

TYPE-SPECIFIC IMMUNOGENICITY OF A
CHEMICALLY SYNTHESIZED PEPTIDE FRAGMENT OF
TYPE 5 STREPTOCOCCAL M PROTEIN*

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The surface M protein of group A streptococci renders the organisms resistant to phagocytosis in the nonimmune host. In the immune host, antibodies against M protein opsonize the organisms, which are then readily phagocytosed and killed. These observations have served for many years as the basis for studies directed toward the development of a safe and effective M protein vaccine that would prevent streptococcal pharyngitis and thus rheumatic fever. Because group A streptococci contain several different antigens that are immunologically cross-reactive with host tissues (1-3), one of the major concerns has been that M protein vaccines may contain harmful antigens able to cause, rather than prevent, rheumatic fever. Indeed, recent studies in our laboratory have shown that a highly purified pepsin extract of type 5 M protein (pep M5) evoked antibodies that cross-reacted with a sarcolemmal membrane protein of human heart (3). The purified heart-reactive antibodies opsonized type 5 streptococci, indicating their reactivity with the surface M protein.

Having demonstrated the presence of heart cross-reactive antigens within the pepsin-derived fragment of type 5 M protein, we undertook the present study to determine the immunogenicity of two chemically synthesized peptides representing only limited regions of the native pep M5 molecule. The two synthetic peptides are copies of the amino terminal region of pep M5, representing residues 1-20 [S-M5(1-20)] and 20-40 [S-M5(20-40)]. We present evidence that neither peptide reacts with purified heart cross-reactive antibodies raised against intact pep M5. When covalently linked to tetanus toxoid and emulsified in complete Freund's adjuvant, S-M5(1-20), but not S-M5(20-40), is shown to evoke high titers of type-specific, opsonic antibodies in rabbits; none of the animals developed antibodies that were cross-reactive with sarcolemmal membranes of human heart. Our data show that immunization with a chemically synthesized peptide representing a limited region of the M protein molecule evokes type-specific, protective antibodies without eliciting tissue cross-reactive antibodies.

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Materials and Methods

Extraction and Purification of M Protein. M protein (pep M) was purified to homogeneity from limited peptic digests of types 5, 6, 19, and 24 group A streptococci as previously described (3-5).

Chemical Synthesis of S-M5(1-20) and S-M5(20-40). Polypeptides identical to the amino-terminal amino acid sequence of pep M5 (5), consisting of 20 amino acids each and overlapping at only the 20th residue, were synthesized by an automated peptide synthesizer at Peninsula Laboratories, Inc., San Carlos, CA and purified by high performance liquid chromatography (HPLC). The peptides were sequenced by automated Edman degradation with a Beckman Sequenator (model 890C; Beckman Instruments, Inc., Fullerton, CA), as previously described (4-6).

Conjugation of Synthetic Peptides to Tetanus Toxoid. Before conjugation to the peptides, the tetanus toxoid was first conjugated to L-lysine. 10 mg of the toxoid was added to 1.0 ml of 0.025% glutaraldehyde in 0.1 M NaHCO₃ and rotated at ambient temperature for 1 h. L-lysine was added to a final concentration of 0.2 M and the mixture was rotated for an additional 48 h. The conjugate was washed with 5 vol of 0.1 M NaHCO₃ using an Amicon flow cell equipped with a PM10 filter (Amicon Corp., Danvers, MA). Lysine conjugation was confirmed by increased reactivity with the ninhydrin reagent (7), and total protein was quantitated according to the principles of Lowry et al. (8). 1 mg of the toxoid and 1 mg of the synthetic peptide were then added to 1 ml of 0.1% glutaraldehyde in 0.1 M NaHCO₃ and rotated for 48 h at ambient temperature. The concentration of glutaraldehyde was then increased to 0.5%, the mixture was rotated for an additional 5 d, and the conjugate was washed with 0.1 M NaHCO₃ as described above. In some experiments, the synthetic polypeptides were covalently linked to poly-L-lysine (mol wt, 70,000; Sigma Chemical Co., St. Louis, MO) in the presence of carbodiimide (hydrogen cyanomide; Sigma Chemical Co.), as previously described (9, 10).

Immunization Procedures. Two groups of three New Zealand white rabbits were injected with 100 µg s.c. of the respective tetanus toxoid-conjugated synthetic peptide, which had been emulsified in complete Freund's adjuvant (9, 10). Blood was obtained immediately before the initial injection and at 2-wk intervals thereafter. At 4 and 10 wk, the animals were boosted with 100 µg of the conjugated peptide in 0.02 M phosphate, 0.15 M NaCl, pH 7.4 (phosphate-buffered saline [PBS]). Rabbit antisera against the native pep M5 molecule were similarly prepared by immunizing with 100 µg doses of pep M5 (5).

Assays for Pep M Antibodies. All sera were tested for the presence of pep M antibodies by an enzyme-linked immunosorbent assay (ELISA) as previously described (9, 11). ELISA inhibition assays were performed by incubating a constant dilution of antiserum with increasing concentrations of synthetic polypeptides or pep M5 as soluble inhibitors (11). Opsonic antibodies were detected by *in vitro* opsonophagocytic assays, as described elsewhere (4, 6, 12).

Detection of Heart Cross-reactive Antibodies. Indirect immunofluorescence tests for heart cross-reactive antibodies were performed using purified sarcolemmal membranes (13) or thin sections of human heart as described elsewhere (3).

Purification of Heart-reactive Antibodies from Pep M5 Antisera. Because previous studies have shown that the heart-reactive antibodies raised in rabbits against pep M5 also cross-reacted with type 19 M protein (3), we used pep M19 that was covalently linked to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) to affinity purify heart-reactive antibodies from the pep M5 antiserum, as described elsewhere (3).

Results

Amino Acid Sequences of S-M5(1-20) and S-M5(20-40). The primary structures of the chemically synthesized peptides were determined by automated Edman degradation to assure their identity with the corresponding regions of the native pep M5 molecule. The sequence of S-M5(1-20) was found to be:

Ala-Val-Thr-Lys-Gly-Thr-Ile-Asn-Asp-Pro-Gln-Ala-Ala-Lys-Glu-Ala-Leu-Asp-Lys-Tyr.

The S-M5(20-40) sequence was:

Tyr-Glu-Leu-Glu-Asn-His-Asp-Leu-Lys-Thr-Asn-Asn-Glu-Gly-Leu-Lys-Thr-Glu-Asn-Thr-Gly.

Both are identical to the corresponding regions of pep M5, according to the amino acid sequence determined in our laboratories (5), but are slightly different from the amino acid sequence previously reported for pep M5 by Manjula and Fischetti (14).

Absence of Heart Cross-reactive Antigenic Determinants on S-M5(1-20) and S-M5(20-40). Initial experiments were designed to determine whether the chemically synthesized peptides of type 5 M protein contained antigens able to bind either heart cross-reactive or type-specific antibodies raised against pep M5. The pep M5 heart-reactive rabbit antiserum (3) was fractionated by affinity chromatography into type-specific and heart cross-reactive antibodies, as described in Materials and Methods. The type-specific fraction reacted in high titer with pep M5, but not with the heart-reactive epitope of pep M19, as measured by ELISA, and was also unreactive in immunofluorescence tests using sarcolemmal membranes from human heart. The heart-reactive fraction of the pep M5 antiserum reacted with pep M5 and pep M19, as measured by ELISA, and also reacted with human heart sarcolemmal membranes by immunofluorescence (3).

To determine the antigenic specificity of the synthetic peptides of type 5 M protein, ELISA inhibition tests were performed using S-M5(1-20), S-M5(20-40), and pep M5 as soluble inhibitors. Inhibition assays were performed with heart-reactive (Fig. 1A) and non-heart-reactive (Fig. 1B) fractions of the pep M5 rabbit antiserum, using pep M5 as the immobilized antigen. Binding of heart-reactive antibodies to pep M5 was not inhibited at all by S-M5(1-20) or S-M5(20-40), but was readily inhibited by the homologous pep M5 antigen (Fig. 1A). However, the binding of type-specific non-heart-reactive antibodies was inhibited 56% by S-M5(1-20), but not by S-M5(20-40) at the highest concentration tested (Fig. 1B). These data suggested that neither of the two synthetic peptides contained antigenic determinants able to bind heart-reactive antibodies raised against the pep M5 molecule. Also, S-M5(1-20), but not S-M5(20-40), contained antigens able to inhibit type-specific antibodies directed against pep M5.

Immunogenicity of Synthetic Peptides of Type 5 Pep M. Because previous studies had shown that a synthetic peptide of type 24 pep M was immunogenic in rabbits after conjugation to poly-L-lysine (9), we first immunized rabbits with 100 μ g of either S-M5(1-20) or S-M5(20-40), covalently linked to poly-L-lysine and emulsified in complete Freund's adjuvant. None of the rabbits developed antibodies against pep M5 as measured by either ELISA or in vitro opsonophagocytic tests.

Two groups of rabbits were then immunized with the synthetic peptides covalently linked to tetanus toxoid and emulsified in complete Freund's adjuvant. All three rabbits immunized with S-M5(1-20) developed type-specific antibody titers against pep M5 as early as 4 wk after the initial injection, as measured by ELISA (Fig. 2A). At 6 wk, all three animals also developed opsonic antibody titers against type 5 streptococci (Fig. 2B), as measured by timed phagocytosis tests. These results were later confirmed by indirect bactericidal tests using type

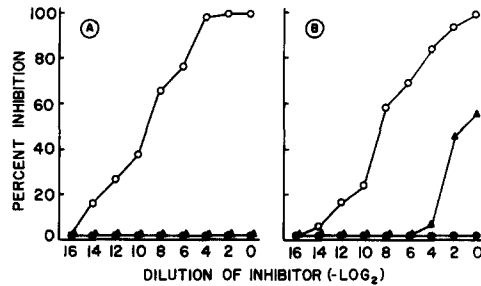


FIGURE 1. ELISA inhibition of heart-reactive and type-specific pep M5 antibodies by pep M5, S-M5(1-20) and S-M5(20-40). Increasing concentrations of pep M5 (○), S-M5(1-20) (▲), and S-M5(20-40) (●) were used to inhibit the binding of heart cross-reactive antibodies (A) and type-specific antibodies (B) to immobilized pep M5. The highest concentration of pep M5 tested was 10 $\mu\text{g}/\text{ml}$ and the highest concentration of the synthetic polypeptides tested was 20 $\mu\text{g}/\text{ml}$.

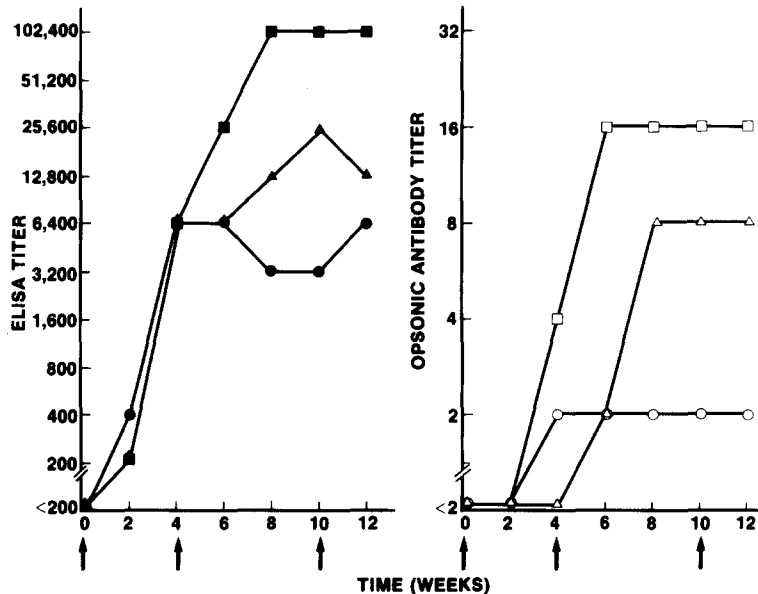


FIGURE 2. ELISA and opsonic antibody titers in serum from rabbits immunized with S-M5(1-20) covalently linked to tetanus toxoid. Three rabbits (▲, ●, ■) were immunized with 100 μg S-M5(1-20) conjugated to tetanus toxoid and emulsified in complete Freund's adjuvant. Booster injections of 100 μg of the conjugated peptide in PBS were given as indicated by the arrows. The assays were performed as described in Materials and Methods.

5 streptococci. None of the S-M5(1-20) immune sera reacted with pep M6, pep M19, or pep M24, as measured by ELISA, nor did they opsonize types 6, 19, or 24 streptococci. None of three rabbits immunized with S-M5(20-40) developed antibodies against type 5 pep M as measured by ELISA or opsonophagocytic tests.

Absence of Heart-reactive Antibodies in the Sera of Rabbits Immunized with S-M5(1-20). To test directly whether S-M5(1-20) contained potentially harmful heart-reactive antigenic determinants, immunofluorescence tests were done using each of the three S-M5(1-20)-immune rabbit sera against thin sections of human heart

(4) and purified sarcolemmal membranes (3). None of the sera reacted with either of these tissue preparations. Also, none of the immune sera reacted in the ELISA with the heart cross-reactive determinant of type 19 pep M (3).

Discussion

Because of the fear that pep M vaccines may contain tissue cross-reactive antigens that might cause, rather than prevent, rheumatic fever, there has been considerable interest in defining the minimum peptide structure of pep M necessary to elicit type-specific protective immunity. This approach allows for the disposal of the majority of the M molecule that may contain potentially harmful tissue cross-reactive determinants. Having previously demonstrated the presence of at least one heart cross-reactive epitope on the pepsin-derived fragment of type 5 M protein (3), we undertook the present study to determine the antigenic specificity and protective immunogenicity of two chemically synthesized peptides of pep M5. Initial experiments showed that neither of the two peptides, S-M5(1-20) nor S-M5(20-40), contained antigens capable of reacting with purified heart cross-reactive antibodies raised against the native pep M5 molecule. S-M5(1-20), but not S-M5(20-40), retained type-specific epitopes, as demonstrated by ELISA inhibition assays. When covalently linked to tetanus toxoid, S-M5(1-20) evoked type-specific, protective antibodies in all three immunized rabbits without producing heart cross-reactive antibodies.

These data are consistent with previous experiments in our laboratory that showed that a cyanogen bromide-derived peptide of type 24 pep M, or its chemically synthesized analogue, consisting of only 35 amino acids covalently linked to polylysine, also produced protective immunity in laboratory animals (9, 10). Taken together, these studies indicate that only a limited region of the M molecule coupled to the appropriate carrier is sufficient to produce protective immunity against group A streptococci, thus limiting the total amount of protein injected and minimizing the possibility of producing potentially harmful tissue cross-reactive immunity. Continued isolation and identification of natural peptides and even chemical synthesis of such peptides from various pep M may yield a safe and effective vaccine against a number of potentially rheumatogenic serotypes of *Streptococcus pyogenes*.

Summary

We determined the antigenic specificity and protective immunogenicity of two chemically synthesized peptides of type 5 streptococcal M protein. The synthetic peptides, designated S-M5(1-20) and S-M5(20-40), represent the amino-terminal amino acid sequence of the native pepsin-extracted M5 molecule, which is known to contain at least one heart cross-reactive epitope. Initial studies showed that neither of the synthetic peptides was able to bind purified heart-reactive M5 antibodies. In addition, S-M5(1-20), but not S-M5(20-40), contained type-specific antigenic determinants as measured by enzyme-linked immunosorbent inhibition assays. When covalently linked to tetanus toxoid, S-M5(1-20), but not S-M5(20-40), evoked significant levels of type-specific, opsonic (and presumably protective) antibodies in rabbits without evoking heart cross-reactive antibodies.

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