

# Renal autoimmune epitope of group A streptococci specified by M protein tetrapeptide Ile-Arg-Leu-Arg

(synthetic peptides/vaccine preparations/protective immunity/glomerulonephritis)

WERNER KRAUS AND EDWIN H. BEACHEY\*

Veterans Administration Medical Center and University of Tennessee, Memphis, TN 38104

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**ABSTRACT** The renal glomerular cross-reactivity of the amino-terminal region of type 1 streptococcal M protein was investigated. Antisera raised in rabbits against a synthetic peptide representing residues 1-26 and a peptide from which residues 20-22 had been omitted during synthesis were capable not only of opsonizing type 1 streptococci but also of reacting in immunofluorescence tests with human renal glomeruli. The cross-reactions were completely inhibited by the immunizing peptides. By using additional synthetic peptides in these inhibition studies, the glomerular cross-reactive epitope was localized to a tetrapeptide sequence Ile-Arg-Leu-Arg at positions 23-26. A number of synthetic M1 peptides containing the tetrapeptide sequence were inhibitory, whereas the M1 peptides lacking the sequence or unrelated tetrapeptides Arg-Gly-Asp-Ser or Arg-Gly-Phe-Ser were without effect. Furthermore, Ile-Arg-Leu-Arg affinity-purified antibodies reacted with renal glomeruli, and the reactivity was inhibited by the tetrapeptide as well as by type 1 M protein. These results indicate that a renal glomerular autoimmune epitope resides in a tetrapeptide Ile-Arg-Leu-Arg near the amino terminus of type 1 streptococcal M protein.

It is becoming increasingly clear that the streptococcal M protein molecules protruding from the surface of virulent group A streptococci contain structural elements capable of evoking autoimmune reactions with certain host tissues. Shared sequences of types 5, 6, and 19 M proteins have been shown to elicit antibodies that react with several myocardial and skeletal muscle proteins, including myosin and other as yet unidentified proteins (1-6). Although these findings have been intriguing in regard to the pathogenesis of postinfectious sequelae of streptococcal infections, they have hampered the development of a safe and effective streptococcal vaccine; it is well established that protective immunity is directed exclusively against the surface M protein of these organisms (7).

The covalent structures of polypeptide fragments of several different M protein serotypes have been established, and the complete amino acid sequences of three different M protein types (types 5, 6, 24) have been deduced from the nucleotide sequences of the structural genes of the respective serotypes (8-10). These primary structural and other physicochemical studies have shown that the M proteins form  $\alpha$ -helical coiled-coil fibrils radiating from the surface of virulent strains of streptococci (11). The fibrils render the organisms resistant to recognition and ingestion by phagocytes of the nonimmune host (7, 12).

The knowledge of the primary structure of M protein has enabled the selection of different regions of the molecule for study of protective as opposed to autoimmune epitopes. In previous studies, we have shown that synthetic peptides

copying amino-terminal sequences of types 5, 6, and 24 M proteins were capable of evoking opsonic and protective antibodies against the related serotypes of streptococci (13-18). The synthetic peptides were devoid of autoimmune epitopes. Synthetic peptides copying other regions of the M protein molecule, however, were shown to evoke not only protective but also tissue cross-reactive antibodies (6).

Our previous studies focused on so-called rheumatogenic serotypes of streptococci. Because of our interest in autoimmune diseases evoked by group A streptococci in general, we decided to investigate the protective and autoimmune epitopes of a strain of streptococci known to be associated with the development of both acute rheumatic fever and acute proliferative glomerulonephritis (19). The M type 1 strain chosen for study has been associated with both streptococcal sequelae (19), and the amino-terminal sequence of this M protein has been established (20). In this paper, we report that synthetic peptides of the amino-terminal region of type 1 M protein contain epitopes that evoke peptide-specific autoimmune antibodies directed against renal glomeruli. The glomerular cross-reactive epitope has been pinpointed to a tetrapeptide sequence Ile-Arg-Leu-Arg.

## MATERIALS AND METHODS

**Extraction and Purification of Streptococcal M Protein.** Polypeptide fragments of type 1 M protein were extracted by limited peptic digestion of whole type 1 group A streptococci as described (20, 21). The crude pepsin digest was partially purified by precipitation with 30-60% saturated ammonium sulfate and was designated pep M1 (20).

**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) (14). Amino acid sequence analysis was performed with a Beckman microsequenator (model 890 C) according to the principles first described by Edman and Begg (22) and as described (14).

**Synthesis and Conjugation of M Protein Peptides.** Several overlapping peptide copies of the amino-terminal region of type 1 M protein (20) were synthesized by the solid-phase method of Merrifield (23) as described (24). The synthetic peptides were cleaved, deblocked with HF, and purified by gel filtration on a column of Sephadex G-10 (Pharmacia, Uppsala, Sweden) as described (14). The purity and composition of the peptides were ensured by HPLC on Ultrasphere ODS2 (Whatman, Clifton, NJ) (14), by quantitative amino acid analysis, and by automated Edman degradation to the penultimate amino acid residue (14). The peptides were synthesized with a carboxyl-terminal cysteine to enable coupling to a carrier molecule with a bifunctional crosslinking



Table 2. Type-specific and kidney cross-reactive antibodies raised in rabbits immunized with SM1(1-26)C, SM1(1-19)(23-26)C, or SM1(1-23)C

Rabbit antiserum	ELISA titer against pep M1	% phagocytosis of type 1 streptococci	Renal glomerular fluorescence
<b>Anti-SM1(1-26)C</b>			
8725	25,600	90	++
8726	51,200	96	+
8727	25,600	94	++
<b>Anti-SM1(1-19)(23-26)C</b>			
8651	12,800	90	+++
8652	12,800	96	++++
8653	25,600	92	++
<b>Anti-SM1(1-23)C</b>			
8710	12,800	80	0
8711	25,600	90	0
8712	25,600	88	0
Preimmune (control)	<200	0	0

Similar patterns and degrees of glomerular immunofluorescence were seen with frozen sections of human, rat, or mouse renal cortex. The preimmune serum of each of the nine rabbits was tested separately; all of the sera showed the represented negative reactivity. The preimmune and the immune sera were each tested at a dilution of 1:4 in PBS.

SM1(1-23)C evoked antibodies to neither of these tissues. These results suggested the possibility that the glomerular cross-reactive epitope resides in the carboxyl-terminal region of peptide SM1(1-26)C.

**Localization of Glomerular Cross-Reactive and Protective Epitopes of SM1(1-26)C.** To pinpoint the glomerular cross-reactive epitope(s), we synthesized additional overlapping peptides, as shown in Table 1, and used them in immunofluorescence inhibition studies. The results of a typical inhibition experiment is illustrated in Fig. 1, in which affinity-purified antibodies against SM1(1-19)(23-26)C were inhibited with the same synthetic peptide. Peptides SM1(1-26)C, SM1(13-26)C, SM1(19-32)C, and SM1(23-36)C, but not SM1(1-12)C, SM1(1-20)C, SM1(1-23)C, and SM1(26-41)C were similarly inhibitory (Table 3). Similar results were

Table 3. Opsonization and immunofluorescence inhibition tests of cross-reactive antiserum 8652

Inhibitory peptide	% phagocytosis of type 1 streptococci	Renal glomerular fluorescence
None	74	++++
SM1(1-26)C	2	0
SM1(1-19)(23-26)C	0	0
SM1(1-23)C	2	++++
SM1(1-20)C	2	++++
SM1(1-12)C	76	++++
SM1(13-26)C	36	0
SM1(19-32)C	22	0
SM1(23-36)C	70	+
SM1(26-41)C	72	++++
SM1(23-26)C, I R L R C*	ND	0
Pep M1	2	0

Each synthetic peptide was tested at a concentration of 100  $\mu$ M, whereas pep M1 was tested at a concentration of 0.5 mg/ml. ND, not done.

\*This peptide (single-letter code) was tested at a final concentration of 5 mM. Similar concentrations of the unrelated tetrapeptides Arg-Gly-Asp-Ser and Arg-Gly-Phe-Ser (representing the cell binding sequence and a substitution peptide thereof of fibronectin) had no inhibitory effect.

obtained when anti-SM1(1-26)C affinity-purified antibodies were substituted for anti-SM1(1-19)(23-26)C in the immunofluorescence inhibition tests (data not shown).

These results suggested that the sequence Ile-Arg-Leu-Arg at positions 23-26 accounted for the renal glomerular cross-reactivity. This notion was confirmed by synthesizing and testing the immunofluorescence inhibitory activity of the tetrapeptide Ile-Arg-Leu-Arg; the tetrapeptide tested at a concentration of 5 mM completely inhibited immunofluorescence, whereas the same concentrations of the unrelated tetrapeptides Arg-Gly-Asp-Ser and Arg-Gly-Phe-Ser had no effect (Table 3); moreover, antibodies from the cross-reactive antisera affinity purified over an Ile-Arg-Leu-Arg-Cys Affigel 10 column reacted with renal glomeruli (Table 4), and the reactivity was again absorbed by Ile-Arg-Leu-Arg-Cys, SM1(1-26)C, and pep M1.

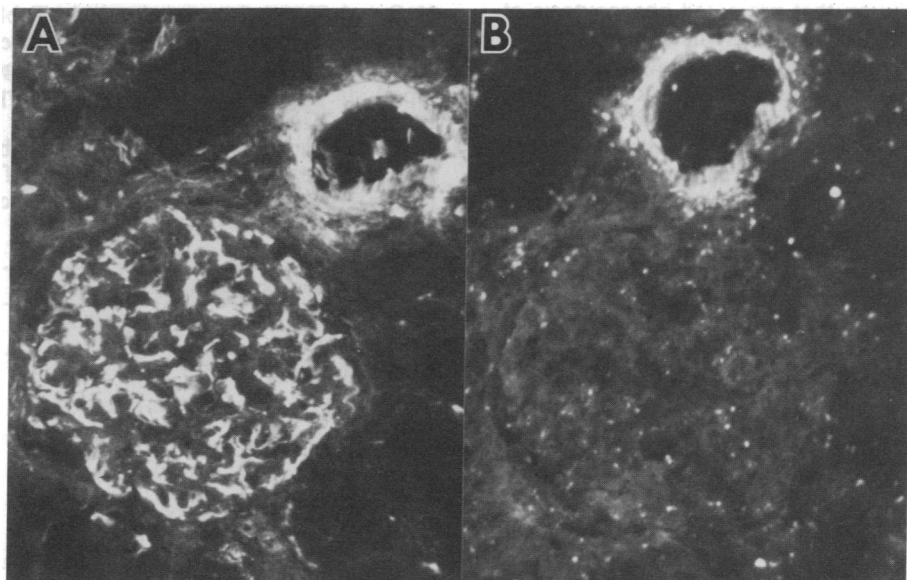


FIG. 1. Immunofluorescence staining of human renal glomeruli by synthetic peptide antiserum. (A) Frozen section of human kidney treated with affinity-purified cross-reactive antibodies raised against SM1(1-19)(23-26)C. (B) Inhibitory effect of the synthetic peptide SM1(1-26)C is shown. The staining of the blood vessels at top of these serial sections was yellow rather than green and is presumed to represent autofluorescence. ( $\times 300$ .)

Table 4. Reactivity of I R L R C affinity-purified antibodies with renal glomeruli

Affinity-purified antibodies inhibited with:	Renal glomerular fluorescence
Uninhibited	+++
Pep M1	±
SM1(1-26)C	-
I R L R C	-

The affinity-purified antibodies concentrated to one-fifth of the original serum volume were preincubated for 30 min at 37°C with pep M1 (2 mg/ml), 100 µM SM1(1-26)C, or 5 mM IRLRC (single-letter code). Control peptides including SM1(1-20)C and Art-Gly-Asp-Ser had no inhibitory effect. To ensure that inhibition by IRLRC was not due to its cationic properties, we tested the same concentration of this peptide for inhibitory activity when preincubated with an unrelated antiserum (7907) that cross-reacts in immunofluorescence tests with cardiac sarcolemmal membranes (1). The peptide had no inhibitory effect on the immunofluorescent reaction of serum 7907 with sarcolemmal membranes.

The ability of pep M1 to inhibit the glomerular cross-reaction demonstrated that the cross-reactive antibodies raised against the synthetic peptide recognized a similar epitope in the native type 1 M protein. A search of the published sequences of serotypes 1 (20), 5 (9, 30), 6 (8), 24 (10), and 49 (31) M proteins revealed that none of them contained the Ile-Arg-Leu-Arg sequence.

Interestingly, the opsonic antibodies against the synthetic peptide SM1(1-19)(23-26)C in rabbit serum 8652 were all directed toward the amino-terminal 20 residues, thus separating potentially protective epitopes from the glomerular cross-reactive determinant (see Table 3). Peptides SM1(1-20)C, SM1(1-23)C, SM1(1-19)(23-26)C and SM1(1-26)C inhibited opsonization, whereas peptides SM1(1-12)C, SM1(23-36)C, and SM1(26-41)C had no inhibitory effect and peptides SM1(13-26)C and SM1(19-32)C inhibited opsonization only partially. At present, we cannot account for the partial opsonic inhibitory effect of peptide SM1(19-32)C; it overlaps the "protective" peptide SM1(1-20)C only by two residues. These results indicate that the glomerular cross-reactive epitope is separable from an anti-opsonic epitope of the amino-terminal region of type 1 M protein.

## DISCUSSION

The development of a group A streptococcal vaccine has been hampered by the discovery that certain M protein preparations evoke antibodies against antigenic determinants in human heart and skeletal muscle tissues. Kaplan (32) described heart cross-reactive antigens associated with cell walls and certain M protein extracts of group A streptococci including the rheumatogenic M types 5 and 19. Krisher and Cunningham (3) prepared a monoclonal antibody against type 5 streptococci that cross-reacted with cardiac myosin. Recent investigations showed that types 5, 6, and 19 M proteins contain epitopes within their molecular structures that are shared with epitopes in cardiac sarcolemmal membranes (1, 2, 6) and muscle myosin (4, 5).

Recent reports (33-35) indicate that immunological cross-reactivity with renal tissues may be evoked by nephritogenic M serotypes. Fitzsimons *et al.* (33) described the preparation of a monoclonal antibody raised against type 12 streptococcal cytoplasmic membranes that cross-reacted with renal glomerular basement membrane antigens. Others have shown the presence of antibodies against basement membrane antigens in sera from patients with poststreptococcal glomerulonephritis (34, 35). The antigens involved were represented by glomerular heparan-sulfate proteoglycan (34), basement membrane collagen (type IV), and laminin (35). The authors speculated that the immunological cross-

reactivity may be related to structural similarities between the streptococcal surface M protein and basement membrane antigens. Most recently, Goroncy-Bermes *et al.* (36) showed that a monoclonal antibody raised against human renal cortex cross-reacted with types 6 and 12 M proteins; the antibody opsonized both serotypes of streptococci providing definitive evidence that M proteins of nephritogenic strains share antigenic determinants with renal glomeruli.

In the present study, we investigated renal tissue cross-reactivity by raising antibodies against defined regions of type 1 M protein. Our studies show that a synthetic peptide representing residues 1-26 of the amino-terminus of type 1 streptococcal M protein evoked not only opsonic antibodies but also antibodies that cross-reacted with human renal glomeruli, whereas peptide 1-23 evoked opsonic but not glomerular cross-reactive antibodies. Furthermore, a synthetic peptide copying residues 1-19 coupled in tandem to residues 23-26 also evoked glomerular cross-reactive antibodies, suggesting that the sequence Ile-Arg-Leu-Arg at positions 23-26 accounted for the cross-reactive epitope. In the latter omission peptide, the sequence Ile-Arg-Leu-Arg was three residues closer to the helix-breaking proline residue at position 18. This may account for the noticeably higher titer of cross-reactive antibodies evoked by this peptide than by the natural sequence 1-26.

Additional evidence indicating that the glomerular cross-reactive epitope resides in the sequence Ile-Arg-Leu-Arg was obtained by immunofluorescence inhibition studies with several overlapping synthetic peptides. Only the peptides containing the sequence Ile-Arg-Leu-Arg were able to inhibit the glomerular cross-reactivity. Furthermore, antibodies affinity purified with immobilized Ile-Arg-Leu-Arg-Cys reacted with renal glomeruli in a peptide-specific manner. We found that at least one anti-opsonic epitope in the amino terminus of type 1 M protein is distinct from the glomerular cross-reactive determinant; the synthetic peptide SM1(1-20)C inhibited the opsonic antibodies raised against SM1(1-26)C or the omission peptide SM1(1-19)(23-26)C but did not inhibit the glomerular cross-reactivity.

A search of the published sequences of serotypes 5, 6, 24, and 49 M proteins (8-10, 20, 30, 31) failed to reveal the Ile-Arg-Leu-Arg sequence. Since type 49 is also a nephritogenic strain it is unlikely that this tetrapeptide is the only sequence defining a renal autoimmune epitope of the streptococcal M proteins.

The identification of cross-reactive antibodies against a defined region of type 1 streptococcal M protein opens new avenues to investigations of the role of such cross-reactive epitopes in the pathogenesis of acute poststreptococcal glomerulonephritis. In preliminary studies (unpublished), the cross-reactive antisera raised against the M1 peptides failed to react with laminin, heparan-sulfate proteoglycan, and type IV collagen in immunoblots, dot blots, or ELISA, suggesting that the glomerular cross-reactivity is most likely not due to a reaction with one of these major basement membrane components. These findings are consistent with lack of a basement membrane pattern of staining observed in the present study. In fact, the pattern of staining is more suggestive of a mesangial cell distribution. Further investigations are underway to identify the cross-reactive glomerular antigens and to determine a possible connection between these autoimmune reactions and the development of acute poststreptococcal glomerulonephritis.

In conclusion, we have shown that synthetic peptides of the amino terminus of type 1 M protein contain opsonic as well as renal glomerular cross-reactive epitopes. At least one protective epitope is separable from the cross-reactive epitope and therefore may be useful in the formulation of vaccine preparations. Our studies provide definitive evidence that at least one of the glomerular cross-reactive

epitopes of type 1 M protein is located within residues 23–26, representing the amino acid sequence Ile-Arg-Leu-Arg. The significance of autoantibodies in the pathogenesis of human poststreptococcal glomerulonephritis remains unknown. Whether such antibodies in combination with complement cause renal damage or whether they play a protective role in masking glomeruli from recognition by cytotoxic T lymphocytes requires further investigation. These results have direct bearing on the development of safe and effective vaccines against group A streptococcal infections and may shed light on the mechanism of autoimmune tissue injury in acute poststreptococcal glomerulonephritis.

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