assume that homogeneous M protein preparations will be free of potentially harmful tissue-cross-reactive epitopes.

These findings emphasize the need to identify precisely the regions of the M proteins responsible for host tissue cross-reactivity so that they may be excluded from vaccine preparations. Considerable progress has recently been made in determining the primary structures of several M protein molecules (10-14, 16-20). In addition, we have shown (16, 21-23) that natural and chemically synthesized peptide fragments of M protein, linked to appropriate carrier molecules, evoke opsonic antibodies, indicating that immunity against the entire M protein is not necessary to protect against infection. Laboratory animals immunized with a chemically synthesized peptide of type 5 M protein, containing only 20 amino acid residues, developed opsonic antibodies that were not heart-cross-reactive (23). Thus, by selecting limited regions of the M protein molecule to use as protective immunogens, the total amount of each M protein represented in a vaccine preparation can be minimized and the risk of evoking potentially harmful tissue-cross-reactive antibodies can be reduced. The antisera described in the present study will provide useful tools to identify fragments of M proteins that contain host tissue-cross-reactive epitopes. The cross-reactive subpeptides, which may be structurally defined and even chemically synthesized, may provide

valuable insights into the pathogenesis of streptococcal infection and possibly the pathogenesis of acute rheumatic fever and rheumatic carditis.

Summary

We present evidence that a highly purified pepsin extract of type 5 streptococcal M protein (pep M5) contains at least three epitopes that are cross-reactive with sarcolemmal membrane proteins of human myocardium. The tissue-cross-reactive determinants of pep M5 are also partially shared with pep M6 and pep M19. Three rabbits immunized with a single 300 μ g dose of pep M5 developed significant levels of heart-cross-reactive antibodies, as determined by indirect immunofluorescence tests. All three sera also contained antibodies that cross-reacted with pep M6 and pep M19. The heart tissue-specific antibodies that were eluted from sarcolemmal membranes opsonized types 5, 6, and 19 streptococci, indicating that they were directed against protective M protein epitopes on the surface of virulent organisms. Immunofluorescence inhibition tests, using purified M proteins as soluble inhibitors of heart-cross-reactive antibodies, revealed the number and M protein serotype distribution of the tissue-cross-reactive epitopes. Immunoblot analyses demonstrated the sarcolemmal membrane proteins containing the various cross-reactive antigenic determinants.

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