SEQUENCE OF MYOSIN-CROSSREACTIVE EPITOPES OF STREPTOCOCCAL M PROTEIN

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The M protein protruding from the surface of group A streptococci is the major virulence factor of these organisms. Only antibodies against M protein opsonize the bacteria and protect mice against challenge infections (1). Recently, we discovered that M proteins from certain serotypes of group A streptococci contain epitopes that crossreact with human cardiac sarcolemma (2, 3) and myosin (4). This has hampered efforts to develop M protein vaccines that would prevent the streptococcal infections that can trigger acute rheumatic fever and rheumatic heart disease, mainly because of the fear that the tissue-crossreactive epitopes within the vaccine preparations may cause, rather than prevent, rheumatic heart disease. This has lead to efforts to fragment M protein molecules, in an attempt to identify protective as opposed to tissue-crossreactive epitopes. In previous studies (5–7), we showed that synthetic peptide copies of the N-terminal regions of several M protein molecules were immunogenic and evoked protective, but not heart-crossreactive antibodies. We now report that another peptide, SM5(84-116) of type 5 M protein, contains the majority of myosin-crossreactive epitopes of the pepsin-extracted M protein.

Materials and Methods

Extraction and Purification of M Protein. M protein was purified from limited peptic digests of type 5 group A streptococci as previously described (8–10). The purified protein, designated pep M5, was judged to be homogeneous by SDS-PAGE and N-terminal sequence analysis (9).

Computer Analyses of Protein Sequences. The amino acid sequence of pep M5 (11, 12) was compared to published sequences of the heavy chain of myosin from nematode (13), rabbit skeletal (14), and rabbit ventricular (15) muscle using the sequence analysis program (International Biotechnologies, Inc., New Haven, CT) developed by Jim Pustell (16). The parameters selected were: range = 1, scale = 1, hash = 1, jump = 1, step = 1, and minimum value plotted = 60.

Chemical Synthesis of Peptides. Polypeptide copies spanning the entire pep M5 molecule (11, 12) were synthesized by an automated peptide synthesizer (Beckman Instruments, Fullerton, CA) by a solid-phase method, as previously described (6, 7). The peptides were purified by HPLC and their compositions and sequences were confirmed by quantitative amino acid analysis and automated Edman degradation, respectively (5, 6, 9). The peptides synthesized for this study are designated SM5(28-54)C, SM5(55-84)C, SM5(84-116)C, SM5(93-116)C, SM5(101-116)C, SM5(117-146)C, SM5(134-163)C, and SM5(164-197)C.

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An additional peptide, SM5(1-35), was synthesized for a previous study (7), and was also used in these experiments.

**Conjugation of SM5(84-116)C.** SM5(84-116)C was conjugated to KLH using succinimidyl 4-(N-maleimido-methyl) cyclohexane-1-carboxylate (Pierce Chemical Co., Rockford, IL) as previously described (7).

**Immunization of Rabbits.** New Zealand White rabbits were injected with 300 μg of pep M5 emulsified in CFA (3, 9). Blood was obtained before the initial injection and at 2-wk intervals thereafter. At 4 and 10 wk, the animals were given 300-μg booster injections in PBS. Rabbit antisera against SM5(84-116)C-KLH were similarly prepared using 100 μg of the conjugate for each dose (7).

**Detection of Myosin-crossreactive Antibodies.** Sera were tested for the presence of myosin-crossreactive antibodies by ELISA, using purified rabbit muscle myosin (Sigma Chemical Co., St. Louis, MO) as the solid-phase antigen (4). Myosin-crossreactive epitopes were detected by ELISA inhibition assays, which were performed by incubating a constant dilution of antiserum with increasing concentrations of synthetic peptides, pep M5 or myosin, as soluble inhibitors (4).

Western immunoblots were performed as previously described (3, 4) using SDS-extracted myocardial proteins from human heart tissue. In immunoblot inhibition experiments, the diluted antisera were first incubated with synthetic peptides or pep M5 before adding the nitrocellulose strips (4).

**Results and Discussion**

Previous studies have shown that pep M5 shares epitopes with the heavy chain of myosin (4, 17), and that streptococcal M proteins and the rod region of myosin both have primary structures that are characteristic of α-helical coiled-coil molecules (18). Therefore, in an attempt to identify the primary structures of pep M5 that might contain myosin-crossreactive epitopes, a computer-assisted comparison of the amino acid sequences of both molecules was performed (16). Although there was a significant number of regions showing primary structural homology between pep M5 and myosin, ranging up to four amino acids in length, there was no single region of pep M5 that contained a majority of the homologous primary structures (J. B. Dale and E. H. Beachey, manuscript in preparation). For this reason, we elected to synthesize peptides spanning the entire pep M5 molecule, which were used in the following studies.

To localize the myosin-crossreactive epitopes of pep M5, ELISA inhibition experiments were performed using the synthetic peptides (see Materials and Methods) as soluble inhibitors of myosin-crossreactive antibodies evoked by pep M5 (Fig. 1). Only SM5(84-116)C inhibited pep M5 antibody binding to myosin. This peptide inhibited ELISA by 75%, as compared to 100% inhibition by pep M5, the immunogen, and 95% by myosin, the ELISA antigen. None of the remaining synthetic peptides inhibited the binding of antibodies to myosin. Because most of the myosin-crossreactive antibodies appeared to be binding to epitopes located in SM5(84-116)C, we also tested the subpeptides SM5(93-116)C and SM5(101-116)C for their ability to inhibit the ELISA reaction. Neither of these peptides inhibited the binding of myosin-crossreactive antibodies (Fig. 1), indicating either that the majority of crossreactive antibodies were recognizing epitopes located between amino acids 84–93, or that the subpeptides were not of sufficient length to retain the conformation required for antibody recognition.

The ELISA inhibition experiments were performed with one of three pep M5 antisera, all of which have previously been shown (4) to contain myosin-cross-
reactive antibodies. To show that the immunogenicity of the crossreactive epitopes within the 84–116 region of pep M5 was not related to individual variations in immune responses, inhibition experiments were also performed using the remaining antisera. In each case, SM5(84-116)C was the only synthetic peptide that inhibited the myosin crossreactions, and the patterns of inhibition were similar to that shown in Fig. 1.

The results obtained by ELISA inhibition tests were confirmed by Western immunoblots using myocardial proteins extracted in SDS from whole human heart tissue (Fig. 2). SM5(84-116)C almost totally blocked the reaction of pep M5 antibodies with a protein corresponding to the M₁ of the heavy chain of myosin (Fig. 2D). The N-terminal peptide SM5(1-35) showed no visible inhibition (Fig. 2E), nor did the remaining synthetic peptides (data not shown).

To test the immunogenicity of the myosin-crossreactive epitopes within SM5(84-116)C, three rabbits were immunized with the synthetic peptide conjugated to KLH. Two of the animals developed antibodies crossreactive with myosin, with titers of 200 and 400, respectively, while the preimmune sera all had titers <100. Thus, the myosin-crossreactive epitopes retained partial immunogenicity; the titers of the crossreactive antibodies were significantly lower than those previously reported (4) after immunization with pep M5, indicating discrepancies between the immunogenicity and antigenicity of the epitopes within the synthetic peptide.

Taken together, these results suggest that the majority of the myosin-crossreactive antibodies evoked by pep M5 are directed against epitopes that are located within a limited region of the molecule. We have previously shown (5–7) that synthetic peptide copies of the N-terminal region of several serotypes of M protein contain protective but not tissue-crossreactive epitopes (5–7). Therefore, it appears that, in some cases, type-specific and heart-crossreactive epitopes
Western immunoblot inhibition of pep M5 myosin-crossreactive antibodies. 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 pep M5 immune serum reacted strongly with a protein band of 230,000 M, (B), corresponding to the heavy chain of myosin (A). The myosin-crossreactive antibodies were totally inhibited by pep M5 (C), almost totally inhibited by SM5(84-116)C (D), and not inhibited at all by SM5(1-35) (E), or any of the remaining SM5 peptides (data not shown). M, ×10^-3 are shown at left.

of M protein are located within different primary structures, which may be identified by using synthetic peptide fragments of the native molecule. The finding that primary structural homologies between pep M5 and myosin were present throughout the M protein molecule yet SM5(84-116)C was the only peptide that inhibited myosin antibodies suggests that the crossreactive epitopes within the 84–116 region may be immunodominant when presented to the immune system in the context of the entire polypeptide fragment. It is possible, therefore, that additional myosin-crossreactive epitopes exist within the primary structure of pep M5 that are not detected by any of the pep M5 antisera tested.

It is of interest to note that the myosin-crossreactive antisera used in this study were raised against a pep M5 fragment from one strain of type 5 streptococci (Manfredo) (3, 4, 12), while the synthetic peptides [except SM5(1-35)] were synthesized according to the primary structure of pep M5 extracted from a different strain (B788) (11). The pep M5 from strain B788 totally inhibited the myosin-crossreactive antibodies evoked by pep M5 from strain Manfredo, as determined by ELISA and Western blot inhibition experiments (data not shown). Thus, the myosin-crossreactive epitopes appear to be conserved from one strain.
to another, and the inability of SM5(84-116)C to totally inhibit the myosin antibodies is probably not due to differences in immunological crossreactivity within this region of pep M5 extracted from the two strains. Therefore, our data do not rule out the presence of additional myosin-crossreactive epitopes within pep M5 that may not be adequately represented by the series of synthetic peptides tested.

Although SM5(84-116)C significantly inhibited pep M5 antibody binding to the heavy chain of myosin in Western immunoblots (see Fig. 2), antibody reactions with additional polypeptides present in human heart tissue were not inhibited. In addition, immunofluorescence inhibition tests using frozen sections of human myocardium showed that SM5(84-116)C only partially inhibited the binding of pep M5 heart-crossreactive antibodies, whereas pep M5 completely inhibited the crossreactions (data not shown). We conclude that pep M5 contains multiple heart-crossreactive epitopes (3), not all of which are myosin-crossreactive (4). Continued efforts to precisely identify the primary structures of M proteins that are immunologically crossreactive with human tissues should not only allow the development of safe and effective M protein vaccines, but also may provide insights into the pathogenesis of rheumatic heart disease.

Summary

Group A streptococcal M proteins contain epitopes that crossreact with sarcolemmal membrane proteins of human myocardium and myosin. In the present study, synthetic peptide copies spanning the entire 197-residue pepsin extracted fragment of type 5 M protein were used to localize the myosin-crossreactive epitopes of the molecule. Peptide 84–116 inhibited by 75% the binding of myosin-crossreactive antibodies evoked by pep M5, as determined by ELISA. Immunoblot inhibition studies confirmed that peptide 84–116 almost totally inhibited the binding of pep M5 antibodies to the heavy chain of human cardiac myosin. None of the remaining synthetic peptides, including peptide 1–35, which contains protective epitopes, inhibited antibodies binding to myosin. Two of three rabbits immunized with peptide 84–116 developed low but significant levels of antibodies crossreactive with myosin. Identification of the primary structures containing tissue-crossreactive as opposed to protective epitopes should not only allow the development of safe and effective M protein vaccines, but may also provide insights into the pathogenesis of rheumatic heart disease.

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