

EPITOPES OF STREPTOCOCCAL M PROTEINS SHARED WITH CARDIAC MYOSIN

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It has been known for many years that group A streptococci contain antigens that are immunologically crossreactive with human tissues, especially the myocardium (1-9). Some of the crossreactive streptococcal antigens are components of the cell wall (2, 5, 6) while others have been localized to the cell membrane (3, 8, 9). We have previously shown that several streptococcal M proteins, which are the protective surface antigens of the organisms, contain epitopes that crossreact with sarcolemmal membrane proteins of human heart (10, 11). In addition, it has recently been shown that group A streptococci contain antigens that crossreact with myosin (12).

The present study was undertaken to determine whether streptococcal M proteins are immunologically crossreactive with cardiac myosin. We present data to show that pepsin-extracted and purified type 5 streptococcal M protein (pep M5)¹ evoked antibodies that crossreacted with cardiac myosin as determined by enzyme-linked immunosorbent assays (ELISA). In ELISA inhibition assays, the myosin-crossreactive antibodies were totally inhibited by pep M5 and partially inhibited by pep M6 and pep M19 but not by pep M24. Furthermore, the crossreactive antibodies, which were affinity purified on a myosin-Sepharose column, opsonized type 5 streptococci, indicating that the myosin-reactive antibodies were directed against protective determinants on the surface of the bacteria.

Materials and Methods

Extraction and Purification of Streptococcal M Proteins. Types 5, 6, 19, and 24 M proteins were purified from limited peptic digests of whole streptococci as previously described (13-16). The purified proteins (pep M) were judged to be homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (13).

Immunization of Animals. Three New Zealand White rabbits were immunized each with a single injection of 300 μ g of pep M5 emulsified in complete Freund's adjuvant (11, 13). Serum was obtained before immunization and at 2-wk intervals thereafter.

Antibody Assays. M protein-specific and myosin-crossreactive antibodies were detected

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

by ELISA, as previously described (10, 17, 18). Rabbit cardiac myosin, myosin light chains, and actin were obtained from Sigma Chemical Co., St. Louis, MO. Human skeletal muscle α -tropomyosin was a generous gift from Dr. A. E. Romero-Herrera (Wayne State University, Detroit, MI). ELISA inhibition experiments were performed by incubating increasing concentrations of antigens with a constant dilution of antiserum and then adding the mixture to the antigen-coated wells (18).

Opsonic antibodies were detected by in vitro opsonophagocytic assays, as reported previously (13). The test mixtures consisted of 0.05 ml of a standard suspension of type 5 streptococci, 0.1 ml test serum, and 0.4 ml whole, heparinized (10 U/ml) blood. After rotating the mixture 45 min at 37°C, the percentage of neutrophils with associated streptococci (percent opsonization) was estimated by microscopic counts of stained smears. Opsonization inhibition assays were performed by preincubating the test serum with 100 μ g pep M5 or myosin for 30 min at 37°C before adding the type 5 organisms (13).

Affinity Purification of Myosin-specific Antibodies. Antibodies evoked by pep M5 that crossreacted with myosin were affinity purified over a column of myosin covalently linked to AH-Sepharose 4B (Pharmacia, Inc., Uppsala, Sweden) by methods previously described (10, 11). The myosin-specific antibodies were eluted with 0.2 M glycine/0.2 M NaCl, pH 2.8, dialyzed against 0.02 M phosphate/0.15 M NaCl, pH 7.4 (PBS) and concentrated to the original serum volume by membrane filtration (YM30 membrane; Amicon Corp., Scientific Systems Div., Lexington, MA).

Transblot Analyses. SDS-PAGE of myocardial proteins and myosin was performed on continuous gradient gels ranging from 10 to 20% or 7.5–15%. Electrophoresed proteins were transferred to nitrocellulose paper (10, 11) which was cut into vertical strips and incubated overnight with pep M5 antiserum 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 proteins (20 μ g/ml) to the diluted antibody before incubating with the nitrocellulose strips. In some experiments the pep M5 antiserum was absorbed with rabbit cardiac myosin that had been electrophoretically transferred to nitrocellulose paper. The horizontal strip containing the myosin heavy chains was cut into small pieces (5 mm), blocked in Tris-BSA, and incubated by constant rotation with the diluted antiserum as above. The absorbed antiserum was then incubated with nitrocellulose strips containing electrophoresed proteins from human myocardial tissue obtained at autopsy.

Myosin Antibodies in Sera from Patients with Acute Rheumatic Fever. Sera from patients with acute rheumatic fever (ARF), their siblings, hospitalized controls with and without inflammatory diseases, and patients with poststreptococcal glomerulonephritis (AGN) were obtained during a separate study between 1981 and 1984 in the Kingdom of Saudi Arabia.² The diagnosis of ARF was based on the modified Jones criteria (19). Rheumatic carditis was assumed to be absent if there were no cardiac murmurs, no evidence of pericarditis, and a normal electrocardiogram. ELISA results are reported as the optical density of the reaction after incubating each serum with cardiac myosin at a dilution of 1:400. Statistical significance was calculated using Student's *t* test for the difference between means of unpaired samples.

Results

Myosin Crossreactive Antibodies Evoked by Pep M5. Each of three rabbits immunized with a single 300 μ g dose of pep M5 developed significant levels of antibody against cardiac myosin (Table I). The pep M5-immune sera also crossreacted with the heterologous antigens pep M6 and pep M19, but not pep M24. Additional antigens that were tested in ELISA with negative results included myosin light chains, actin, and α -tropomyosin (data not shown).

Because the pep M5 antibodies crossreacted with myosin, pep M6, and pep M19, ELISA inhibition experiments were performed with one of the antisera to

² Norton, W. L., J. B. Dale, A. L. Bisno, and E. H. Beachey. Manuscript in preparation.

TABLE I
M Protein and Myosin Antibodies Evoked in Rabbits Immunized with Pep M5

Antiserum	ELISA titer against:				
	Pep M5*	Pep M6*	Pep M19*	Pep M24*	Myosin
8329	102,400	25,600	6,400	<200	3,200
8331	25,600	25,600	1,600	<200	800
8332	25,600	12,800	3,200	<200	1,600
Preimmune pool	<200	<200	<200	<200	<200

* These data are similar to those reported previously (11).

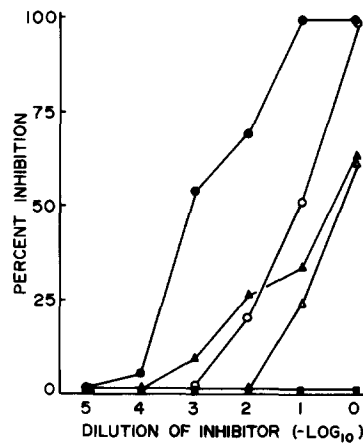


FIGURE 1. M protein specificity of myosin-crossreactive antibodies evoked by pep M5, as determined by ELISA inhibition assays. Inhibition of antibody binding to myosin was measured with increasing concentrations of myosin (O), pep M5 (●), pep M6 (Δ), pep M19 (▲), or pep M24 (■). The highest concentration of soluble inhibitor was 40 $\mu\text{g}/\text{ml}$.

determine the M protein specificity of the myosin-crossreactive antibodies (Fig. 1). The binding of all of the myosin-crossreactive antibodies was inhibited by pep M5, the immunogen, and myosin, the solid phase test antigen (Fig. 1). Binding was partially inhibited by pep M6 and pep M19 but not at all by pep M24. These data show that pep M5 evokes antibodies that crossreact with cardiac myosin; some of the myosin antibodies are inhibited by pep M6 and pep M19, indicating that all three M proteins share epitopes with myosin.

Opsonization of Type 5 Streptococci by Myosin-crossreactive Antibodies. To determine whether the pep M5 antibodies that crossreacted with myosin were evoked by protective epitopes on the M protein molecule, opsonization tests were performed with antibodies that were affinity purified over a myosin-Sepharose column. The myosin-crossreactive antibodies eluted from the column opsonized type 5 streptococci (Table II). The opsonization was totally inhibited by pep M5 and myosin, confirming the specificity of the affinity-purified antibodies. In the control experiment, myosin failed to inhibit the opsonization of type 5 streptococci by the unfractionated pep M5 antiserum, indicating that the majority of the antibodies were M protein specific and not myosin crossreactive. These results show that at least some of the myosin-crossreactive antibodies evoked by

TABLE II
Opsonization of Type 5 Streptococci by Affinity-purified Myosin-crossreactive Antibodies

Antiserum	Inhibitor	Percent opsonization of type 5 streptococci
8329 (16 wk)	None	100
	Pep M5	4
	Myosin	98
8329 eluted from myosin	None	98
	Pep M5	2
	Myosin	6
8329 preimmune	None	0

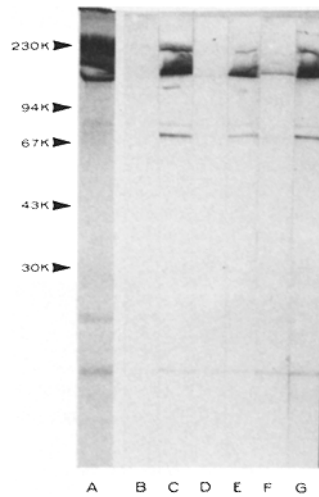


FIGURE 2. M protein specificity of myosin-crossreactive antibodies evoked by pep M5, as determined by immunoblot analyses. Purified rabbit cardiac myosin was electrophoresed under reducing conditions on an SDS-polyacrylamide continuous gradient gel, ranging from 10 to 20%. A portion of the gel was stained with Coomassie Blue (A), and the rest was transferred to nitrocellulose strips (B-G). Preimmune rabbit serum was negative (B) and the immune serum reacted strongly with the heavy chain of myosin (C). The antibodies were totally inhibited by pep M5 (D), partially inhibited by pep M6 (E) and pep M19 (F), and not at all by pep M24 (G).

pep M5 are directed against protective epitopes on the native type 5 M protein that are exposed on the surface of the organism.

Transblot Analyses of Myosin-crossreactive Antibodies. Immunoblot analyses were performed to demonstrate directly the binding of pep M5 antibodies to myosin (Fig. 2). Purified rabbit cardiac myosin was electrophoresed (Fig. 2A) and transferred to nitrocellulose strips (Fig. 2, B-G). The preimmune serum did not react with myosin (Fig. 2B), while the immune serum was strongly reactive (Fig. 2C). Inhibition studies showed that pep M5 totally inhibited antibody binding to the heavy chain of myosin (Fig. 2D). Although the antiserum reacted weakly with one of the light chains of myosin (Fig. 2C), the antibody was not inhibited by pep M5. Pep M6 and pep M19 partially inhibited the crossreactive

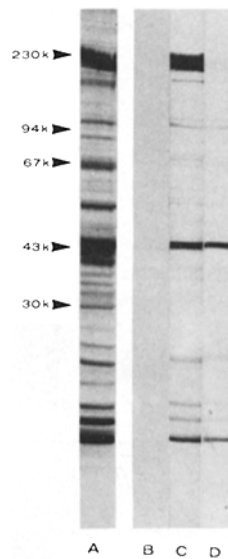


FIGURE 3. Immunoblot analysis of epitopes shared between rabbit and human cardiac myosin. SDS-extracted human myocardial proteins were electrophoresed under reducing conditions on an SDS-polyacrylamide continuous gradient gel, ranging from 7.5 to 15%. Multiple protein bands were observed on the stained gel (A). Immunoblot analyses revealed that the preimmune serum did not react with human myocardial proteins (B) and the pep M5 immune serum reacted with several proteins (C). Absorption of the immune serum with rabbit myosin removed the antibodies binding to the heavy chain of human myosin but not to several other myocardial proteins (D).

antibodies (Fig. 2, E and F, respectively), whereas pep M24 had no effect (Fig. 2 G).

To show that rabbit and human cardiac myosin both contain the pep M5-crossreactive epitopes, we performed immunoblots with SDS-extracted human myocardial proteins (Fig. 3A). The preimmune serum did not react with myocardial proteins (Fig. 3B). As previously shown (11), pep M5 antibodies cross-reacted with a number of human heart proteins (Fig. 3C). Absorption of the immune serum with rabbit cardiac myosin removed the antibodies that reacted with a protein equal in size to the myosin heavy chain (M_r 230,000) (Fig. 3D). These data indicate that the pep M5-crossreactive epitopes are shared by human and rabbit cardiac myosin. In addition, pep M5 antibodies crossreact with several other human myocardial proteins, and these antibodies were not absorbed by myosin.

Myosin Antibodies in the Sera of ARF Patients. The finding that several streptococcal M proteins contained epitopes that crossreacted with cardiac myosin prompted us to test sera from patients with acute rheumatic fever for the presence of myosin antibodies. Controls were sera from healthy siblings of ARF patients, unrelated children who were hospitalized at the same time as the ARF patients, and children with AGN. The ELISA reaction against myosin of each serum diluted 1:400 was compared for each group (Fig. 4). The mean ELISA reading of the sera from ARF patients (Fig. 4A) was 0.56, which was significantly higher than the mean values obtained with sera from siblings, hospital controls, and

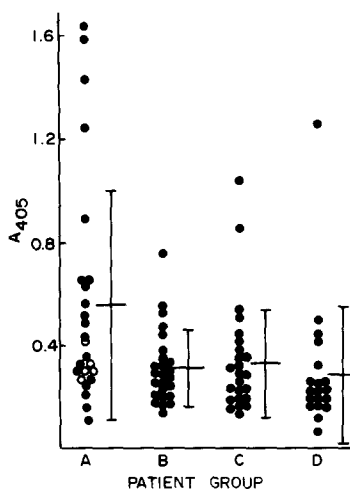


FIGURE 4. Myosin antibodies in sera from patients with acute rheumatic fever. Sera were diluted 1:400 and the absorbance of the ELISA reaction was recorded for ARF patients (A), their healthy siblings (B), hospitalized control patients (C), and patients with AGN (D). Mean values \pm SD are indicated by the bars. Open circles represent ARF patients without clinical evidence of carditis.

AGN patients; the latter resulted in mean readings of 0.31 ($P < 0.01$), 0.34 ($P < 0.025$), and 0.30 ($P < 0.025$), respectively. Sera from patients with ARF who had no clinical evidence of carditis (Fig. 4A, open circles) all showed values less than the mean for the ARF group. Although there was considerable overlap between the experimental and control groups, it is clear that sera from some ARF patients showed the highest reactivities with cardiac myosin.

Discussion

In the present study we have shown that M proteins from three different serotypes of group A streptococci contain epitopes that crossreact with cardiac myosin. The affinity-purified myosin antibodies opsonized type 5 streptococci, indicating that they were directed against protective M protein epitopes on the surface of the organism. The pep M5 antibodies reacted with intact myosin in ELISA, but not myosin light chains, suggesting that the crossreactive epitopes reside in the heavy chain region of the molecule. Western blot analyses showed that all of the myosin-crossreactive antibodies were directed against pep M5, but not all of the pep M5 heart-crossreactive antibodies were directed against myosin. Rabbit myosin absorbed the antibodies directed against human myosin, indicating that the M protein-crossreactive epitopes are present in the myosin molecules from each species.

Immunological similarities between group A streptococci and host tissues have been recognized for many years (1-8). In most of these studies, neither the streptococcal nor the host tissue antigens responsible for the crossreactions were identified. Previous studies in our laboratory (10, 11) have shown that types 5, 6, and 19 streptococcal M proteins crossreacted with sarcolemmal membrane proteins of human heart. Most recently, Krisher and Cunningham (12) have

described a murine monoclonal antibody raised against type 5 streptococci that crossreacts with myosin. The authors provide evidence that this antibody is not reactive with the surface M protein. Thus, there may be at least two different streptococcal components capable of evoking crossreactive immunity against myosin: one, the surface M protein, and the other, a membrane-associated antigen (9).

The immunological crossreactions reported here between myosin and M protein may be directly related to certain structural similarities between the two molecules. Recent determinations of the primary structures of several M proteins obtained from different streptococcal serotypes (13–17, 20–25) revealed that a common feature was a seven residue periodicity with respect to nonpolar and charged amino acids (21, 24). The heptapeptide repeats, which extend through the majority of the M protein molecules for which primary structures have been determined, are characteristic of alpha-helical coiled-coil structures (25). The primary structure of the rod region of myosin, which constitutes ~50% of the heavy chain (26), also shows heptapeptide repeats that would predict an alpha-helical coiled-coil three-dimensional structure (26–28). Thus, a protein derived from a prokaryote and one derived from a eukaryote, each with seemingly unrelated functions, share similar configurations that may be responsible, at least in part, for immunological crossreactions. An exhaustive comparison of the amino acid sequences of the M proteins and myosin heavy chains from mammalian muscle may reveal regions of primary structural homology that could represent crossreactive epitopes.

The significance of immunological crossreactions between group A streptococci and host tissues, in relation to the pathogenesis of acute rheumatic fever and rheumatic carditis is unknown. In the present study we showed that some sera from acute rheumatic fever patients reacted very strongly with cardiac myosin. We do not mean to imply a pathologic role for these crossreactions. Previous studies (29–32) have demonstrated heart-reactive antibodies in the sera of patients suffering other forms of myocardial injury. To our knowledge, the reactivity of these antibodies with myosin has not been reported. Further studies are necessary to determine the significance of myosin-crossreactive antibodies, whether they are found in the sera of patients with rheumatic heart disease or patients with other forms of myocardial damage. It is known that many patients with ARF have exaggerated immune responses (19), and our data may simply reflect the general immune status of the individual rather than an abnormal immunologic response to myosin in particular. Nonetheless, we believe that continued efforts to identify streptococcal and host tissue antigens that are immunologically crossreactive may provide valuable insights into the host-pathogen relationship and, possibly, the pathogenesis of autoimmune diseases such as acute rheumatic fever.

Summary

We present evidence that M proteins from three different serotypes of group A streptococci share epitopes with cardiac myosin. Rabbit antisera evoked by a purified fragment of type 5 M protein crossreacted with myosin, but not α -tropomyosin, actin, or myosin light chains. In enzyme-linked immunosorbent

assays, the myosin-crossreactive antibodies were totally inhibited by type 5 M protein and partially inhibited by types 6 and 19 M proteins. The affinity-purified myosin antibodies opsonized type 5 streptococci, indicating that they were directed against protective M protein epitopes on the surface of the organisms. Immunoblot analyses demonstrated the binding of the crossreactive antibodies to myosin heavy chains. Sera from patients with acute rheumatic fever showed significantly stronger reactions with myosin than did sera from their siblings, hospitalized controls, or patients with poststreptococcal glomerulonephritis.

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