Phosphorylase-Cross- Reactive Antibodies Evoked by Streptococcal M Protein

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Rabbit antisera evoked by type 5 streptococcal M protein (M5) were screened by enzyme-linked immunosorbent assay (ELISA) for immunological cross-reactivity with purified rabbit muscle phosphorylases a and b. Of 10 pep M5 antisera tested, 3 showed significant cross-reactivity with both forms of the enzyme. ELISA inhibition studies using one of the pep M5 antisera showed that all of the phosphorylase b antibodies were inhibited by pep M5, the immunogen, and phosphorylase b, the ELISA antigen. All of the antibodies were also inhibited by pep M6 and pep M19, but not by pep M24, indicating that the cross-reactive epitopes were shared by multiple serotypes of M protein. Western blot (immunoblot) analyses showed that pep M5 antisera reacted strongly with the subunit of phosphorylase b. In addition, purified phosphorylase partially inhibited the binding of pep M5 antibodies to a 95-kilodalton protein of human myocardium. One of the three cross-reactive pep M5 antisera inhibited the enzymatic activity of phosphorylase a in a dose-related fashion, reaching a maximum inhibition of 75%. The enzymatic activity in the presence of antibody was totally restored when the antisera was first incubated with pep M5.

A number of recent studies have shown that M proteins of certain serotypes of group A streptococci contain epitopes that cross-react with myocardial antigens, including sarcolemmal membrane proteins, myosin, and several unidentified antigens (4-10, 23). It has long been recognized that rheumatic fever and rheumatic heart disease are triggered by antecedent group A streptococcal infections. Therefore, immunological cross-reactions between streptococcal antigens and tissue antigens have been of considerable interest because of the role that autoimmunity may play in the pathogenesis of the nonsuppurative sequelae of streptococcal infections.

Phosphorylase is an enzyme that is present in heart, skeletal muscle, liver, and brain; it catalyzes the first step in the degradation of glycogen (12). The role of phosphorylase is to provide glucose 1-phosphate, which in muscle is linked to the energy needs of contraction (12). In the present study, we show that type 5 M protein of group A streptococci (M5) evokes antibodies that cross-react with muscle phosphorylase. Moreover, some of the antibodies inhibit the activity of the enzyme. Our findings may have bearing on the pathogenesis of autoimmune sequelae of streptococcal infections.

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 (3). The purified proteins (pep M) were judged to be homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (3).

Immunization of animals. New Zealand White rabbits were each immunized with 300 μg of pep M5 emulsified in complete Freund adjuvant (CFA) (3). Serum was obtained before immunization and at 2-week intervals thereafter.

Detection of phosphorylase-cross-reactive antibodies. Sera were tested for the presence of phosphorylase-cross-reactive antibodies by enzyme-linked immunosorbent assay (ELISA) by using purified rabbit muscle phosphorylases a and b (Sigma Chemical Co., St. Louis, Mo.) as solid-phase antigens (11). The M protein serotype specificity of phosphorylase-cross-reactive antibodies was determined by ELISA inhibition assays, which were performed by incubating a constant dilution of antisera with increasing concentrations of pep M protein or phosphorylase as soluble inhibitors (11).

Western blotting (immunoblotting) was performed as previously described (7, 9) by using either sodium dodecyl sulfate-extracted myocardial proteins from human heart tissue or purified rabbit muscle phosphorylase. Immunoblot inhibition experiments were performed by first incubating the diluted antisera with the pep M protein or phosphorylase before the nitrocellulose strips were added (9).

Assay of phosphorylase activity. The enzymatic activity of phosphorylase a was measured by using a colorimetric assay kit (Sigma) according to the manufacturer’s instructions. Inhibition of phosphorylase activity by pep M5 antisera was assayed by adding increasing concentrations of antisera diluted in phosphate-buffered saline (PBS) to the reaction mixture and comparing the maximum rate of enzyme activity to control values obtained with PBS or preimmune sera.

Sequence comparison of pep M5 and phosphorylase. Dot-matrix analysis of the primary structure of pep M5, which was deduced from the emm 5 gene sequence encoding the first 207 amino acids of the structural protein (22) and phosphorylase (12), was performed by using BIONET software.

RESULTS

Phosphorylase-cross-reactive antibodies evoked by pep M5. Previous studies using pep M5 antisera reacted with immunoblots of human myocardial tissue revealed multiple cross-reactive proteins, one of which migrated adjacent to the molecular mass marker phosphorylase b (8, 9). For this reason, we tested pep M5 rabbit antisera for the presence of phosphorylase-cross-reactive antibodies by ELISA. All 10

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antiseras contained significant levels of pep M5 antibodies (7, 9). Three of the ten sera tested contained low but significant levels of antibodies against phosphorylase b (Table 1).

Identical levels were obtained when phosphorylase a (the phosphorylated and more active form of the enzyme) was used as the ELISA antigen (data not shown). The remaining antiseras and the preimmune sera all showed titers of <50.

**TABLE 1.** Phosphorylase-cross-reactive antibodies evoked in rabbits immunized with Pep M5

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>ELISA titer against:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pep M5</td>
</tr>
<tr>
<td>8205</td>
<td>25,600</td>
</tr>
<tr>
<td>8329</td>
<td>102,400</td>
</tr>
<tr>
<td>8331</td>
<td>25,600</td>
</tr>
<tr>
<td>Preimmune</td>
<td>&lt;50</td>
</tr>
</tbody>
</table>

* ELISA titers represent the reciprocal of the last dilution of antiserum resulting in an A405 of >0.1.
* The preimmune titers for each of the three antiseras against both antigens were all <50.

**M protein specificity of phosphorylase-cross-reactive antibodies.** We have previously shown that some of the heart-cross-reactive epitopes of pep M5 were also shared with pep M6 and pep M19 (9). Therefore, we performed ELISA inhibition experiments with one of the antisera (8329) to determine the M protein specificity of the cross-reactive antibodies (Fig. 1). The binding of the phosphorylase-cross-reactive antibodies was completely inhibited by soluble pep M5, the immunogen, and phosphorylase b, which was also the solid-phase ELISA antigen (Fig. 1). In addition, pep M6 and pep M19 completely inhibited the cross-reactive antibodies, while pep M24 had no effect.

These data were confirmed by immunoblot inhibition experiments using purified rabbit muscle phosphorylase b (Fig. 2). The preimmune serum did not react with phosphorylase subunits (Fig. 2, lane A), while the pep M5 immune serum was strongly reactive (Fig. 2, lane B). The cross-reactive antibodies were completely inhibited by phosphorylase b (Fig. 2, lane C) and by pep M5, pep M6, and pep M19 (Fig. 2, lanes D to F, respectively) but not at all by pep M24 (Fig. 2, lane G). These data show that pep M5 evokes antibodies that cross-react with rabbit phosphorylase; all of the phosphorylase-cross-reactive antibodies are inhibited by pep M5, pep M6, and pep M19, indicating that all three M proteins contain a similar cross-reactive epitope(s).

In previous studies we have used a series of synthetic peptides that span the entire pep M5 molecule in order to localize tissue-cross-reactive epitopes (8, 9). Similar experiments were performed in the present study in an attempt to identify the primary structure of the phosphorylase-cross-reactive epitope(s). None of the synthetic peptides inhibited the pep M5 phosphorylase-cross-reactive antibodies, as determined by ELISA inhibition studies (data not shown), indicating that the epitope(s) was not adequately represented by the series of synthetic peptides.

**Comparison of covalent structures of pep M5 and phosphorylase.** To identify homologous covalent structures that might represent potential cross-reactive epitopes between pep M5 and phosphorylase b, we performed a dot-matrix analysis comparing the amino acid sequences of the two proteins (12, 18, 22). The longest homologous segments were QENK and TIGT. Because all of the phosphorylase-cross-reactive antibodies evoked by pep M5 were also completely inhibited by pep M6, we compared the three sequences and found that only one region, TIGT, was present in all three proteins.

**Immunoblot analysis of pep M5 antibodies that cross-react with human cardiac phosphorylase.** The experiments described above were performed with purified rabbit muscle phosphorylase. In previous studies, we have shown that pep
M5 evokes antibodies against multiple proteins in human myocardium, and the immunoblot patterns were completely inhibited by pep M5, indicating the M protein specificity of the heart-cross-reactive antibodies (9). To determine whether phosphorylase was one of the myocardial antigens recognized by pep M5 antibodies, we performed immunoblot analyses using sodium dodecyl sulfate-extracted human myocardial proteins (Fig. 3). The pep M5 antiserum contained antibodies that reacted with multiple proteins in human cardiac tissue (Fig. 3, lane B). One of these proteins migrated adjacent to purified rabbit phosphorylase b that was electrophoresed on the same gel (Fig. 3, lane A). When the pep M5 antiserum was preincubated with rabbit phosphorylase as a soluble inhibitor, antibody binding to only this myocardial protein was reduced (Fig. 3, lane C [arrow]). These data suggest that phosphorylase is one of the human myocardial antigens that cross-reacts with pep M5. The inability of even high concentrations of rabbit phosphorylase (400 μg/ml) to totally inhibit the binding of pep M5 antibodies to human cardiac phosphorylase suggests that human phosphorylase may contain additional pep M5-cross-reactive epitopes that are not present in the rabbit enzyme.

Inhibition of phosphorylase a activity by pep M5 antibodies. To determine whether the phosphorylase-cross-reactive antibodies evoked by pep M5 had functional activity, we tested the antisera for their ability to inhibit the enzyme in a standard colorimetric assay. One of the three cross-reactive antisera (8329) significantly inhibited phosphorylase activity in a dose-dependent manner (Fig. 4). The preimmune serum had no effect on enzymatic activity, nor did the remaining two antisera. To show that pep M5 antibodies were respon-

FIG. 3. Immunoblot analysis of pep M5 antibodies that cross-react with human cardiac phosphorylase. Purified phosphorylase b (lane A) and whole human myocardium (lanes B and C) were electrophoresed under reducing conditions on a sodium dodecyl sulfate-polyacrylamide gel, ranging from 7.5 to 15% polyacrylamide, and transferred to nitrocellulose paper. The pep M5 antiserum reacted with multiple myocardial antigens (lane B), one of which migrated adjacent to purified phosphorylase b (lane A). When the antiserum was preincubated with 400 μg of phosphorylase b per ml, the intensity of the antibody reaction with only the 95-kilodalton (kDa) protein was diminished (lane C, arrow). The heavily stained band at 230 kDa represents the heavy chain of myosin (9).

FIG. 4. Inhibition of phosphorylase a activity by pep M5 rabbit antiserum. Increasing concentrations of pep M5 antiserum (C) were added to a standard colorimetric assay of phosphorylase activity. Inhibition of enzyme activity reached a maximum of 75% at a concentration in serum of 13% (400 μl in a 3-ml reaction mixture). The preimmune serum (○) had no effect on enzyme activity. The addition of 200 μg of pep M5 to 200 μl of antiserum completely restored the enzymatic activity to control levels (data not shown).

Possible for the inhibition, we preincubated 200 μl of antiserum with 200 μg of pep M5 for 30 min at 37°C and then added the antiserum to the reaction mixture. The activity of the enzyme returned to the control value in the presence of the inhibited antiserum (data not shown), indicating that the pep M5 antibodies were responsible for enzyme inhibition.

DISCUSSION

Previous studies have shown that M proteins from several serotypes of group A streptococci contain epitopes that cross-react with human tissues (4–10, 23). Type 5 M protein contains multiple heart-cross-reactive epitopes that evoke antibodies against sarcolemmal membrane antigens (23) and myosin (5, 8). Some of these epitopes are also shared with types 6 and 19 M proteins (4, 8–10, 23). Type 1 M protein contains at least one epitope that cross-reacts with renal glomerular antigens (15, 16). In the present study we have shown that types 5, 6, and 19 M proteins are immunologically cross-reactive with phosphorylase, and one of the antisera inhibited the activity of the enzyme. In addition, immunoblot inhibition experiments suggest that phosphorylase may be one of the M protein-cross-reactive antigens of human myocardium.

Recent studies have focused on the structural characteristics of M proteins (1, 2, 13–22, 24), and a common feature is a high alpha helix potential throughout most of their lengths (13, 20, 21). The rod region of myosin has a secondary structure that is almost wholly alpha helical, yet comparison of the primary structures of pep M5 and myosin did not reveal areas of significant homology, even though most of the myosin-cross-reactive epitopes were localized to peptide 84-116 of pep M5 (10). In the present study, immunological cross-reactivity between pep M5 and phosphorylase was not easily explained on the basis of primary structural similarities. Because all of the phosphorylase-cross-reactive antibodies evoked by pep M5 were inhibited by pep M6, we compared all three sequences and found only a single homologous region greater than three amino acids in length, TIGT, which occurs twice in pep M5 (22) and three
times in pep M6 (13). However, the synthetic peptides of pep M5 that contain this sequence, SM5(134-163)C and SM5(164-197)C, did not inhibit the phosphorylase-cross-reactive antibodies. These data suggest either that this primary structure is not the cross-reactive epitope or that it does not retain the appropriate conformation within the synthetic peptide. The latter case may result in very low avidity of the cross-reactive antibodies for epitopes within the synthetic peptides compared with their binding to the native pep M5 molecule. Alternatively, the cross-reactive epitopes of M protein and phosphorylase may be conformational, that is, not completely determined by primary structural homologies, and therefore they would be difficult to identify on the basis of sequence alone. Previous studies have shown that the phosphorylase subunit contains 44% alpha helix (12). This structural similarity between phosphorylase and M protein may account for shared epitopes that are determined by both primary structure and three-dimensional conformation.

Although our results indicate a clear immunological relationship between streptococcal M proteins and muscle phosphorylase, it is possible that the cross-reactive antibodies were actually evoked by antigens expressed by the mycobacteria in the CFA. We believe that this is unlikely because the phosphorylase-cross-reactive antibodies were completely inhibited by pep M5, pep M6, and pep M19. These results indicate that there were no phosphorylase-specific antibodies evoked by the immunogen, which might be more likely if the immunogen were a third cross-reactive molecule. In addition, all 10 rabbits were immunized with pep M5 emulsified in CFA, yet sera from only 3 rabbits contained antibodies against phosphorylase. Nevertheless, we conclude that streptococcal M protein shares at least one epitope with muscle phosphorylase.

The relationship of the immunological cross-reactions between phosphorylase and M protein to the pathogenesis of rheumatic fever and rheumatic carditis is not at all clear. Phosphorylase and myosin are both intracellular proteins that would not be expected to be available for antibody binding. Previous studies, however, have shown that M proteins also evoke antibodies that bind to sarcosomal membrane antigens (9). Antibodies binding to the surface of myocardiocytes could theoretically activate complement and cause cell lysis, thus exposing cross-reactive intracellular antigens that then form in situ immune complexes that further promote tissue damage. Alternatively, intracellular antigens that are released after initial tissue injury might function as autoantigens that stimulate T-cell-mediated immune injury. Whether humoral or cell-mediated autoimmune mechanisms play any role in the pathogenesis of acute rheumatic carditis is not known. We believe that continued efforts to define the structures of M protein tissue-cross-reactive epitopes and the host antigens containing these epitopes may provide valuable tools to understand the autoimmune pathogenesis of nonsuppurative poststrepococcal sequelae.

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LITERATURE CITED


