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PRIMARY STRUCTURAL SIMILARITIES BETWEEN TYPES 5 AND 24 M PROTEINS OF *Streptococcus pyogenes*

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SUMMARY: Type 5 M protein was extracted from whole *S. pyogenes* organisms by limited digestion with pepsin. The peptic extract was purified to physicochemical homogeneity by ammonium sulfate fractionation and ion exchange chromatography. The purified product, designated pep M5, migrated as a single band in sodium dodecyl sulfate (SDS)-gels at a position consistent with a molecular weight of ~ 21,000. Subcutaneous injections of rabbits with a single 100 μg dose of pep M5 emulsified in complete Freund's adjuvant resulted in the production of peak levels of type specific precipitating and opsonic antibodies in each of the animals at 8 weeks after the immunizing dose.

The amino acid composition and the amino acid sequence of pep M5 showed repeating sequences and striking similarities with the known amino acid sequences of peptide fragments of the heterologous serotype 24 pep M protein. The antisera raised in rabbits against pep M5 reacted only with the homologous pep M5 and not with the heterologous pep M24 in enzyme linked immunosorbent assays (ELISA). One serum from a rabbit immunized with pep M24, however, exhibited a high titer of antibody against the heterologous pep M5. These results indicate that pep M5 similar to pep M24 is composed of repeating covalent structures and suggest that these heterologous serotypes of M protein share certain antigenic determinants which may reside in the regions of the molecule showing structural homologies.

INTRODUCTION:

The type-specific M proteins located on the surface of virulent group A streptococci confer resistance to phagocytosis by host cells. Antibodies directed towards these proteins are opsonic, thus neutralizing the organism's defenses against the host (1). Recent investigations have shown that limited pepsin digestion of the intact organism removes this M protein (pep M) which retains antiopsonic activity (2). Antibodies produced in laboratory animals and humans immunized with purified pep M have been found to be mostly type-specific with opsonic activity for the parent streptococci (2-4). Recent studies have been directed towards understanding the relationship between the primary structure of M proteins and the immune response to virulent group A streptococci (2-5).

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A highly purified protein (pep M24) has recently been obtained and chemically characterized (3). Its partial covalent structure was determined from intact pep M24 and seven of its CNBr derived peptides. A large number of repeating internal amino acid sequences were identified (3). This report represents initial studies with another type-specific M protein (pep M5) and demonstrates a repeating covalent structure with a high degree of similarity and amino acid sequence homologies between pep M5 and pep M24. We present data which suggest that the structural homologies between these heterologous serotypes of M protein occasionally may give rise to immunological cross-reactions with each other.

MATERIALS AND METHODS:

Preparation of Pep M5. The group A streptococci (Strain Manfredo) were grown in Todd-Hewitt broth and the M protein removed by limited pepsin digestion (50 mg/ml, Worthington) at pH 5.8 for 1 hr at 37°C as previously described (4). The protein was purified by 60% saturated ammonium sulfate precipitation, and fractionated by QAE Sephadex ion exchange chromatography. The protein was applied to columns (1.6 x 32 cm) previously equilibrated with 0.05M Tris HCl, pH 7.4, and then eluted with a stepwise gradient of NaCl of 0 to 0.5M. Material eluting at 0.1M NaCl was collected, dialyzed against distilled water, lyophilized and used for the following structural and immunological studies.

Chymotrypsin Digestion of Pep M5. The pep M5 was digested with chymotrypsin (50 mg/0.25 mg) in 10 ml 0.1M NH_4HCO_3, pH 8.0 at 30°C for 30 min. The reaction was halted by adjusting the pH to 3.5 with acetic acid and chymotryptic peptides were separated by Sephadex G50S gel filtration (3.0 x 110 cm column) in 0.1M acetic acid. The peptides were further fractionated using 0.9 x 20 cm columns of CM-cellulose equilibrated with 0.01M sodium acetate, pH 4.8, using a linear NaCl gradient from 0 to 0.1M NaCl in a total volume of 500 ml. The fractions were collected as indicated, lyophilized, desalted with columns of Sephadex G25 in 0.1M acetic acid and relyophilized.

Analytical Methods. Polyacrylamide gel electrophoresis on 7.5% gel ($NaDodSO_4$-PAGE) of the various fractions were used to follow the purification. The molecular weight was estimated by $NaDodSO_4$-PAGE and amino acid content. Amino acid sequence analysis was performed using an Automatic Protein Sequencer (Model 890 C Beckman Instrument) according to the principles of Edman and Begg (6). The peptide-DMAA program (071472 of Beckman Instrument) was used and the pth amino acids were identified by high pressure liquid chromatography (Waters Inst.) (7), or hydrolysis with 55% HI to their parent amino acids (8). Amino acid analyses were performed after hydrolyzing the protein under N_2 in double distilled, constant boiling HCl for 24 hrs at 105°C (9).

Assays for M Antigen. Rabbits were immunized intracutaneously with 100 ug of pep M5 or pep M24 emulsified in complete Freund's adjuvant (2,10). Type specific opsonic antibody tests were performed on the immune sera using heterologous and homologous M serotypes of group A streptococci as described previously (2). Dilutions of opsonic antiserum which produced phagocytosis of 10% or greater after 30 min of incubation at 37°C compared with less than 2% for the non-immune sera were considered positive. Type specificity was assured by failure

of the antisera to opsonize heterologous M serotypes of streptococci (2). Enzyme-linked immunosorbent assays (ELISA) were performed as previously described in detail (4,11).

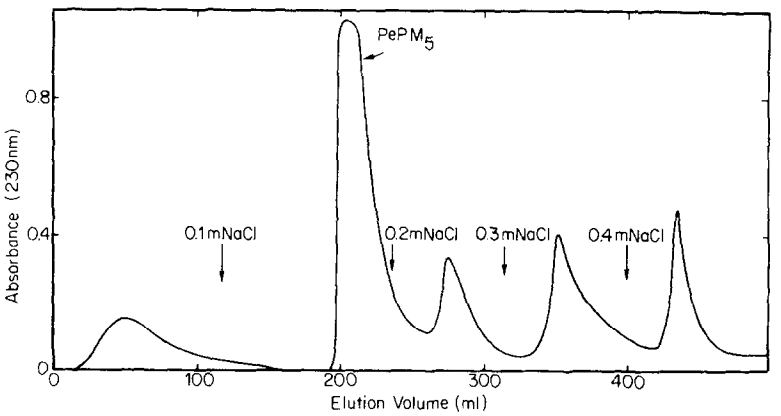

Figure 1. QAE-Sephadex ion-exchange chromatography of pepsin solubilized pep M5 from type 5 group A streptococci. Material eluting at 0.1 M NaCl was identified as pep M5 (see Methods and Materials for details).

TABLE 1

Amino Acid Composition of Pep M5, the Chymotrypsin Derived Peptide C1 and Pep M24[a]

Amino Acid	Pep M5	C1	Pep M24[b]
Aspartic Acid	28	6.8 (7)	55
Threonine	20	4.2 (4)	17
Serine	2.6 (3)	-	25
Glutamic Acid	38	8.1 (8)	54
Proline	2.0 (2)	-	-
Glycine	11	2.8 (3)	7.0
Alanine	12	1.2 (1)	73
Cysteine	0	-	-
Valine	5.6 (6)	0.6 (1)	3.6
Methionine [c]	0.2 (0)	-	6.4
Isoleucine	7.6 (8)	0.6 (1)	6.8
Leucine	24	4.7 (5)	47
Tyrosine	1.6 (2)	0.7 (1)	-
Phenylalanine	0.4 (0)	-	10
Lysine	41	7.2 (7)	56
Histidine	2.0 (2)	1.1 (1)	2.6
Arginine	4.6 (5)	1.0 (1)	11
Total	201	40	376

[a] Values expressed as residues per molecule. A dash indicates the level was less than one residue per molecule. Residues expressed to the nearest 0.1 except for values greater than 10. Numbers in parenthesis indicate assumed integral values.
[b] Taken from Beachey et al. (1978) Proc. Natl. Acad. Sci. USA 75, 3163.
[c] Includes methionine sulfone.

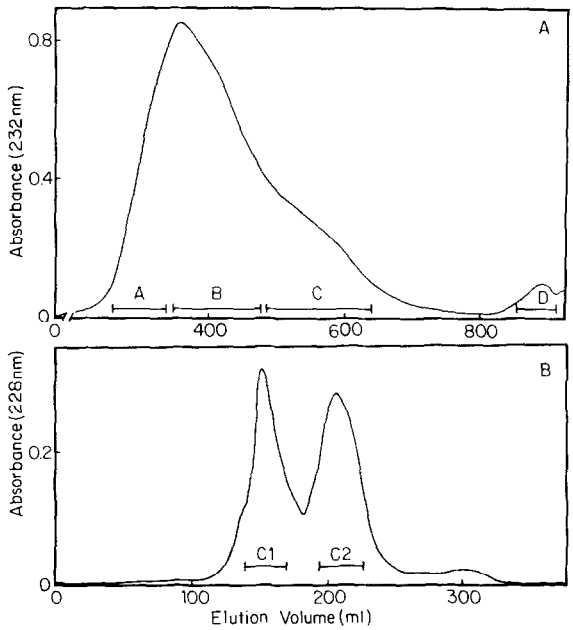

Figure 2a. Sephadex G50S gel filtration of chymotryptic peptides of pep M5. (See Methods and Materials for details). Areas indicated by bars were collected and A and C were further characterized.

Figure 2b. CM-cellulose chromatography of the chymotryptic peptides (C of Figure 2) isolated after Sephadex G50S gel filtration of a chymotryptic peptide of pep M5. The chromatographic conditions are presented in Methods and Materials. The material indicated by the bar under C1 was collected, desalted, lyophilized and used for amino acid sequence analyses.

RESULTS:

The type-specific M antigen from type 5 streptococci was purified from pepsin extracts of the whole organism. Pep M5 eluted from QAE Sephadex at 0.1M NaCl (Figure 1). The pep M5 was homogenous as judged by NaDodSO$_4$-PAGE and the presence of Ala as the single NH_2-terminus by amino acid sequence analysis. The amino acid composition, assuming a molecular size of 21,000 obtained by the above procedures, is presented in Table 1. High quantities of charged amino acids, lysine, glutamic acid, and aspartic acid as well as leucine and threonine were present similar to that previously found for pep M24, a 33,000 molecular weight streptococcal M protein (3).

Chymotryptic peptides of pep M5 were separated by Sephadex G50S gel filtration and CM-cellulose chromatography (Figure 2a). The larger peptide, (A in

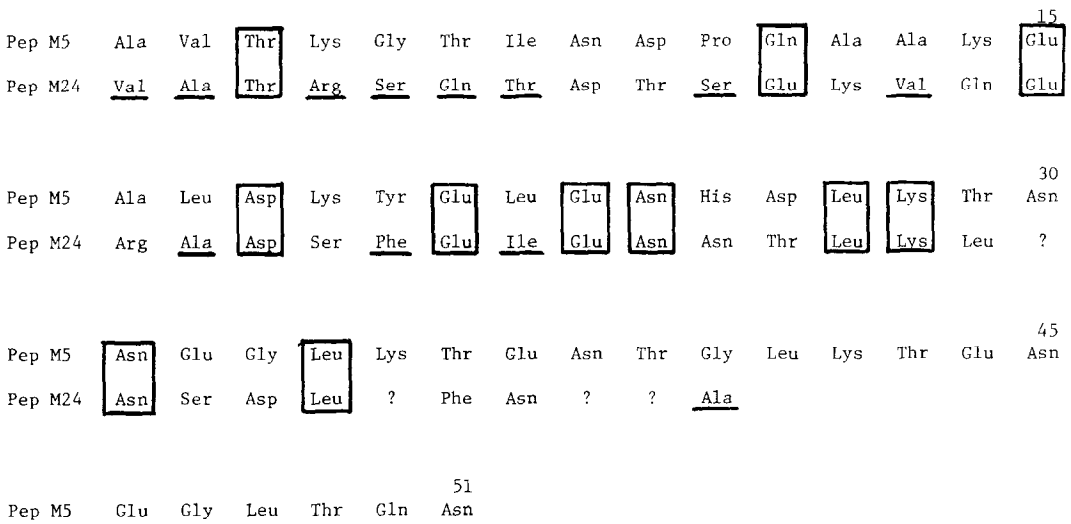

Figure 3. The primary structure of the initial 51 residues of pep M5 isolated by limited pepsin digestion of group A streptococci. Identification of residue 21-51 was obtained using the NH_2 terminus of the chymotryptic peptide, C1. For comparison, the NH_2-terminal segment of pep M24 identified previously (6) was included. Boxed in areas represent sequence identity and the lined residues represent sequence similarities not involving either acid or basic amino acid substitutions.

Figure 2a) contained an amino terminal sequence identical with the NH_2-terminus of pep M5. The smaller peptides, (C in Figure 2a) were further separated by CM-cellulose (Figure 2b) and its amino acid composition is recorded in Table 1. The sequence of the first 51 residues from the NH_2 terminus are depicted in Figure 3. Ala was the amino terminus. Using intact pep M5, the sequence was determined through residue 29. The chymotryptic peptide, C1, contained an NH_2 terminal amino acid sequence identical with residues 21-29 of pep M5 and is presented tentatively as an overlapping sequence of pep M5, thereby enabling determination of the covalent structure through residue 51. A comparison of pep M5 with the known covalent structure of pep M24 revealed striking sequence homologies with identities at positions 3, 15, 18, 21, 23, 24, 27, 28, 31 and 34 (Figure 3).

Three rabbits were immunized with 100 ug doses of pep M5 emulsified in Freunds' complete adjuvant. Each of the rabbits developed high titers of type-specific opsonic antibodies reaching peak levels of 1:256 8 wks after

TABLE 2

Type Specific and Cross-reactions Among Rabbit Antisera Against Pep M5 and Pep M24

	Serum antibody titer[a]	
Antiserum	Pep M5	Pep M24
Pep M5 (Pool of 3 rabbit sera)	>102,400	<200
Pep M24		
Rabbit #1	12,800	12,800
" #2	<200	12,800
" #3	<200	12,800

[a]Serum antibody titers were determined by ELISA as previously described in detail (4,11).

immunization. ELISA assays indicated that a pool of all three antisera reacted with pep M5 but not with the heterologous, though structurally related, pep M24 (Table 2). A serum obtained from one of three rabbits immunized with pep M24 (2), however, showed cross-reactivity at a titer of 1:12,800 with pep M5 indicating some degree of immunological cross-reactivity between these structurally related M protein molecules.

DISCUSSION:

The amino acid composition of pep M5 was strikingly similar to that previously found in pep M24 (3) both showing a high concentration of charged amino acids. The major amino acids in both cases were aspartic acid, glutamic acid, leucine and lysine. In contrast, with pep M5, the threonine content was high while alanine was high in pep M24. Further similarities were found when the amino acid sequence of pep M5 was compared with pep M24. Ten amino acids were identical within the initial 34 residues. Thirteen of the remaining thirty four amino acids involved only conservative substitutions not involving charged amino acid substitutions. Furthermore, it was noted that similar to pep M24, pep M5 contained internal repeating sequences including a hexapeptide at residue 33-38 which was identical to a hexapeptide at residues 41-45. In addition, the tripeptide Leu-Lys-Thr encompassed in the hexapeptide is again repeated at residues 27-29.

Pep M24 previously has been fragmented into seven distinct peptides and repeating amino acid sequences were identified in each (3). Two peptides, CB1 and CB2, contained sequences identical to each other and to the amino terminus of pep M24 through residue 29 and, therefore, regions in the intact molecule are closely related to pep M5. This similarity is especially evident between residues 15-31 (8 identical residues) and may be related to their cross-reactive properties in some immunized rabbits and humans (11). Although opsonic antibodies directed against these two proteins were type-specific, one rabbit immunized with pep M24 showed a high titer antibody cross-reactive with pep M5 in ELISA. These results indicate that closely related covalent structures are shared by M5 and M24 and yet the immune response to these antigens is mostly type specific, suggesting that the common regions may be less accessible to recognition by the immune system. Recently, evidence has been obtained that pep M24 may contain "buried" cross-reactive antigenic determinants that are only occasionally recognized in immunized animals and humans (11). Separation and characterization of these two biologically distinct molecular domains, therefore, may provide valuable information towards developing a common immunogen for various virulent streptococci.

During the preparation of this manuscript, a second pep M5 protein from a different strain of type 5 streptococci was isolated (12) and amino acid substitutions between the two pep M5 molecules were found at residues 1,4,8 and 12. These results suggest that different strains of the same serotype of streptococci may possess slightly different primary structures. Highly significant, perhaps, was the fact that no substitutions were found between residues 13-29, the region containing the greatest identity with pep M24.

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