

## PROTECTIVE ANTIGENIC DETERMINANT OF STREPTOCOCCAL M PROTEIN SHARED WITH SARCOLEMMA MEMBRANE PROTEIN OF HUMAN HEART\*

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The finding that group A streptococci contain antigens that are immunologically cross-reactive with human heart tissue (1-8) has been of considerable concern to investigators attempting to develop protective vaccines against strains of group A streptococci that are capable of initiating acute rheumatic fever and rheumatic heart disease. Indeed, the presence of such cross-reactive antigens in various streptococcal products (1-8) has hampered efforts to vaccinate humans because of the theoretical danger that the cross-reactive antigens may evoke heart-reactive immune responses and thereby elicit, rather than prevent, rheumatic heart disease. Most of the cross-reactive antigens appear to be associated with components of the streptococcal cell wall (2, 5, 6) or cell membrane (3, 8), rather than with the surface M protein, the type-specific antigen of group A streptococci that evokes protective immunity. Thus, Zabriskie and Freimer (3) described heart cross-reactive antibodies in the sera of rabbits immunized with streptococcal protoplast membranes. Because the antibodies could be removed by absorption with membranes from streptococci of heterologous as well as homologous serotypes, it was assumed that heart cross-reactive antibodies were not associated with the type-specific M protein. That more than one protoplast membrane antigen may be involved in these cross-reactions was suggested by the studies of van de Rijn et al. (8), who showed that the antibodies in the sera of membrane-immunized rabbits reacted with different antigenic determinants of heart tissue than did the sera from some subjects with rheumatic fever. Similar non-type-specific heart cross-reactive antigens were shown to be associated with cell wall preparations of group A streptococci (5).

In addition to these studies, however, Kaplan (2, 5, 6) demonstrated an additional heart cross-reactive antigenic determinant(s) associated with the cell wall that was restricted to types 5 and 19 streptococci. Furthermore, this heart cross-reactive antigen was copurified with acid extracts of the M proteins from these serotypes (2). Similar type-specific cross-reactions were reported by Lyampert et al. (7), who immunized rabbits with types 1 and 5 streptococci. In none of these studies, however, was it shown that the cross-reactive determinants resided in the M protein molecule itself. Indeed, Kaplan concluded they did not, because type-specific M antigens could be separated

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from the heart cross-reactive antigen, although the heart-reactive antigen seemed inseparable from an additional type-specific antigen (2).

Now that new methods of extraction and purification have yielded homogeneous preparations of M protein and it has been demonstrated that native and synthetic peptide fragments of the M protein molecule are immunogenic (9–14), we have undertaken studies to examine in detail the structural relationships of the M protein molecule to heart tissue cross-reactive antigens. In this report, we present definitive evidence that at least one protective antigenic determinant of a structurally defined polypeptide fragment of type 5 M protein evoked antibody that cross-reacted with sarcolemmal membranes of human heart muscle; the cross-reactive antibody eluted from the sarcolemmal membranes opsonized both types 5 and 19 streptococci, indicating that the heart cross-reactive determinant of type 5 M protein is shared with type 19 M protein. The purified antibody was used in an affinity column to isolate a sarcolemmal membrane protein ( $\approx 215,000$  mol wt) shown to be cross-reactive with types 5 and 19 M proteins.

### Materials and Methods

*Extraction and Purification of M Protein.* M proteins were purified from limited peptic digests of types 5, 6, 19, and 24 streptococci as previously described (9–11). The purified products, designated pep M proteins, were judged to be homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (9).<sup>1</sup> The details of the purification and characterization of type 19 M protein (pep M19), not previously reported, will be reported elsewhere (E. H. Beachey and J. M. Seyer, manuscript in preparation).

*Preparation of Cell Walls and Protoplast Membranes.* Cell walls were prepared by rupturing whole type 6 streptococci with glass beads in a Braun MSK homogenizer (Arthur H. Thomas Co., Philadelphia, PA) followed by differential centrifugation and washing as described by Bleiweis et al. (15). Cell membranes were prepared from protoplasts produced by treating whole type 30 streptococci with group C bacteriophage-associated lysin as described by Zabriskie and Freimer (3).

*Immunization of Animals.* New Zealand White rabbits were immunized intracutaneously with 100  $\mu$ g pep M5 emulsified in complete Freund's adjuvant (12). Serum was obtained before immunization and at 2-wk intervals thereafter. Some animals received additional injections of 100  $\mu$ g pep M5 in incomplete Freund's adjuvant, and serum was again collected at 1- and 2-wk intervals.

*Detection of M Protein Antibodies.* All sera were tested for type-specific and cross-reactive M antibodies by an enzyme-linked immunosorbent assay (ELISA) as previously described (13, 14). ELISA titers are expressed as the reciprocal of the highest dilution of serum giving an absorbance of  $>0.1$  at 405 nm.

Type-specific and cross-reactive opsonic antibodies were detected by in vitro opsonophagocytic assays as described elsewhere (9). Briefly, the test mixture consisted of 0.4 ml fresh human blood supplemented with 10 U/ml of heparin, 0.05 ml of a standard suspension of streptococci, and 0.1 ml of test serum. The percentage of neutrophils with associated streptococci (percent phagocytosis) was estimated by microscopic counts of stained smears prepared from the assay mixtures after rotating at 37°C for 45 min. Indirect bactericidal tests were performed using the same test mixtures as described above except fewer colony-forming units of streptococci (CFU) were added (16). The mixtures were rotated at 37°C for 3 h, after which pour plates were prepared by adding the test mixtures to 20 ml of 5% sheep blood agar (16).

*Detection of Tissue Cross-Reactive Antibodies.* Sarcolemmal membrane sheaths were prepared from fresh human heart by the method of van de Rijn et al. (8). The lyophilized membranes

<sup>1</sup> Abbreviations used in this paper: pep M, purified pepsin extracts of types 5, 6, 19 and 24 streptococci; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; CFU, colony-forming units of streptococci; PBS, 0.02 M phosphate/0.15 M NaCl, pH 7.4; Tris-BSA, 0.05 M Tris/0.15 M NaCl, pH 7.4, with 1% bovine serum albumin.

were mixed with distilled water, and one drop was placed on microscope slides, allowed to dry, and stored at  $-70^{\circ}\text{C}$ . Before use, the samples were fixed in 1% paraformaldehyde for 3 min. Sarcolemmal cross-reactive antibodies were detected by indirect immunofluorescence as described previously (17). Briefly, preimmune and immune rabbit sera were diluted 1:4 with 0.02 M phosphate/0.15 M NaCl, pH 7.4 (PBS) and allowed to react with sarcolemmal membranes for 30 min at ambient temperature. After washing three times for 5 min in PBS, the antibody-treated tissue was incubated with a 1:40 dilution of fluorescein-labeled goat anti-rabbit IgG (N. L. Cappel Laboratories Inc., Cochranville, PA), again washed three times in PBS, and then mounted with one drop of 1% Gelvatol, pH 7.0 and a coverslip. The tissue was examined under a fluorescence microscope (E. Leitz, Inc., Rockleigh, NJ). In addition, the specificity of tissue cross-reactive antibodies was determined by indirect immunofluorescence as described above using frozen sections (4- $\mu\text{m}$  thick) of human lung, kidney, liver, brain, skeletal muscle, and heart, which were obtained at autopsy.

*Immunoabsorption of Test Sera.* In some experiments, the test sera were first absorbed with an equal packed volume of freshly cultured and PBS-washed streptococci or sarcolemmal membranes. Serum was also absorbed with cell membranes of type 30 streptococci and cell walls of type 6 organisms, each at a concentration of 4 mg/ml. All immunoabsorptions were performed at  $37^{\circ}\text{C}$  for 1 h, then  $4^{\circ}\text{C}$  for 18 h. Inhibition experiments were performed by first adding purified M protein (1 mg/ml) or  $\alpha$  tropomyosin (4 mg/ml) to the test sera before performing opsonophagocytic or immunofluorescence assays.

Cross-reactive antibodies were eluted from sarcolemmal membranes by the following procedure. 3 ml of immune rabbit serum was absorbed with 250 mg lyophilized sarcolemmal membranes as described above. The mixture was centrifuged at 500 *g* for 15 min, and the absorbed serum was retained. The membrane pellet was washed three times with PBS and then rotated in 5 ml of 0.2 M glycine/0.2 M NaCl, pH 2.8 for 30 min at ambient temperature. The membranes were removed by centrifugation, and the supernatant containing eluted antibodies was dialyzed against PBS overnight. The sample was concentrated to a final volume of 1 ml by membrane filtration (YM 30 membrane; Amicon Corp., Scientific Systems Div., Lexington, MA).

*Purification of Heart-reactive Antibodies.* Because previous immunoabsorption experiments had shown that only heart-reactive antibodies in the pep M5 antiserum reacted with the heterologous pep M19 antigen, heart-reactive antibodies were purified by affinity chromatography using pep M19 immobilized on agarose beads.

Pep M19 (10 mg) was conjugated to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) by the method of March et al. (18). Free antigen was removed from the beads by washing alternately with 0.1 M acetate buffer, pH 4.0, and 0.1 M borate buffer, pH 9.4. The beads were suspended in PBS and packed into a  $1 \times 30$ -cm column (Pharmacia). After equilibrating the column with PBS, 3 ml of immune serum was applied and allowed to incubate for 30 min at ambient temperature. Serum proteins were eluted with PBS until the protein concentration, as measured by ultraviolet absorption at a wavelength of 230 nm, reached baseline. Specific pep M19 antibody was eluted with 0.2 M glycine/0.2 M NaCl, pH 2.8. The antibody-containing fractions were dialyzed against 0.15 M NaCl and concentrated by membrane filtration (YM 30 membrane; Amicon Corp.). The titer of antibody against pep M19 was determined by ELISA, and the presence of heart-reactive antibodies was confirmed by indirect immunofluorescence using sarcolemmal membrane sheaths as described above.

*Purification of Human Heart Antigen.* The purified antibody obtained above was immobilized on agarose beads by the following method. 10 ml of CH-Sepharose 4B was mixed with 5 ml purified antibody, which had been adjusted to pH 4.5. To this was added 100 mg carbodiimide, which was dissolved in 5 ml distilled water and adjusted to pH 4.5. The mixture was rotated for 18 h at ambient temperature, after which the beads were washed extensively with distilled water and packed into a  $1 \times 30$ -cm column (Pharmacia). The antibody-Sepharose column was then equilibrated with PBS.

Sarcolemmal membrane antigens were extracted by adding 1 ml of 1% SDS to 250 mg of sarcolemmal membranes. The mixture was placed in a boiling water bath for 2 min and then centrifuged for 5 min in an Eppendorf microfuge (Brinkmann Instruments, Inc., Westbury, NY). The supernatant was removed, diluted with 30 ml PBS, and applied to the antibody-

Sephacose column. Excess proteins were eluted with PBS, and after the protein concentration reached baseline, specific antigen was eluted with 0.2 M glycine/0.2 M NaCl, pH 2.8. The fraction containing the eluted antigen was dialyzed against PBS and concentrated by membrane filtration.

Purity of the sarcolemmal membrane antigen was assessed by SDS-PAGE as described previously (9). Some samples were subjected to reducing conditions by boiling in sample buffer containing 1%  $\beta$ -mercaptoethanol. Polyacrylamide gels were stained with silver nitrate reagent (Bio-Rad Laboratories, Richmond, CA) according to the manufacture's instructions with minor modifications. For autoradiographic analysis, the proteins were transferred to nitrocellulose paper using an Electroblood apparatus (E-C Apparatus Corp., St. Petersburg, FL) by the method of Towbin et al. (19). The nitrocellulose paper was preincubated in 0.05 M Tris/0.15 M NaCl, pH 7.4 with 1% bovine serum albumin (Tris-BSA) at 37°C for 1 h to reduce nonspecific antibody binding. The paper was treated with heart-reactive antiserum, diluted 1:250 in Tris-BSA for 2 h at 37°C, and washed extensively with 0.15 M NaCl. The nitrocellulose was then reacted with  $^{125}$ I-protein A (New England Nuclear, Boston, MA) (sp. act. 70–100  $\mu$ Ci/mg, diluted in Tris-BSA to 200,000 CPM/ml) at 37°C for 2 h. After extensive washing, the paper was dried and exposed to Kodak X-ray film (Eastman Kodak Co., Rochester, NY) for 36 h.

## Results

*Heart Cross-Reactive Antibodies Evoked by Immunization with pep M5.* One of the first three rabbits immunized with pep M5 developed antibody cross-reactive with human sarcolemmal membranes (Table I). All three rabbits showed strong M5 antibody responses by both ELISA and opsonophagocytic tests (Table I). None of an additional six rabbits immunized with pep M5 developed heart-reactive antibodies, although each showed strong M5 immune responses (data not shown), suggesting that the heart

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

| Immune rabbit serum | ELISA titer against pep M5 | Percent phagocytosis of type 5 streptococci* | Sarcolemmal fluorescence |
|---------------------|----------------------------|--|--------------------------|
| 7907                | 51,200                     | 92   | +++                      |
| 7908                | 102,400                    | 62   | 0                        |
| 7909                | >102,400                   | 80   | 0                        |
| Preimmune pool‡     | <200                       | 2  | 0                        |

\* Percent phagocytosis equals the percentage of neutrophils that ingested streptococci.

‡ Preimmune sera from rabbits 7907, 7908, and 7909.

TABLE II  
*Specificity of Tissue Cross-Reactive Antibodies Raised against Type 5 M  
Protein Measured by Indirect Immunofluorescence*

| Tissue                     | Immunofluorescence |
|----------------------------|--------------------|
| Heart                      | +++                |
| Skeletal muscle            | +++                |
| Smooth muscle (arteriolar) | 0                  |
| Kidney                     | 0                  |
| Brain                      | 0                  |
| Lung                       | 0                  |
| Liver                      | 0                  |

cross-reactive determinant in pep M5 preparations is a minor antigenic determinant.

*Tissue Specificity of Heart Cross-Reactive Antibody.* Immunofluorescence tests of frozen sections of human heart, skeletal muscle, kidney, liver, lung, and brain were positive only with heart and skeletal muscle (Table II). These tissues immunofluoresced in a sarcolemmal distribution similar to that previously reported for other streptococcal cross-reactive antibodies (2, 3).

*Opsonization of Type 19 Streptococci by Heart Cross-Reactive Antibody.* Because earlier studies by Kaplan (2) had suggested a relationship between tissue cross-reactive antigens of types 5 and 19 streptococci, the pep M5 antisera were tested for their ability to opsonize type 19 streptococci (Table III). Only the heart cross-reactive antiserum (7907) was able to opsonize the heterologous type 19 organisms. Nevertheless, none of six rabbits immunized with 100  $\mu$ g of pep M19 emulsified in complete Freund's adjuvant developed heart cross-reactive antibodies, although each developed type 19 opsonic antibodies (E. H. Beachey and J. H. Seyer, manuscript in preparation). The pep M5 heart cross-reactive antiserum (7907) was used in the following studies.

*Absorption of Opsonic Antibodies by pep M Proteins and Sarcolemmal Membranes.* The above studies suggested that the pep M5 antibodies that cross-reacted with human heart tissue may be directed against opsonic antigenic determinants on types 5 and 19 M proteins. To test this hypothesis, the cross-reactive serum was absorbed with human heart tissue, pep M5 or pep M19, and then assayed in opsonophagocytic tests with type 5 or type 19 streptococci (Table IV). As expected, opsonic antibodies against type 19 streptococci were totally inhibited by pep M5, the immunizing antigen, and

TABLE III  
*Cross-Reactive Opsonic Antibodies against Type 19 Streptococci in pep M5 Immune Rabbit Serum*

| Immune rabbit serum | Percent phagocytosis of: |                      |
|---------------------|--------------------------|----------------------|
|                     | Type 5 streptococci      | Type 19 streptococci |
| 7907                | 92                       | 96                   |
| 7908                | 62                       | 6                    |
| 7909                | 80                       | 10                   |
| Preimmune pool      | 2                        | 2                    |

TABLE IV  
*Absorption of Heart Cross-Reactive Opsonic Antibodies from Rabbit Antiserum 7907 with Purified pep M Proteins and Human Heart Tissue*

| Phagocytosis test organism | Serum absorbed with: | Percent phagocytosis |
|----------------------------|----------------------|----------------------|
| Type 19 streptococci       | Unabsorbed           | 78                   |
|                            | pep M5               | 2                    |
|                            | pep M19              | 0                    |
|                            | Human Heart          | 4                    |
| Type 5 streptococci        | Unabsorbed           | 72                   |
|                            | pep M5               | 2                    |
|                            | pep M19              | 86                   |
|                            | Human heart          | 72                   |

by pep M19, the homologous test antigen. Absorption with sarcolemmal membranes similarly reduced opsonization of type 19 streptococci, suggesting that the heart cross-reactive antibodies were raised against a protective antigenic determinant of type 5 M protein that is shared with type 19 M protein. In contrast, opsonic antibodies directed against type 5 streptococci were inhibited only by pep M5 (Table IV), indicating the presence on type 5 M protein of additional protective antigenic determinants that are not heart cross-reactive.

*Absorption of Heart-reactive Antibody by pep M Proteins, Streptococcal Cells, and Heart Tissue.* The specificity of the cross-reactive antibodies raised against pep M5 was assessed by the ability of various immunoabsorbents to inhibit the fluorescent antibody reaction against sarcolemmal membranes (Table V). Of the many whole group A streptococci tested, only types 5 and 19 absorbed the tissue cross-reactive antibody. In agreement with opsonic inhibition tests (see above), pep M5 and pep M19 completely inhibited the sarcolemmal immunofluorescence, whereas pep M6 and pep M24 had no effect (Table V). Absorption of the serum with cell walls of type 6 and cytoplasmic membranes of type 30 streptococci failed to inhibit immunofluorescence. Finally, because previous studies have indicated structural similarities between  $\alpha$  tropomyosin of heart tissue and streptococcal M proteins (20), we tested the ability of purified  $\alpha$  tropomyosin derived from rabbit heart to inhibit the binding of heart-reactive antibody to sarcolemmal membranes. Concentrations up to 4 mg/ml failed to alter the immunofluorescence reaction (Table V).

*Elution of Opsonic Antibodies Adsorbed to Sarcolemmal Membranes.* To provide further evidence that the heart-reactive antibodies raised against pep M5 were opsonic, the serum was absorbed with sarcolemmal membranes, and the adsorbed antibodies were then eluted at pH 2.8 and tested for opsonophagocytic activity against types 5 and 19 streptococci (Table VI). Absorption of the pep M5 antiserum with heart tissue totally removed opsonic antibodies against type 19 streptococci, but only partially removed antibodies directed against type 5 organisms (Table VI). Antibodies eluted from sarcolemmal membranes opsonized both types 5 and 19 organisms (Table VI). Bactericidal activity of the eluted antibody was retained as indicated by the total

TABLE V  
*Type-specific Absorption of Heart-reactive Antibodies from Rabbit Antiserum 7907 by Type 5 Streptococci, Type 19 Streptococci, pep M5, and pep M19*

| Serum absorbed with:          | Sarcolemmal fluorescence |
|-------------------------------|--------------------------|
| Unabsorbed                    | +++                      |
| Type 5 streptococci           | 0                        |
| Type 19 streptococci          | 0                        |
| Other serotypes*              | +++                      |
| Type 6 cell walls             | +++                      |
| Type 30 cytoplasmic membranes | +++                      |
| pep M5                        | 0                        |
| pep M19                       | 0                        |
| pep M6                        | +++                      |
| pep M24                       | +++                      |
| Heart tissue                  | 0                        |
| $\alpha$ tropomyosin          | +++                      |

\* Group A streptococcal serotypes 1, 2, 3, 6, 12, 14, 24, 30, 31, 49, 55, and 56.

TABLE VI  
*Opsonization of Types 5 and 19 Streptococci by Heart-reactive Antibody Eluted from Human Heart Tissue*

| Serum   | Percent phagocytosis of: |                      |
|---|--------------------------|----------------------|
|   | Type 5 streptococci      | Type 19 streptococci |
| Normal rabbit serum   | 10                       | 4                    |
| 7907  | 82                       | 96                   |
| 7907 absorbed with sarcolemmal membranes                    | 66                       | 4                    |
| Heart-reactive antibodies eluted from sarcolemmal membranes | 62                       | 94                   |

TABLE VII  
*Bactericidal Activity against Type 19 Streptococci of Heart-reactive Antibodies Eluted from Human Heart Tissue*

| Serum   | CFU of type 19 streptococci surviving 3-h rotation in test mixture |             |
|---|--|-------------|
|   | Inoculum 194   | Inoculum 85 |
| Normal rabbit serum   | >2,000   | >2,000      |
| 7907  | 0  | 0           |
| 7907 absorbed with sarcolemmal membranes                    | 444  | 113         |
| Heart-reactive antibodies eluted from sarcolemmal membranes | 0  | 0           |

killing of type 19 organisms after 3 h incubation in the bactericidal test mixture (Table VII).

*Purification of the Cross-Reactive Antigen from Sarcolemmal Membranes.* The sarcolemmal membrane antigen that cross-reacted with antibody raised against pep M5 was isolated by affinity chromatography. Because immunoabsorption studies using serum from rabbit 7907 indicated that heart tissue and pep M19 shared the same cross-reactive antigenic determinant(s), heart cross-reactive antibodies were first purified by affinity chromatography on a column of pep M19 covalently linked to Sepharose beads. Specific antibody eluted from the column had an ELISA titer of 12,800 against both pep M19 and pep M5, and a four plus immunofluorescence reaction against sarcolemmal membranes. In contrast to the parent antiserum, the purified antibody failed to precipitate pep M5 as determined by agar gel diffusion. Neither the original serum nor the purified antibody precipitated pep M19, suggesting that the cross-reactive antibody was directed against a single, nonrepeating antigenic determinant on each pep M protein (21). The purified heart-reactive antibodies were then covalently linked to CH-Sepharose, which was used to prepare an affinity column for the isolation of the cross-reactive antigen from human heart tissue.

The affinity-purified cross-reactive sarcolemmal membrane antigen migrated as a single polypeptide of  $\approx 215,000$  mol wt in the unreduced state (Fig. 1 B). The purified antigen corresponded to a major protein band in the crude detergent extract of

sarcolemmal membranes (Fig. 1 A). Under reducing conditions, the antigen migrated as four distinct polypeptides ranging from 58,000 to 78,000 mol wt (Fig. 1 D). Three of these polypeptides reacted with the pep M5 cross-reactive antiserum after electroblot transfer (Fig. 1 E). Taken together, these initial studies indicate that the cross-reactive antigen of sarcolemmal membranes is a complex protein composed of four peptide subunits linked by disulfide bonds; furthermore, three of the four subunits were capable of binding the opsonic pep M5 heart cross-reactive antibodies.

### Discussion

In these studies, we have demonstrated that type 5 streptococcal M protein bears at least one protective antigenic determinant that is shared by human heart tissue. Rabbit antiserum raised against a highly purified fragment of type 5 M protein cross-reacted with sarcolemmal membranes of human heart and type 19 M protein. All of the cross-reactive antibodies directed against human heart were absorbed by type 5 as well as type 19 streptococci, and conversely, all of the opsonic antibodies reactive with type 19 organisms were absorbed by sarcolemmal membranes, indicating that the opsonic antibodies in the cross-reactive antiserum were directed against a common antigenic determinant shared by sarcolemmal membranes and types 5 and 19 M proteins. The most definitive evidence that the tissue cross-reactive determinant is located within the M protein molecule itself, rather than a contaminant, was the finding that the cross-reactive antibodies eluted from sarcolemmal membranes opsonized types 5 and 19 streptococci.

The finding that the heart cross-reactive determinant was located on types 5 and

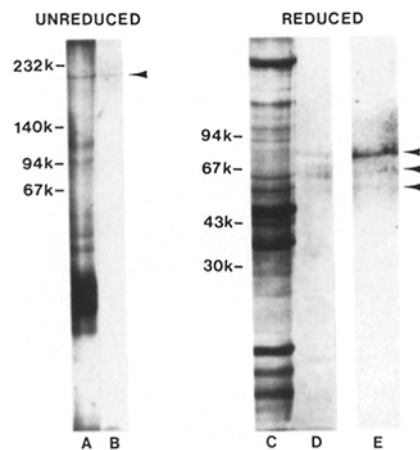


FIG. 1. SDS-PAGE of affinity-purified sarcolemmal membrane antigen of human heart that cross-reacts with pep M5 antibody. Detergent-extracted sarcolemmal membrane proteins (A) and the purified cross-reactive antigen (B) were prepared as described in Materials and Methods and electrophoresed in the unreduced state on 7.5% SDS-polyacrylamide. The molecular weight of the purified antigen (arrow) was calculated to be  $\approx 215,000$ . Electrophoresis of the detergent extract (C) and purified antigen (D) on 10% SDS-polyacrylamide after treatment with  $\beta$ -mercaptoethanol revealed that the purified antigen was composed of four subunits of 78,000, 71,000, 65,000, and 58,000 mol wt, respectively. Autoradiography of electroblot transfer of the reduced subpeptides after incubating with pep M5 antiserum and  $^{125}\text{I}$ -protein A (E) showed that three of the polypeptides (arrows) bound heart-reactive antibodies. Polyacrylamide gels were stained with silver nitrate reagent as described in Materials and Methods.



19 M proteins but on no other serotypes tested is in agreement with earlier studies by Kaplan (2, 6), who demonstrated that rabbits immunized with type 5 streptococci developed heart cross-reactive antibodies that were absorbed only by types 5 and 19 cell walls: he concluded that the tissue cross-reactive antigen was distinct from M protein, even though it copurified with the type-specific M antigen (2). This conclusion was based on the finding that type-specific moieties of type 5 M protein could be separated from the tissue cross-reactive antigen, although the cross-reactive moiety appeared to be inseparable from type-specific determinants (2). Because subsequent studies (11, 13, 14, 21–26) have shown that the M protein molecule contains several distinctly different type-specific protective determinants, these data can now be interpreted more clearly. Kaplan's studies (2) demonstrating M type-specific heart cross-reactivity used hot HCl extracts of M protein. It is known that such extracts contain a number of polypeptides, each of which contain type-specific antigenic determinants (22). Thus, it is probable that Kaplan separated these peptides into two populations, one of which contained type-specific determinants that were heart cross-reactive and the other of which contained additional type-specific determinants that were not. This interpretation is also consistent with our data, which clearly show that types 5 and 19 M proteins contain type-specific protective antigens in addition to the protective, heart cross-reactive antigen. The evidence is as follows. First, only one of nine rabbits immunized with pep M5 and none of six immunized with pep M19 developed heart cross-reactive antibodies, even though all of the rabbits developed type-specific opsonic antibodies against the immunizing serotype. Second, absorption of the pep M5 cross-reactive antiserum (7907) with sarcolemmal membranes reduced opsonization of type 5 streptococci only minimally, indicating that the cross-reactive antiserum contained large amounts of type 5 opsonic antibodies in addition to the opsonic, cross-reactive antibodies. Finally, the purified heart cross-reactive antibody failed to precipitate pep M5 or pep M19, suggesting that this antibody is directed against a distinct, nonrepeating antigenic determinant (21).

In addition to these serotype-restricted, heart cross-reactive antigens, group A streptococci contain heart cross-reactive antigens associated with structures other than the M protein. Zabriskie and Freimer (3) described heart-reactive antibodies raised against purified protoplast membranes of many different serotypes of group A streptococci. Furthermore, the cross-reactive antibodies that could be absorbed by heterologous serotypes of group A streptococcal membranes also could be absorbed by membranes from groups C and G streptococci. Kaplan (5) also reported heart-reactive antibodies raised against whole group A streptococci that were absorbed by cell walls derived from many heterologous serotypes. In a recent study, van de Rijn et al. (8) isolated a family of four protoplast membrane proteins that reacted with antibodies in the sera of rheumatic subjects. Taken together, these studies suggest that group A streptococci contain several antigens capable of evoking antibodies that cross-react with cardiac myofibers; a protective antigenic determinant shared by types 5 and 19 M proteins, a common protoplast membrane antigen(s), and a common cell wall antigen(s). The extent to which these antigenic determinants are different from each other remains to be elucidated.

We have used purified pep M5 heart-reactive antibodies to isolate a cross-reactive antigen from sarcolemmal membranes of human heart. The antigen consists of a large polypeptide ( $\approx 215,000$  mol wt) composed of subunits apparently cross-linked by

disulfide bonds; three of the reduced subunits reacted with the pep M5 cross-reactive antiserum. Although there is no evidence by any means that heart cross-reactive antibodies raised by streptococcal products are injurious to heart tissue *in vivo*, the continued studies of the immunological cross-reactions between structurally defined protein antigens of bacteria and specific membrane proteins of the animal host may provide new insights into the pathogenesis of autoimmune diseases in general.

In conclusion, our studies provide the most definitive evidence that a heart cross-reactive antigenic determinant is inherent in the structure of certain streptococcal M proteins. It is clear, however, that these M proteins contain additional protective antigenic determinants (probably the majority) that are not heart cross-reactive. These studies emphasize the need to isolate and identify subpeptides of M proteins that contain protective, as opposed to tissue cross-reactive, antigenic determinants. Identification and perhaps synthesis of protective and tissue cross-reactive peptides would be important not only for the development of safe streptococcal vaccines, but also in elucidating the pathogenesis of acute rheumatic heart disease.

### Summary

We present definitive evidence that at least one protective antigenic determinant on type 5 M protein of group A streptococci evokes antibody that is cross-reactive with human heart tissue. One of nine rabbits immunized with a peptide fragment of type 5 M protein (pep M5) produced antibody that cross-reacted by immunofluorescence with sarcolemmal membranes of human heart. The cross-reactive antibody could be removed by absorbing the antiserum with sarcolemmal membranes, types 5 and 19 streptococci, or their pepsin-extracted M proteins, but with no other serotypes tested. Although each of the pep M5 immune sera was opsonic for type 5 streptococci, only the heart-reactive antiserum opsonized type 19 streptococci. The opsonization of type 19 streptococci was abolished by absorbing the antiserum with sarcolemmal membranes isolated from human heart tissue. Purified heart-reactive antibodies eluted from sarcolemmal membranes opsonized both types 5 and 19 streptococci, indicating that the heart cross-reactive determinant of type 5 M protein is cross-protective. The cross-reactive antigen was purified by affinity chromatography from detergent extracts of sarcolemmal membranes and determined to be a complex protein composed of four subunits apparently linked by disulfide bonds.

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