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Repeating covalent structure of streptococcal M protein

(vaccines/M antigen/cyanogen bromide peptides/opsonic inhibition)

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ABSTRACT **We have attempted to identify the covalent structure of the M protein molecule of group A streptococci that is responsible for inducing type-specific, protective immunity. M protein was extracted from type 24 streptococci, purified, and cleaved with cyanogen bromide. Seven cyanogen bromide peptides were purified and further characterized. Together, the peptides account for the entire amino acid content of the M protein molecule. Each of the purified peptides possessed the type-specific determinant that inhibits opsonic antibodies for group A streptococci. The primary structures of the amino-terminal regions of each of the purified peptides was studied by automated Edman degradation. The partial sequences of two of the peptides were found to be identical to each other and to that of the uncleaved M protein molecule through at least the first 27 residues. The amino-terminal sequences of the remaining five peptides were identical to each other through the twentieth residue but completely different from the amino-terminal region of the other two peptides. However, the type-specific immunoreactivity and the incomplete analysis of the primary structure of the seven peptides suggest that the antiphagocytic determinant resides in a repeating amino acid sequence in the M protein molecule.**

The opsonic and presumably protective antibodies against group A streptococci are directed exclusively against the type-specific M protein antigen located on the surface of virulent organisms. Attempts to immunize humans against streptococcal infections have been hampered by toxic reactions to vaccines prepared from almost any streptococcal product, including various M protein preparations (1). Crossreactive toxic moieties are often closely associated with the M protein molecule; the conventional methods of extraction and purification have failed to consistently separate the M protein from these moieties, which account for toxic reactions in the skin (2–5) and in the blood (6, 7).

Several years ago Cunningham and Beachey (8) showed that limited peptic digestion abolished the toxic properties while retaining type-specific immunogenicity of purified preparations of M protein. Subsequently, M protein was extracted directly from group A streptococci by subjecting the intact organisms to limited digestion with pepsin (9). The peptic extracts of M protein (pep M) were then purified to homogeneity, and the antigen was readily separable from crossreactive toxic materials (10). In its purified form the M antigen was immunogenic in laboratory animals, producing high titers of type-specific opsonic antibodies without producing crossreactive antibodies against M-associated antigens (10, 11). Furthermore, the purified antigen was well tolerated in skin tests of guinea pigs and humans (11, 12).

Having obtained satisfactory yields of purified pep M protein, we thought it of interest to study the primary structure of the M antigen in an attempt to define those structural features of the molecule that account for antigenic type specificity and protective immunity. In the present study, we have isolated seven cyanogen bromide (CNBr) peptides which together account for the entire amino acid content of the type 24 M protein molecule. Each of the seven peptides is shown to possess type-specific antiopsonic activity. A comparison of the partial amino acid sequence of each of the CNBr peptides demonstrates homologous regions among the peptides.

MATERIALS AND METHODS

Extraction and Purification of Streptococcal M Protein. Group A streptococci were grown in Todd–Hewitt broth, intermittently passaged through human blood (13) to enhance optimal production of M protein, and stored lyophilized or frozen in 20% serum broth at −70° as described (6). The organisms were grown in Todd–Hewitt broth for 16 hr in 60-liter batches, sedimented by centrifugation, washed twice in 20 mM phosphate/0.15 M NaCl, pH 7.4 (P_i/NaCl) and once in 67 mM phosphate, pH 5.8. The organisms were finally resuspended in 3 vol of the buffer at pH 5.8 containing 50 μg of pepsin per ml (Worthington Biochemical Corp., Freehold, NJ) and incubated for 1 hr at 37°. Digestion was stopped by adding 7.5% $NaHCO_3$ to raise the pH to 7.5. The organisms were then sedimented by centrifugation at 10,000 × *g* (Dupont Instruments, Sorvall Operations, Newton, CT) for 20 min, and the cells were extracted a second time with pepsin under identical conditions. The supernates were combined, filtered through a 0.45-μm membrane filter (Millipore), dialyzed against P_i/NaCl, lyophilized, redissolved in 10 ml of distilled water, further dialyzed against distilled water, and relyophilized. The lyophilized material was redissolved in P_i/NaCl at a concentration of 10 mg/ml and precipitated with 30% saturated ammonium sulfate (10). The ammonium sulfate precipitates were redissolved, digested with ribonuclease, dialyzed, and relyophilized as described (14).

The ribonuclease-digested pep M was redissolved in 50 mM Tris-HCl buffer at pH 7.5 and fractionated by ion-exchange chromatography on columns of quaternary aminoethyl (QAE) Sephadex A50 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) followed by isoelectric focusing as described (10).

Cleavage with CNBr. Fifty-milligram samples of purified pep M were dissolved in 20 ml of 70% formic acid and digested under N_2 with CNBr at 40° for 4 hr. The digests were diluted with 20 vol of cold distilled water and lyophilized.

Molecular Sieve and Ion-Exchange Chromatography. CNBr peptides were partially separated by gel filtration through a 4 × 120 cm column of Sephadex G50 SF (Pharmacia

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Abbreviations: CB, cyanogen bromide peptide; CM, carboxymethyl; P_i/NaCl, 20 mM phosphate/0.15 M NaCl at pH 7.4; pep M24, purified type 24 M protein extracted from streptococci with pepsin.

Table 1. Immunoreactivity of CNBr peptides of type 24 M protein

M protein preparation	Precipitin reaction	Opsonic inhibition,* nmol
CNBr peptide mixture	+++	1.3
Uncleaved pep M24	+++	0.05

* Minimal amount required to inhibit phagocytosis by 50%.

Fine Chemicals, Inc., Piscataway, NJ). The peptides were further separated at 43° by ion-exchange chromatography on a 0.9 × 20 cm column of carboxymethyl (CM)-cellulose (Whatman CM52). The samples were dissolved in starting buffer (20 mM sodium acetate, pH 4.8), applied to the column, and eluted with a linear gradient of NaCl from 0 to 0.12 M over a total volume of 800 ml at a flow rate of 40 ml/hr.

Analytical Methods. Amino acid analyses were performed as described (15). Samples were hydrolyzed in doubly distilled, constant boiling HCl under an atmosphere of nitrogen for 24 hr at 108°. The hydrolyzed samples were then analyzed on a Beckman 121 automatic amino acid analyzer (Beckman Instruments, Inc., Fullerton, CA) by a single column technique with a four-buffer elution system (16). No corrections were made for the loss of labile amino acids (threonine, serine, methionine, and tyrosine) or the incomplete release of valine.

Automated Edman degradations were performed with a Beckman Sequencer (model 890C, Beckman Instruments, Inc.) according to the principles first described by Edman and Begg (17). The protein-Quadrol (10 mM) program (122974) of Beckman Instruments was used (18). The phenylthiohydantoin-amino acids were identified by high-pressure liquid chromatography (19). Arginine derivatives were identified as their parent amino acid by amino acid analysis after hydrolysis with 55% HI (20). Repetitive yields of 97% were obtained during the automated Edman degradation.

Tests for Type-Specific M Antigen. The antisera used in these tests were prepared by immunizing rabbits intracutaneously with a 100-μg (dry weight) dose of M protein emulsified in complete Freund's adjuvant (21). Rabbits were bled 6–8 weeks after injection. In some cases, rabbits were given booster intraperitoneal (10 μg) and intravenous (100 μg) injections of M protein dissolved in P_i/NaCl on consecutive days, and sera were then collected 6–8 days after the intravenous injection. Sera were stored at 4°.

Capillary precipitin tests of serial 2-fold dilutions of M protein in P_i/NaCl were performed according to Swift *et al.* (22) and as described (6). Tests for the inhibition of type-specific opsonization of homologous M serotypes of group A streptococci were performed as described (10, 23). Samples of opsonic antiserum diluted in P_i/NaCl to the highest dilution that produced 40–60% phagocytosis of homologous-type streptococci were absorbed with serial twofold dilutions of M protein or its derived peptides.

Table 2. Inhibition of indirect bactericidal test by CNBr peptides by type 24 M protein

Rabbit serum	No. of colonies* Inoculum 40	Inoculum 8
Preimmune serum	1545	200
Anti-pep M24		
Unabsorbed	0	0
Absorbed† with uncleaved pep M24	1500	200
Absorbed with mixture of CNBr peptides of M24	600	64

* Type 24 streptococci after 3 hr of growth in test mixture.

† Rabbit antiserum against pep M24 was absorbed with an equal volume of a 1 mg/ml solution of uncleaved pep M24 or of pep M24 cleaved with CNBr.

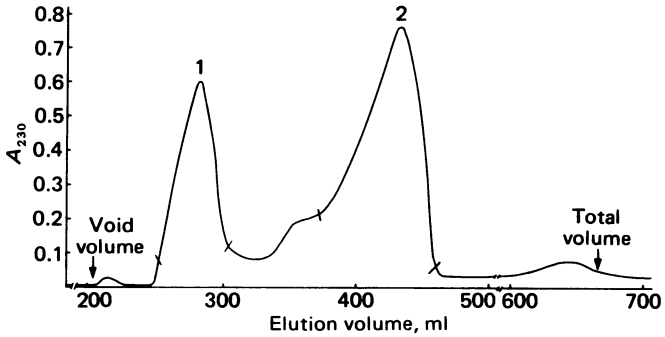

FIG. 1. Molecular sieve chromatography of CNBr peptides of type 24 M protein on column of Sephadex G50-SF.

After precipitates were removed that may have formed by centrifugation, the absorbed and unabsorbed antiserum samples were used to opsonize homologous-type streptococci. It should be emphasized that in this system the opsonized streptococci were thoroughly washed in ice-cold P_i/NaCl before they were added to samples of fresh human blood. Washing removes excess antigen and soluble antigen–antibody complexes that may inhibit phagocytosis non-type specifically (24), either by depleting the fresh human blood of complement or by mediating cytotoxic effects upon polymorphonuclear leukocytes (6). Results are expressed as the minimal amount in nanomoles of M protein or its derived peptides that is capable of inhibiting opsonization and phagocytosis of homologous-type streptococci by 50% or more. The type specificity of opsonic inhibition was assured by absorbing heterologous antisera with the M preparations. In no case were the opsonic effects of the absorbed heterologous antisera reduced against their respective serotypes of streptococci. Control experiments demonstrated that the streptococci used in these tests were phagocytosed less than 5% in the presence of normal rabbit serum. The results of the phagocytosis tests were confirmed by indirect bactericidal tests (6) by using the unabsorbed and M protein-absorbed antisera to opsonize homologous-type streptococci.

RESULTS

Purification of the pepsin-extracted type 24 M antigen by ammonium sulfate precipitation, ion-exchange chromatography, and isoelectric focusing resulted in a homogeneous protein preparation free of crossreactive antigens or toxicity (10–12). Since the purified pep M24 molecule contains six methionyl residues (10), we decided to cleave the molecule with cyanogen

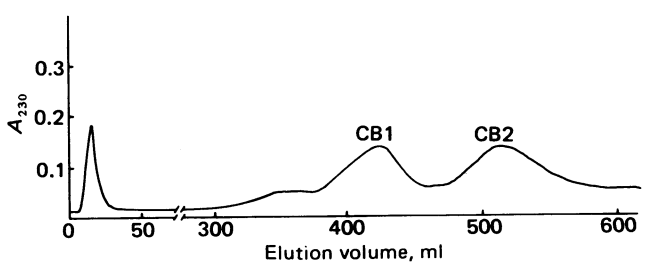

FIG. 2. Ion-exchange chromatography on column of CM-cellulose of fraction 1 eluted from Sephadex G50 (see Fig. 1).

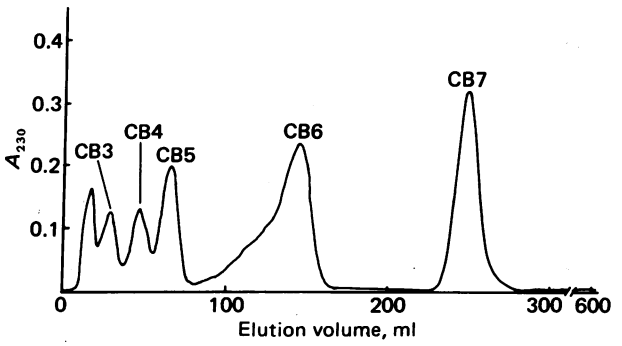

FIG. 3. Ion-exchange chromatography on column of CM-cellulose of fraction 2 eluted from Sephadex G50 (see Fig. 1).

bromide. The mixture of CNBr peptides retained type-specific immunoreactivity, as judged by precipitin reactions and by inhibition of opsonic antibody in phagocytosis (Table 1) and bactericidal tests (Table 2). The results indicate that CNBr digestion reduces but does not abolish antigenicity of pep M24.

Separation of CNBr Peptides. The lyophilized CNBr peptides were separated into two major peptide fractions by gel filtration through Sephadex G50-SF (Fig. 1). Amino acid analysis of the material in the shoulder preceding fraction 2 was similar to that of the material in fraction 2, and it was assumed that the material represented larger peptides that were incompletely digested by CNBr.

Each of the major peptide fractions was further fractionated by gradient salt elution from CM-cellulose. The first fraction eluted from Sephadex G50 was separated into two homogeneous peptides designated CB1 and CB2 (Fig. 2), and the second fraction (peak 2 eluted from Sephadex) was separated into five additional peptides, designated CB3 through CB7 (Fig. 3). Each of the peptides was desalted by passing it through a 2.5 × 100 cm column of polyacrylamide gel beads (Bio-Gel P2) equilibrated with 10 mM HCl and lyophilized.

Peptide Analysis. Quantitative amino acid analysis of each of the CNBr peptides demonstrated the presence of homoserine in each of the peptides except CB5 (Table 3), indicating that CB5 probably represents the carboxyl-terminal peptide of the intact pep M24 molecule. The absence of methionine in any of the peptides indicates complete hydrolysis by CNBr. Assuming an average molecular weight of 100 for each amino acid residue, the molecular weights of CB1 and CB2 are approximately 9000 while those of CB3–CB7 range between 4000 and 5000. The sum of the assumed integral values of the amino acids calculated for each of the peptides agrees within experimental error with values obtained for the uncleaved pep M24 molecule, indicating that all of the CNBr peptides were recovered.

Table 4. Immunoreactivity of purified CNBr peptides of pep M24

Peptide	Amino acid residues/peptide	Precipitin reactions*	Peptide required to inhibit phagocytosis,† nmol
CB1	88	++	0.2
CB2	90	++	0.2
CB3	45	–	0.4
CB4	44	–	0.4
CB5	40	–	1.6
CB6	35	–	1.6
CB7	34	–	1.6
Pep M24	376	++++	0.05
Pep M6			>50

* Capillary precipitin tests performed using antiserum to intact pep M24 (see *Materials and Methods*).

† Minimal amount to inhibit phagocytosis of type 24 streptococci by 50%.

Immunoreactivity of Purified CNBr Peptides. Before proceeding with further chemical analysis of the peptides, it was of interest to determine the type-specific immunoreactivity of each peptide. Capillary precipitin tests with homologous M antiserum demonstrated the presence of precipitating antigen in CB1 and CB2 but the absence of precipitating antigen in the remaining smaller peptides (Table 4). Nevertheless, each of the seven CNBr peptides was able to inhibit type 24 opsonic antibody, ranging from minimal inhibitory amounts of 0.2 nmol for CB1 and CB2, 0.4 nmol for CB3 and CB4, and 1.6 nmol for

Table 3. Amino acid composition of CNBr peptides of pep M24*

Amino acid	Pep M24	CB1	CB2	CB3	CB4	CB5	CB6	CB7	Total†
Aspartic acid	55.41 (55)	16.8 (17)	17.7 (18)	6.1 (6)	4.8 (5)	4.4 (4)	2.1 (2)	3.0 (3)	55
Threonine	16.86 (17)	3.8 (4)	2.8 (3)	1.6 (2)	1.8 (2)	1.8 (2)	1.8 (2)	1.6 (2)	17
Serine	25.01 (25)	5.6 (6)	6.3 (6)	2.7 (3)	2.1 (2)	3.1 (3)	2.7 (3)	2.4 (2)	25
Glutamic acid	53.86 (54)	13.3 (13)	12.6 (13)	6.2 (6)	6.2 (6)	5.1 (5)	6.8 (7)	4.1 (4)	54
Proline	—	—	—	—	—	—	—	—	0
Glycine	7.04 (7)	1.4 (1)	0.7 (1)	1.1 (1)	0.9 (1)	0.6 (1)	1.1 (1)	1.0 (1)	7
Alanine	72.82 (73)	9.8 (10)	10.8 (11)	12.2 (12)	12.7 (13)	10.4 (10)	7.9 (8)	8.8 (9)	73
Valine	3.60 (4)	1.7 (2)	1.5 (2)	—	—	—	—	—	4
Methionine	6.41 (6)	—	—	—	—	—	—	—	—
Isoleucine	6.84 (7)	1.2 (1)	1.1 (1)	1.1 (1)	0.8 (1)	1.1 (1)	0.8 (1)	1.2 (1)	7
Leucine	47.06 (47)	11.1 (11)	12.6 (13)	5.1 (5)	5.2 (5)	5.4 (5)	3.9 (4)	3.8 (4)	47
Tyrosine	—	—	—	—	0.3	—	0.3	—	0
Phenylalanine	10.13 (10)	2.2 (2)	1.8 (2)	1.1 (1)	1.3 (1)	2.4 (2)	1.1 (1)	1.2 (1)	10
Lysine	55.80 (56)	16.1 (16)	14.4 (14)	6.2 (6)	6.4 (6)	4.8 (5)	3.6 (4)	5.1 (5)	56
Histidine	2.54 (3)	1.3 (1)	1.1 (1)	—	—	1.0 (1)	—	—	3
Arginine	11.62 (12)	3.3 (3)	4.2 (4)	0.9 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	12
Homoserine‡	—	1.2 (1)	0.9 (1)	0.8 (1)	0.9 (1)	—	1.0 (1)	1.2 (1)	6
Total	376	88	90	45	44	40	35	34	376

* Values expressed as residues per peptide. A dash indicates the level was less than one residue per peptide. Numbers in parentheses indicate assumed integral values.

† Total of CB1–CB7.

‡ Includes homoserine lactone.

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                    5                   10                  15                  20
M24  Val-Ala-Thr-Arg-Ser-Gln-Thr-Asp-Thr-Ser-Glu-Lys-Val-Gln-Glu-Arg-Ala-Asp-Ser-Phe-

CB1  Val-Ala-Thr-Arg-Ser-Gln-Thr-Asp-Thr-Ser-Glu-Lys-Val-Gln-Glu-Arg-Ala-Asp-Ser-Phe-

CB2  Val-Ala-Thr-Arg-Ser-Gln-Thr-Asp-Thr-Ser-Glu-Lys-Val-Gln-Glu-Arg-Ala-Asp-Ser-Phe-

                    25                  30                  35                  40
M24  Glu-Ile-Glu-Asn-Asn-Thr-Leu-Lys-Leu-

CB1  Glu-Ile-Glu-Asn-Asn-Thr-Leu-

CB2  Glu-Ile-Glu-Asn-Asn-Thr-Leu-Lys-Leu- ? -Asn-Ser-Asp-Leu- ? -Phe-Asn- ? - ? -Ala-
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FIG. 4. Amino acid sequences of the amino-terminal regions of CB1 and CB2 of type 24 M protein compared to the amino-terminal region of the uncleaved molecule. ?, Unidentified amino acid.

CB5, CB6, and CB7 (Table 4). These values compare to a minimal inhibitory concentration of 0.05 nmol for the uncleaved pep M24 molecule and no inhibition with as much as 50 nmol of a heterologous pep M6 preparation. These results indicate that the type-specific opsonic, and presumably, protective antigenic determinant resides in each of the derived CNBr peptides.

Comparison of Amino Acid Sequences of CNBr Peptides. Since each of the peptides possessed type-specific immunoreactivity, it was of interest to compare the covalent structures of the peptides. Each of the peptides was subjected to automatic Edman degradation to determine the primary structure of their amino-terminal regions. As can be seen in Fig. 4, the amino acid sequences of CB1 and CB2 were identical to each other through at least residue 27 and to the amino-terminal region of the uncleaved pep M24 molecule. The amino acid sequences of CB3, CB4, CB5, CB6, and CB7 were identical to each other through amino acid number 20 but different from CB1 and CB2 (Fig. 5). The peptides CB4 and CB6 are identical to each other at least through residue 25. The sequences of the amino acids of CB3, CB5, and CB7 vary from each other and the remaining peptides after the twentieth residue. These results indicate that the pep M24 molecule is composed of repeating covalent structures.

DISCUSSION

Our data demonstrate that the type-specific antiphagocytic determinant of the type 24 M protein antigen of group A streptococci is composed of repeating covalent structures. The observation that each of the peptides inhibits opsonization of homologous-type streptococci suggests that the protective M antigen is associated with these covalent structures.

Several investigations have suggested that streptococcal M protein is composed of heterogeneous subunits each of which possesses antigenic type specificity (5, 25–29). Fox and Wittner (25) showed that although the purified HCl extracts of M proteins prepared from three serotypes, 12, 14, and 24, exhibited homogeneity by immunodiffusion analysis, each of the M proteins was separable into at least four major fractions by molecular sieve chromatography and into 10–15 bands by polyacrylamide gel electrophoresis. The bands from the individual serotypes were antigenically identical when tested by immunodiffusion against the homologous serotype of M antiserum. M proteins extracted with caustic soda produced only one or two slowly migrating bands in gel electrophoresis, indicating a larger molecular size (26). Brief acid hydrolysis partially degraded these larger-size molecules into smaller peptides similar to those in M protein extracted with HCl. Since the electrophoretic patterns of M proteins extracted with HCl from homologous serotypes were nearly identical, it was concluded that the acid cleaved the molecule nonrandomly into smaller peptides with similar, though not identical, amino acid content. Amino acid analysis and peptide mapping of the larger and smaller molecules suggested that the larger peptides were composed of repeating subunits (26). The multiple molecular structure of M protein has been substantiated by several other investigators (6, 27, 28).

Recently, Fischetti *et al.* (29) presented further evidence for a subunit structure of streptococcal M protein. These investigators used nonionic detergents to extract M protein from type 6 streptococci. The extracts were composed of multiple molecular species ranging from 6000 to 35,000 daltons. Although each of the molecules precipitated type-specific M antiserum, only the larger molecules (28,000–35,000 daltons) were able to inhibit opsonization of streptococci of the homologous type. The similarity in the amino acid content among the various molecules suggested that the type 6 M protein molecule, in its native form, is composed of repeating subunits. By radiolabeling techniques, the same investigators obtained evidence to suggest that the antiphagocytic molecules of M protein are assembled on the surface of the streptococcus by crosslinkage of the smaller peptides which, as monomers, lack the ability to inhibit opsonic antibodies against the homologous serotype.

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                    5                   10                  15                  20                  25
CB3  Asn-Phe-Ser-Thr-Ala-Asp-Ser-Ala-Lys-Ile-Lys-Thr-Leu-Glu-Ala-Glu-Lys-Ala-Ala-Leu- ? -Ala- ? -Lys-Ala-

CB4  Asn-Phe-Ser-Thr-Ala-Asp-Ser-Ala-Lys-Ile-Lys-Thr-Leu-Glu-Ala-Glu-Lys-Ala-Ala-Leu-Glu-Ala-Arg-Lys-Ala-

CB5  Asn-Phe-Ser-Thr-Ala-Asp-Ser-Ala-Lys-Ile-Lys-Thr-Leu-Glu-Ala-Glu-Lys-Ala-Ala-Leu-Glu-Ala-Glu-Lys-Ala-

CB6  Asn-Phe-Ser-Thr-Ala-Asp-Ser-Ala-Lys-Ile-Lys-Thr-Leu-Glu-Ala-Glu-Lys-Ala-Ala-Leu-Glu-Ala-Arg-Gln-Ala-

CB7  Asn-Phe-Ser-Thr-Ala-Asp-Ser-Ala-Lys-Ile-Lys-Thr-Leu-Glu-Ala-Glu-Lys-Ala-Ala-Leu-Ala-Ala-Arg-Lys-Ala-
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FIG. 5. Amino acid sequences of the amino-terminal regions of CB3, CB4, CB5, CB6, and CB7 of type 24 M proteins. Areas of amino acid substitutions are enclosed in boxes. ?, Unidentified amino acid.

Our data provide the most definitive evidence that the M protein molecule is composed of repeating primary structures. In contrast to the concept that M protein is assembled from smaller peptides that lack antiopsonic activity, we show that the type 24 molecule is made up of seven antiopsonic peptides. The type 24 M protein molecule contained six residues of methionine, which allowed fragmentation into smaller specific peptides by cleavage with CNBr. Each of the purified peptides possessed type-specific, antiopsonic activity and showed homologies among their covalent structures.

Thus, the composition of the type 24 M protein molecule described in the present study appears to differ from that of the type 6 M protein described by Fischetti and his coworkers (29). Recent studies in our laboratories of peptic extracts of type 6 M protein have shown that the purified extracts were separable into two molecular species, each 20,000 daltons in size (11). Although the amino acid contents of the two molecules were similar, the partial amino acid sequences showed no similarities. Both precipitated type-specific M antiserum, but only one was able to inhibit the opsonic antibody of the homologous type. The partial amino acid sequences of these type 6 M protein molecules were totally different from that of the type 24 molecule. It is of interest that protein molecules of different serotypes, which share the function of conferring resistance to phagocytosis, appear to possess totally different molecular structures. Perhaps the M protein on the surface of group A streptococci serves simply to mask a streptococcal ligand specific for receptors on the membranes of phagocytic cells (E. H. Beachey and I. Ofek, unpublished data).

In the type 24 molecule described in the present study, the amino-terminal regions of CB1 and CB2, although identical to each other, share no common amino acid sequences with the amino-terminal regions of CB3, CB4, CB5, CB6, and CB7; the latter five peptides show identity through at least the twentieth residue. It would appear, therefore, that the antiopsonic determinant resides elsewhere in the derived peptides. In fact, preliminary studies of the tryptic peptides of each of the CNBr peptides demonstrate the presence of a common sequence of six amino acids, the only sequence shared by all seven of the CNBr peptides (unpublished data). We do not know whether the antiopsonic determinant resides in the common structure.

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