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Vimentin-Cross-Reactive Epitope of Type 12 Streptococcal M Protein

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The NH_2-terminal amino acid sequence of type 12 M protein was determined by automated Edman degradation of a 38-kilodalton polypeptide fragment purified from a limited pepsin digest of intact type 12 streptococci. The sequence of the first 13 amino acid residues of the polypeptide confirmed that predicted by the nucleotide sequence of the mature type 12 M protein. A chemically synthesized peptide copying the NH_2-terminal 25 residues, SM12(1-25)C, evoked opsonic antibodies against type 12 streptococci as well as renal glomerular cross-reactive antibodies. The serum from one of six rabbits reacted in immunofluorescence tests with human glomeruli in a mesangial staining pattern. The cross-reactive antibodies were completely inhibited by the immunizing peptide and absorption with type 12 streptococci. Subpeptides of the 25-residue synthetic peptide were without inhibitory effect, suggesting that the cross-reactive antibodies are directed against a conformational epitope of SM12(1-25)C. Anti-SM12(1-25)C antisera reacted specifically with the intermediate filament protein vimentin extracted from mesangial cells. None of the cross-reactions of anti-SM12(1-25)C were inhibited by a synthetic peptide SM1(1-26)C of type 1 M protein, which was previously shown to share a cross-reactive epitope with vimentin. These results indicate that type 12 M protein contains at least one vimentin cross-reactive epitope that is clearly distinct from the tetrapeptide epitope shared with vimentin by type 1 M protein.

The M protein molecules of group A streptococci form α-helical coiled-coil fibrils radiating from the surface of virulent strains of *Streptococcus pyogenes* (24). The fibrils confer on the organisms resistance to recognition and ingestion by phagocytes of the nonimmune host (28). Only antibodies directed towards protective epitopes on the M protein molecule render the organisms susceptible to phagocytosis and killing in the immune host (22). In addition to its protective epitopes, certain M protein molecules contain epitopes capable of eliciting autoimmune reactions with specific host tissues. Sequences of rheumatogenic serotypes 5, 6, and 19 M proteins have been shown to evoke antibodies that react with myosin, tropomyosin, and other as yet unidentified myocardial and skeletal muscle proteins (8, 10–12, 14, 20, 27). Recently, it has become clear that M proteins of nephritogenic types 1 and 12 share antigenic determinants with renal glomeruli (16, 17). These autoimmune properties of various M proteins have hampered the development of a safe and effective vaccine against streptococcal infections.

Previous studies have shown that synthetic peptides copying NH_2-terminal sequences of types 5, 6, and 24 M proteins contain protective but not autoimmune epitopes (1–5, 13). These findings suggest that the NH_2-terminal regions of various M proteins may serve as ideal components of a multivalent streptococcal vaccine. Recently, however, Bronze et al. (8) found heart-cross-reactive as well as opsonic epitopes in the NH_2-terminal sequence of type 19 M protein, and Kraus and Beachey (17) found renal glomerular cross-reactive epitopes in the NH_2-terminal sequence of type 1 M protein, indicating that these regions of M proteins are not always devoid of autoimmune epitopes.

In our previous studies (19), we demonstrated that an epitope of type 1 M protein specified by a tetrapeptide, Ile-Arg-Leu-Arg, is shared with vimentin, an intermediate filament protein extracted from renal glomerular mesangial cells. In this study, we report that the NH_2-terminal region of the M protein of nephritogenic type 12 streptococci specifies vimentin cross-reactive epitopes that are distinct from those specified by the NH_2-terminal region of type 1 M protein.

MATERIALS AND METHODS

Proteins. Purified bovine vimentin (57 kilodaltons) and purified chicken desmin (53 kilodaltons) were purchased from ICN Immunobiologicals (Lisle, Ill.). They were judged to be pure by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as previously described (19). Pepsin-extracted M protein of type 24 streptococci (pep M24) was purified in a previous study (6).

Extraction and purification of streptococcal M protein. Polypeptide fragments of type 12 M protein were extracted from intact type 12 group A streptococci (strain SF42) by limited pepsin digestion at pH 5.8 and were partially purified by ammonium sulfate precipitation and ion-exchange chromatography over DEAE-cellulose as previously described (6). To affinity purify pep M12 fragments containing the NH_2 terminus of the mature type 12 M protein, the immunoglobulin G fraction of antiserum raised against the synthetic peptide SM12(1-25)C was purified over DEAE-cellulose. The purified immunoglobulin G fraction was coupled to Affi-Gel 10 (Bio-Rad Laboratories, Rockville Centre, N.Y.) by following the instructions given by the manufacturer, and partially purified pep M12 fragments were applied to the immunoglobulin G-Affi-Gel 10 column. The bound material was eluted with 0.2 M glycine-0.5 M NaCl, pH 2.3, neutralized, dialyzed extensively against 0.01 M NH_4HCO_3, and lyophilized. The homogeneity of the purified pep M12 was judged by SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis and Western blots. SDS-polyacrylamide gel electrophoresis was performed on 12% polyacrylamide slab gels according to the method of

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Laemmli (21), and Western blots were performed as previously described by Towbin et al. (29).

Analytical methods. Quantitative amino acid analysis was performed on peptide samples hydrolyzed in constant boiling HCl with an automatic amino acid analyzer (Beckman, Model 121 MB). Amino acid sequence analysis was performed with a Beckman microsequenator (Model 890C) according to the principles first described by Edman and Begg (15) and as previously described (4).

Synthesis and conjugation of M protein peptides. Several overlapping peptide copies of the NH_2-terminal region of type 12 M protein (25) were synthesized by the solid-phase method of Merrifield (23) as described (7). The synthetic peptides were cleaved, deblocked, and purified by gel filtration on a column of Sephadex G10 (Pharmacia Fine Chemicals, Uppsala, Sweden) as previously described (4). The purity and composition of the peptide were assured by high-pressure liquid chromatography on Ultrasphere ODS2 (Whatman Laboratory Products, Inc., Clifton, N.J.), by quantitative amino acid analyses, and by automated Edman degradation to the penultimate amino acid residue (4). The peptides were synthesized with a COOH-terminal cysteine to enable coupling to a carrier molecule with a bifunctional cross-linking reagent. The peptides synthesized for this study are designated SM12(1-25)C, SM12(1-12)C, SM12(13-25)C, and SM12(7-18)C. The peptides SM1(1-26)C, SV(63-73)C, and SV(178-188)C were synthesized in previous studies (17, 19).

The peptide SM12(1-25)C was conjugated through its COOH-terminal cysteine residue to keyhole limpet hemocyanin or pep M24 (6) as described (30) with the modification of Rothbard et al. (26).

Immunization of rabbits. Sets of three New Zealand White rabbits (2 kg) were injected subcutaneously with 100-nmol doses of synthetic peptide SM12(1-25)C conjugated to keyhole limpet hemocyanin or pep M24 and emulsified in complete Freund adjuvant as described (13). Booster injections consisting of the same dose in 0.02 M phosphate-0.15 M NaCl, pH 7.4, were given at 4, 8, and 10 weeks. Rabbits were bled before and at 2-week intervals after the initial injection. All sera were heat inactivated at 56°C for 30 min and stored in sterile vials at 4°C. Antiserum 8652 raised against SM1(1-19) (23-26)C in a previous study (18) was also used in this study.

Assays for anti-M protein antibodies. The immune rabbit sera were assayed for antibodies against M protein by enzyme-linked immunosorbent assay (ELISA) (17), opsonophagocytosis, and indirect bactericidal tests (4). The peptide specificity of the opsonic antibodies directed against type 12 streptococci was tested by opsonization inhibition tests (4). Samples of opsonic antisera, diluted in 0.02 M phosphate-0.15 M NaCl, pH 7.4, to the highest dilution that promoted phagocytosis of homologous type 12 streptococci, were preincubated for 30 min at 37°C with synthetic peptides at a concentration of 100 μM or with 1 mg of vimentin or desmin per ml before addition to the opsonization test mixture. Phagocytosis was expressed as the percentage of polymorphonuclear leukocytes associated with streptococci in 50 counted leukocytes after rotating the test mixtures end-over-end at 37°C for 45 min.

Detection of tissue cross-reactive antibodies. Preimmune and immune sera were tested for the presence of tissue cross-reactive antibodies with frozen sections (4 μm thick) of normal human renal cortex or human myocardium by indirect immunofluorescence assays (9, 10). The peptide specificity was assessed by immunofluorescence inhibition assays. The immune serum 86142 was preincubated at a dilution of 1:4 in 0.02 M phosphate-0.15 M NaCl, pH 7.4, for 30 min at 37°C with synthetic peptides at a concentration of 100 μM or with 1 mg of vimentin or desmin per ml and then was used in indirect immunofluorescence tests as described (9, 10).

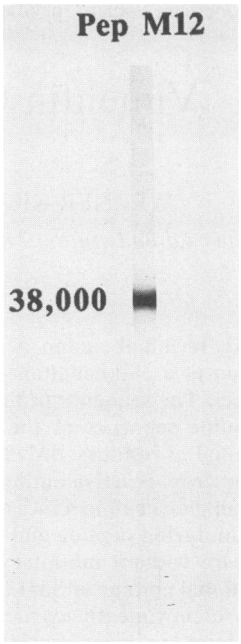

FIG. 1. Coomassie blue-stained SDS gel of the purified pep M12 showing an apparent molecular weight of 38,000.

RESULTS

Purification and NH_2-terminal sequence analysis of pep M12. Type 12 streptococci were extracted by limited digestion with pepsin at pH 5.8 (6). The crude digest was purified by ammonium sulfate precipitation, ion-exchange chromatography over DEAE-cellulose (6), and affinity chromatography over Affi-Gel 10 to which the IgG fraction of antiserum raised against SM12(1-25)C had been coupled. The purified protein migrated as a single polypeptide on an SDS-polyacrylamide gel with an apparent molecular weight of 38,000 (Fig. 1). The amino acid sequence of the purified protein was determined by automated Edman degradation (4), and the NH_2-terminal 13 residues were found to be D H S D L V A E K Q R L E, in agreement with that predicted by the nucleotide sequence encoding the NH_2-terminal region of the mature type 12 M protein (25).

Immunogenicity of a synthetic peptide of the NH_2 terminus of type 12 M protein. In an attempt to identify protective versus cross-reactive epitopes of type 12 M protein, we synthesized peptides copying the NH_2 terminus of pep M12 (Table 1). To determine the immunogenicity of SM12(1-25)C, the peptide was conjugated to either highly purified pep M24 or keyhole limpet hemocyanin, emulsified in complete Freund adjuvant, and injected into rabbits. The synthetic peptide evoked high titers of antibodies as measured by ELISA against the unconjugated synthetic peptide or pep M12 (Table 2). In addition, the SM12-pep M24 conjugate evoked high titers of antibodies against pep M24 (Table 2). Moreover, the immune sera of all six rabbits opsonized type 12 streptococci in opsonization and opsonobactericidal tests

TABLE 1. Covalent structure of synthetic peptides of type 12 M protein

Synthetic peptide	Amino acid sequence[a]
	5 10 15 20 25
SM12(1-2)C	D H S D L V A E K Q R L E D L G Q K F E R L K Q R C[b]
SM12(1-12)C	D H S D L V A E K Q R L C
SM12(13-25)C	E D L G Q K F E R L K Q R C
SM12(7-18)C	A E K Q R L E D L G Q K C

[a] The sequence of the NH_2-terminal region of type 12 M protein was determined partially by automated Edman degradation and was predicted by nucleotide sequence analysis of the type 12 M protein gene (25).
[b] Underlined cysteine residues were added for the purpose of coupling to a carrier.

against M type 12 streptococci (Table 3). The SM12-pep M24 conjugate also evoked antibodies that opsonized type 24 streptococci, indicating that pep M24 can serve as both carrier and antigen.

Although none of the six antisera reacted with sarcolemmal membranes of human myocardium, one of three antisera raised against SM12(1-25)C conjugated to keyhole limpet hemocyanin reacted with renal glomeruli in a mesangial cell pattern as determined by immunofluorescence tests of frozen sections of human renal cortex (Table 3). Thus, the NH_2-terminal 25 residues of type 12 M protein contain type-specific opsonic epitopes as well as kidney cross-reactive epitopes.

Localization of protective and glomerular cross-reactive epitopes of SM12(1-25)C. To identify the protective and glomerular cross-reactive epitopes of the NH_2 terminus of type 12 M protein, we used overlapping peptides in opsonization and immunofluorescence inhibition tests. Opsonization of type 12 streptococci by antiserum 86142 (diluted 1:32) was totally inhibited by preincubation with type 12 streptococci or SM12(1-25)C, the immunogen (Table 4). The subpeptides SM12(1-12)C, SM12(13-25)C, and SM12 (7-18)C were also inhibitory, suggesting the presence of several protective epitopes. The synthetic peptide SM1(1-26)C, copying the NH_2-terminal region of type 1 M protein (17), had no inhibitory effect (Table 4), indicating the type specificity of the type 12 peptide antisera.

The renal glomerular cross-reactivity was completely inhibited by preincubation of the antiserum with whole type 12 streptococci (Table 4), demonstrating that the cross-reactive antibodies raised against the synthetic peptide recognized a similar epitope on the native type 12 M protein. In contrast to the opsonization inhibition tests, only SM12(1-25)C inhibited the glomerular cross-reaction; none of the overlapping subpeptides showed any inhibitory effect (Table 4). In addition, preincubation with a mixture of synthetic peptides SM12(1-12)C and SM12(13-25)C failed to inhibit immunofluorescence (Table 4), suggesting that the cross-reactive antibodies are directed against a conformational epitope of SM12(1-25)C.

Identification of cross-reactive antigen. The mesangial staining pattern of cross-reactive antiserum 86142 with renal glomeruli (Fig. 2) was similar to that previously described for autoimmune antibodies evoked against SM1(1-26)C (17), suggesting that the same protein in the glomeruli may account for the cross-reactivity. We had recently shown that antibodies directed against the amino acid sequence Ile-Arg-Leu-Arg at residues 23 through 26 of the NH_2 terminus of type 1 M protein reacted with the intermediate filament protein vimentin extracted from glomerular mesangial cells (19). Although type 12 M protein does not contain this sequence, bovine vimentin, nevertheless, completely inhibited renal glomerular fluorescence of antiserum 86142. In contrast, desmin, another intermediate filament protein present in renal glomeruli, had no inhibitory effect (Table 4), suggesting that vimentin is the cross-reactive antigen accounting for immunofluorescence of glomeruli.

Antiserum 86142 raised against SM12(1-25)C reacted with mesangial cells cultured from isolated rat glomeruli in a fibrillar staining pattern identical to that previously reported

TABLE 2. ELISA titers of antibodies raised in rabbits immunized with SM12(1-25)C

Rabbit antiserum	ELISA titer[a] against:		
	SM12(1-25)C	pep M12	pep M24
Anti-SM12(1-25)C–pep M24			
8695	>102,400	25,600	>102,400
8696	>102,400	12,800	>102,400
8697	102,400	3,200	>102,400
Anti-SM12(1-25)C–KLH			
86140	>102,400	25,600	<200
86141	>102,400	51,200	<200
86142	>102,400	25,600	<200
Preimmune[b]	<200	<200	<200

[a] The ELISA titers are recorded as the highest reciprocal dilution of antiserum that resulted in an absorbancy reading of 0.1 or greater at a wavelength of 450 nm.
[b] The preimmune serum of each of the six rabbits was tested separately; all of the sera showed the represented negative reactivity.

TABLE 3. Type-specific and kidney cross-reactive antibodies evoked in rabbits immunized with SM12(1-25)C

Rabbit antiserum	% Phagocytosis[a] of streptococci		No. of colonies of type 12 streptococci after 3-h rotation in test mixture:		Renal glomerular fluorescence[c]
	Type 12	Type 24	Inoculum 25[b]	Inoculum 7[b]	
Preimmune[d]	2	2	3,140	870	0
Anti-SM12-pep M24					
8695	94	98	0	0	0
8696	92	98	15	0	0
8697	90	98	0	0	0
Anti-SM12-KLH					
86140	90	0	80	0	0
86141	90	0	5	0	0
86142	94	2	0	0	+++

[a] Determined as explained in Materials and Methods.
[b] Inoculum contained 25 or 7 CFU, respectively, at the beginning of the bactericidal test.
[c] +++, Maximum fluorescence.
[d] The preimmune serum of each rabbit was tested separately. Inasmuch as each of the sera gave essentially the same results, one serum was chosen for the experiments recorded in this table.

TABLE 4. Opsonization and immunofluorescence inhibition tests of cross-reactive antiserum 86142

Inhibitor of antiserum 86142[a]	% Phagocytosis[b] of type 12 streptococci	Renal glomerular fluorescence[c]
None	64	+++
SM12(1-25)C	2	0
SM12(1-12)C	6	+++
SM12(13-25)C	16	+++
SM12(7-18)C	8	+++
SM12(1-12)C + SM12(13-25)C	2	+++
SM1(1-26)C	66	+++
M12 streptococci[d]	0	0
Vimentin	ND	0
SV(63-73)C	ND	+++
SV(178-188)C	ND	+++
Desmin	ND	+++

[a] Each synthetic peptide was tested at a concentration of 100 μM, whereas bovine vimentin and chicken desmin were each tested at a concentration of 1 mg/ml.
[b] Determined as described in Materials and Methods.
[c] +++, Maximum fluorescence.
[d] Antiserum 86142 was absorbed twice at 37°C for 30 min with pellets collected from 40 ml of an 18-h culture of M type 12 streptococci.

for anti-SM1(1-19) (23-26)C (19). In Western blots, antiserum 86142 reacted with authentic bovine vimentin as well as with vimentin extracted from cultured mesangial cells in a manner identical to that previously reported for anti-SM1 (19). These results provide strong evidence that the intermediate filament protein vimentin shares autoimmune epitopes with type 12 as well as with type 1 M protein. However, preincubation of antiserum 86142 with the type 1 M protein peptide SM1(1-26)C had no effect on glomerular immunofluorescence (Table 4). Similarly, SM12(1-25)C had no effect on the glomerular cross-reactivity of antiserum 8652 (13) raised against an NH_2-terminal synthetic peptide of type 1 M protein (data not shown). Furthermore, the synthetic peptides of human vimentin SV(63-73)C and SV(178-188)C, which totally inhibited the glomerular cross-reactivity of the anti-SM1-antiserum (19), did not inhibit renal glomerular fluorescence of antiserum 86142 (Table 4). These results indicate that SM12(1-25)C contains a vimentin cross-reactive epitope distinct from that specified by SM1(1-26)C.

DISCUSSION

Previous studies from our laboratories have demonstrated the presence of an autoimmune epitope in type 1 streptococcal M protein capable of eliciting immune responses to the intermediate filament protein vimentin extracted from the mesangial cells of renal glomeruli (19). The cross-reactive epitope was localized in a tetrapeptide sequence Ile-Arg-Leu-Arg shared by type 1 M protein and vimentin (17, 19). In this study, we identified a vimentin cross-reactive epitope in type 12 M protein that appears to be distinct from that specified by the type 1 M protein peptide. The evidence that the two are distinct is as follows: (i) the respective synthetic peptides evoke antibodies that react only with the homologous serotype of M protein; (ii) the vimentin cross-reactive antibodies raised against the M1 and M12 peptides are inhibited only by the respective serotype of synthetic peptides; (iii) the M12 peptide is devoid of the autoimmune tetrapeptide sequence Ile-Arg-Leu-Arg of type 1 M protein; and (iv) the synthetic peptides copying Arg-Leu-Arg sequences of vimentin inhibit the vimentin cross-reactive antibodies raised against the type 1 peptide but not those raised against the type 12 peptide.

In this study, we were unable to ascribe the autoimmune responses evoked by SM12(1-25)C to any particular primary sequence within the 25-residue peptide. A computer comparison of the peptide sequence with that of vimentin failed to reveal significant primary sequence homology. Overlapping SM12 subpeptides failed to react with the vimentin cross-reactive antibodies, suggesting that a tertiary structural determinant may specify the autoimmune epitope of this peptide.

The first evidence that streptococcal M proteins may share epitopes with renal antigens was reported by Goroncy-Bermes et al. (16). They showed that a monoclonal antibody raised against human renal cortex opsonized M types 6 and 12 streptococci. The monoclonal antibodies were directed against a 43-kilodalton protein of renal cortex, an antigen distinct from the 57-kilodalton vimentin molecule. Thus, it appears that the cross-reactions between M protein and renal antigens are quite complex. Different M proteins may contain distinctly different epitopes shared with the same tissue protein; however, the same M proteins may contain yet other epitopes that are shared with distinctly different tissue antigens.

The role of autoimmune responses to streptococcal M protein in the pathogenesis of the sequelae after streptococcal infections has not been established nor has it been established that humans recognize the same vimentin cross-reactive epitopes of type 12 M protein as those recognized by rabbits. Whether or not the tissue antigens or epitopes within defined proteins of given cells are accessible to antibodies or immune T-lymphocytes also will require further investigation. Such studies are now under way to determine the accessibility of vimentin epitopes in mesangial cells in culture and in vivo.

In summary, we have demonstrated that the NH_2-terminal region of type 12 M protein contains an epitope(s) that evokes immune responses to a vimentin epitope(s) distinct

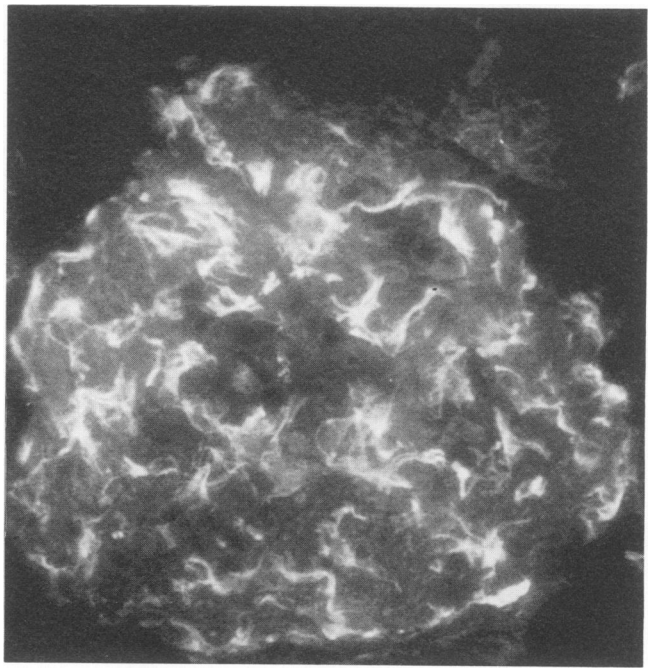

FIG. 2. Immunofluorescence staining of human renal glomerulus by synthetic peptide antiserum 86142. Magnification, ×400.

from that evoked by the NH_2-terminal region of type 1 M protein. The epitope appears to reside in a tertiary conformation rather than in a primary sequence. The autoimmune epitope appears to be less dominant than the protective epitopes of the same peptide inasmuch as the immune response to the protective epitopes was universal, whereas that to the cross-reactive epitope occurred in only one of six immunized rabbits. Whether or not the failure of any of the rabbits immunized with the pep M24-conjugated peptide to recognize the autoimmune epitope was due to immunosuppressive effects of pep M24 requires further investigation.

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