

PURIFICATION AND PROPERTIES OF M PROTEIN
EXTRACTED FROM GROUP A STREPTOCOCCI WITH
PEPSIN: COVALENT STRUCTURE OF THE
AMINO TERMINAL REGION OF TYPE 24 M ANTIGEN*

By EDWIN H. BEACHEY, GENE H. STOLLERMAN, EDNA Y. CHIANG, THOMAS M.
CHIANG, JEROME M. SEYER, AND ANDREW H. KANG

*(From the Veterans Administration Hospital and the Departments of Medicine and Biochemistry,
University of Tennessee Center for the Health Sciences, Memphis, Tennessee 38104)*

The type-specific M protein on the surface of group A streptococci renders the organisms resistant to the phagocytic defenses of the nonimmune host. In the immune host, antibodies which are opsonic for streptococci in vitro and protective against streptococcal challenge infections in vivo are directed toward the M protein antigen located on the surface of virulent organisms. Efforts to immunize humans against streptococcal infections have been frustrated by toxic reactions to almost any streptococcal product introduced into the human host (1). "Purified" M protein vaccines have apparently contained tenaciously associated nontype-specific antigens which have accounted, at least in part, for the toxic effects of various M protein preparations (2, 3).

Since conventional methods have failed to separate the nontype-specific antigens from the type-specific M antigen, various new methods for the extraction and purification of M protein have been tried by a number of different investigators (4-10). Fox and Wittner (4) used a treatment with caustic soda or hydroxylamine to extract a molecular species of M protein that was larger and apparently more immunogenic than the conventional HCl-extracted M protein. In our hands (5), a purified preparation of an alkaline extract of type 24 streptococci was found to be free of immunotoxic or cross-reactive properties while retaining the type-specific M determinant as judged by its anti-opsonic effect in blocking the homologous M-anti-M opsonic system. The purified antigen, however, lacked immunogenicity in rabbits even though the mol wt was 36,500 daltons. Fischetti et al. (6) extracted an immunogenic M protein from type 6 streptococci and found that the purified protein appeared to be composed of subunits less than 10,000 daltons in size; the subunits precipitated type-specific M antiserum but lacked anti-opsonic activity. Radiolabeling studies of viable streptococci suggested that the subunits constitute the structural elements of a larger, 33,000 dalton M protein molecule(s) which possessed type-specific anti-opsonic activity. Russell and Facklam (7) extracted M protein with 6.0 M guanidine and further purified it by hydroxyapatite column chromatography. Whether or not the guanidine-extracted material contained cross-reactive antigens, however, was not reported. Vosti and Williams (8) extracted whole type 12 group A streptococci with cyanogen bromide. After further purification they obtained a relatively homogenous fraction

* These studies were conducted under V. A. Research project 0805-02, 0805-04, and 4826-02, and by Research Grants AI-10085, AI-13350, and AM-16506 from the U. S. Public Health Service.

which, however, contained both type-specific and nontype-specific antigens. Havlicek (9) extracted M protein by sonic disruption of whole streptococci followed by ammonium sulfate precipitation and isoelectric focusing. The purified material contained two precipitating moieties but produced only traces of nontype-specific reactions. Widdowson (10) compared the isoelectric-focusing patterns of opacity factor (OF)¹-negative and OF-positive streptococci. They found that the ability to separate the non-type-specific antigens from the type-specific M antigen varied among the different serotypes tested.

Recent studies in our laboratories (11) have demonstrated that purified M proteins lose much of their toxic properties without losing immunogenicity after they have been digested under suboptimal conditions with pepsin. We took advantage of the finding and have subsequently prepared immunogenic protein from mild pepsin digests of whole group A streptococci (12). In the present study we further purified the pepsin-extracted M protein (pep M) to homogeneity by ion-exchange chromatography and isoelectric focusing. We present data to indicate that the type-specific M (TSM) antigen which was obtained in high yields, was readily separable from cross-reactive, toxic material, and that the purified material remained immunogenic producing high titers of type-specific opsonic and precipitating antibodies in immunized rabbits.

We also present the amino acid sequence of the first 29 amino acids from the amino-terminus. Only one new amino acid at each step of Edman degradation confirmed the homogeneity of the purified pep M24. The NH₂-terminal amino acid was found to be valine.

Materials and Methods

Culturing and Extraction of Group A Streptococci. Group A streptococci were cultured in Todd-Hewitt broth (THB), intermittently passaged through mice or fresh human blood (13) to stimulate optimal M protein production, and stored lyophilized or frozen in 20% serum broth at -70°C as previously described (2). The organisms were grown in THB for 16 h in 60-liter batches, sedimented by centrifugation, washed twice in 0.02 M phosphate-0.15 M NaCl, pH 7.4 (PBS) and once in 0.067 M phosphate, pH 5.8. The organisms were finally resuspended in three volumes of the latter buffer containing 50 µg/ml of pepsin (Worthington Biochemical Corp., Freehold, N. J.) and incubated for 1 h at 37°C. Digestion was stopped by adding sufficient 7.5% NaHCO₃ to raise the pH to 7.5. The streptococci were sedimented by centrifugation at 10,000 g (DuPont Instruments, Sorvall Operations, Newtown, Conn.) 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 PBS, lyophilized, redissolved in 10 ml distilled water, further dialyzed against distilled water, and relyophilized. The lyophilized material was redissolved in PBS at a concentration of 10 mg/ml and precipitated with 30% saturated and 60% saturated ammonium sulfate. The ammonium sulfate precipitates were redissolved and digested with ribonuclease and lyophilized as previously described (12). M protein was also prepared by the conventional method of extracting washed streptococci with HCl at 95°C and was partially purified by ribonuclease digestion and ammonium sulfate precipitation by the method of Lancefield and Perlmann (14) as previously described (15).

Ion-Exchange Chromatography. The ribonuclease-digested, pep M was redissolved in 0.05 M Tris-HCl buffer at pH 7.5 and applied to a 1.6 × 32 cm column of quaternary amino ethyl (QAE)-Sephadex A50 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) equilibrated with the same

¹ Abbreviations used in this paper: CF, complement fixation; LTA, lipoteichoic acid; NTSM, nontype-specific M-associated; OI, opsonization inhibition; OF, opacity factor; PBS, 0.02 M phosphate-0.15 M NaCl, pH 7.4; pep M, pepsin-extracted M protein; PHA, passive hemagglutination; ppt'n, precipitinogen; QAE, quaternary amino ethyl; SDS, sodium dodecyl sulfate; THB, Todd-Hewitt broth; TSM, type-specific M.

buffer. The protein was eluted in a stepwise fashion with increasing concentrations of NaCl. The immunoreactive protein peaks were pooled, lyophilized, redissolved and dialyzed against distilled water, and relyophilized.

Isoelectric Focusing. 10- and 50-mg samples of the pep M purified by QAE-Sephadex chromatography were dissolved in a 5% Ampholyte solution (LKB Produkton AB, Bromma, Sweden) and pumped into 110-ml or 440-ml isoelectric-focusing columns (LKB Instruments, Inc., Rockville, Md.) (5), and electrofocused at 450 V until the flow of current approached zero. Usually 48 h for the 110-ml column or 72 h for the 440-ml column was required. Fractions were collected and analyzed for pH, absorbance at 280 nm, and immunoreactivity with M24 antiserum. Active peaks were pooled, exhaustively dialyzed against distilled water, and lyophilized.

Analytic Methods. Protein content was estimated by the method of Lowry et al. (16). Hexose was determined by the method of Dubois et al. (17) and methyl pentose by the cysteine-sulfuric acid method of Dische and Shettles (18).

Polyacrylamide gel electrophoresis was performed in 7% gel according to the method of Davis (19) as previously described (11). Molecular weights of the purified pep M were estimated by electrophoresis in 10% polyacrylamide gels containing sodium dodecyl sulfate (SDS) according to the method of Weber and Osborn (20).

Amino acid analyses were performed as previously described (21). 1-mg samples were hydrolyzed in constant boiling 6 N HCl in an atmosphere of nitrogen for 24 h at 108°C. The samples were then analyzed with a Beckman 121 automatic amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.) by a single column technique (22).

Automatic Edman degradations were performed with a Beckman Sequencer (Model 890C; Beckman Instruments, Inc.) according to the principles first described by Edman and Begg (23). The protein-Quadrol (0.01 M) program (122974) of Beckman Instruments was employed (24). The phenylthiohydantoin-amino acids were identified by high pressure liquid chromatography (25). Arginine derivatives were identified as their parent amino acid by amino acid analysis after hydrolysis with 55% HI (26). Repetitive yields of 97% were obtained during the automated Edman degradation.

Streptococcal Antisera. Antisera to whole group A streptococci were prepared by immunizing rabbits with heat-killed or ultraviolet-irradiated streptococci as previously described (5). Standard, M type-specific antisera were obtained from the Center for Disease Control, Atlanta, Ga.

The immunogenicity of the purified pep M was studied by injecting rabbits intracutaneously with a 100 μ g (dry weight) dose of M protein emulsified in complete Freund's adjuvant (27) as previously described (5). Rabbits were bled at 2-wk intervals for 8 wk after immunization. Sera were collected and stored at 4°C. Rabbits were also immunized with purified lipoteichoic acid (LTA) prepared from a strain (1RP41) of group A streptococci, and precipitated with methylated bovine serum albumin according to the method of Fiedel and Jackson (28) as previously described (29).

Streptococcal Opsonization Tests. In vitro timed phagocytosis tests for type-specific opsonic M antibody were performed as previously described (5, 30). The test mixtures consisted of 0.4 ml fresh heparinized (10 U/ml) human blood, 0.05 ml of a standard suspension of phagocytosis-resistant streptococci, and 0.05 ml of test serum. The ratio of streptococcal units per leukocyte was approximately 10:1. The percentage of neutrophilic leukocytes which had ingested streptococci (percent phagocytosis) was estimated by microscopic examination of stained smears prepared from a drop of test mixture at 15 and 30 min of incubation. The opsonic antibody titer is expressed as the reciprocal of the highest twofold dilution of test serum that in three separate tests of each serum produced phagocytosis of 10% or greater at 30 min of incubation at 37°C while phagocytosis of the same organisms in the presence of nonimmune rabbit serum was less than 2% in each test. M antisera exhibiting titers greater than 1:4 all produced phagocytosis in the range of 40-70% when tested undiluted. The results of phagocytosis tests were confirmed by indirect bactericidal tests performed as previously described (2). Type specificity was assured by failure of antisera to opsonize heterologous M serotypes of streptococci.

Titration of TSM Antigen. Capillary precipitin tests of serial twofold dilutions of M protein in PBS were performed by the method of Swift et al. (31) as previously described (2). The reciprocal of the highest dilution of a 1 mg/ml solution of M protein producing a type-specific precipitate was designated the TSM precipitinogen (ppt'n) titer.

Tests of the inhibition of type-specific opsonization were performed as previously described in

detail (5, 30). Samples of opsonic antiserum diluted in PBS to the highest dilution producing 40-60% opsonization were absorbed with serial twofold dilutions of M protein solutions. After removing precipitates by centrifugation the absorbed and unabsorbed antiserum samples were used to opsonize homologous type streptococci that were resistant to phagocytosis. It must be stressed that in this system the opsonized streptococci are thoroughly washed before adding them to fresh human blood samples. Washing removes excess antigen and antigen-antibody complexes that might inhibit phagocytosis nonspecifically either by depleting the fresh human blood of complement or by mediating cytotoxic effects upon polymorphonuclear leukocytes as previously reported (2). The reciprocal of the highest dilution of a 1 mg/ml solution of the absorbing antigen that prevented opsonization was designated the TSM opsonic inhibitory (OI) titer. The type specificity of preopsonic inhibition was assured by absorbing heterologous antisera with the M preparations. In no case were the preopsonic effects of the absorbed heterologous antisera reduced against their respective serotypes of streptococci.

Tests for Nontype-Specific M (NTSM)-Associated Antigens. Complement fixation (CF) tests were performed by a microtechnique as previously described (21). NTSM antigen titers were expressed as the reciprocal of the highest dilution of a 1 mg/ml solution of M protein preparation that produced 50% lysis of antibody-coated sheep erythrocytes in the presence of rabbit antiserum against whole type 30 streptococci. In addition to M30-specific antibody the serum had a known high titer of NTSM antibody. The type 30 antiserum was analyzed for opsonic activity against type 24 streptococci to confirm the absence of M24-specific antibody which might have interfered with the test for NTSM.

Tests for the presence of LTA antigen were performed as previously described (21, 32). Human erythrocytes were incubated with serial dilution of a 1 mg/ml solution of the M protein preparations, washed, and mixed with a dilution of antiserum containing high agglutinating titers of anti-LTA. Control erythrocytes were incubated with serial dilutions of a 1 mg/ml solution of purified LTA or with PBS. The reciprocal of the highest dilution of antigen capable of modifying erythrocytes was expressed as the passive hemagglutination (PHA) titer of LTA antigen. To assure the absence of teichoic acid antigen that may have lost its erythrocyte-sensitizing activity, the antigen preparations were tested for their ability to inhibit the PHA activity of anti-LTA against LTA-modified erythrocytes as previously described (32).

Delayed skin reactivity of the various M protein fractions were tested in adult guinea pigs as previously described (5, 15). 100- μ g doses of the antigen preparations were dissolved in 0.1 ml PBS and injected intradermally at a shaved area of the back. Diameters of induration were recorded at 24 h.

Results

The crude pepsin extracts of type 24 M protein were fractionated first by ammonium sulfate precipitation. Most of the immunoreactive material precipitated at 30% saturation (Table I). Although immunoreactive material was precipitated also at 60% the fraction was more heterogeneous (see below). Therefore, the 30% fraction was employed in the following studies.

Ion-Exchange Chromatography and Isoelectric Focusing of Pep M24. After ribonuclease digestion, the ammonium sulfate (30% saturation) precipitated material was applied to a column of QAE-Sephadex equilibrated with 0.05 M Tris-HCl and was eluted in step-wise fashion with increasing concentrations of NaCl (Fig. 1). The type-specific immunoreactive material eluted with 0.3 M NaCl.

The protein in the 0.3 M NaCl eluate was pooled, dialyzed against distilled water, and lyophilized. It was then redissolved in the appropriate ampholine-sucrose solution and loaded onto an isoelectric-focusing column (see Materials and Methods). A typical elution pattern is depicted in Fig. 2. Most of the type-specific immunoreactive material was located in a broad ultraviolet (280 nm) absorbing area with a peak pH of 7.15. The opsonic inhibitory activity was

TABLE I
Immunoreactivity of Ammonium Sulfate Fractions of Crude pep M24

Ammonium sulfate Fraction	TSM antigen titers*	
	Precipitinogen	Opsonic Inhibition
30% Saturation	1:256	1:16
60% Saturation	1:128	1:8
Supernate	<1:2	<1:2

* Antigen titers are expressed as the highest dilution of a 1 mg/ml solution producing a positive reaction.

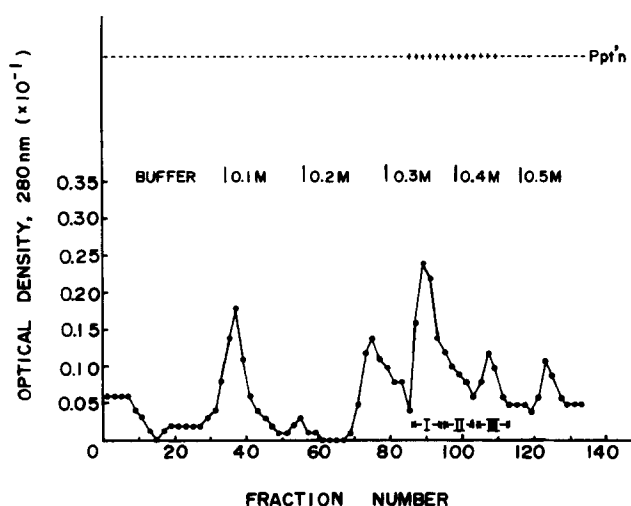


FIG. 1. Stepwise elution of pep M from a QAE-Sephadex column. 50 mg of pep M24 was applied to a 1.6×32 cm column equilibrated with 0.05 M Tris-HCl, pH 7.0 (buffer). Samples were eluted with stepwise increases in NaCl concentrations as indicated. Samples were pooled into fractions I, II, and III as indicated. Most of the immunoreactive material was located in fraction I. Ppt'n, precipitation with type-specific M24 antiserum; +, positive reaction; -, negative reaction.

located in the same peak indicating that the type-specific opsonogen and ppt'n are closely associated in pep M24. The final yield of electrofocused M protein was 84 mg/60 liters of streptococcal culture (Table II).

Immunochemical Purity and Properties of Pep M24. Methylpentose, which is associated with cell walls, decreased from 1.4 to less than 0.5%, and NTSM antigens, estimated by CF in the presence of antiserum to type 30 streptococci, decreased from a titer of 1:256 to less than 1:2 while the type-specific antigenic activity increased from a ppt'n titer of 1:128 to 1:1,024 and an anti-opsonic titer of 1:4 to 1:64 (Table II). The purified material in concentrations of 1 mg/ml was unable to spontaneously modify group O erythrocytes to enable them to agglutinate in the presence of antiserum against purified LTA; it also was unable to inhibit anti-LTA in passive hemagglutination tests of LTA-modified erythrocytes, indicating the absence of LTA or teichoic acid antigens in the purified M protein.

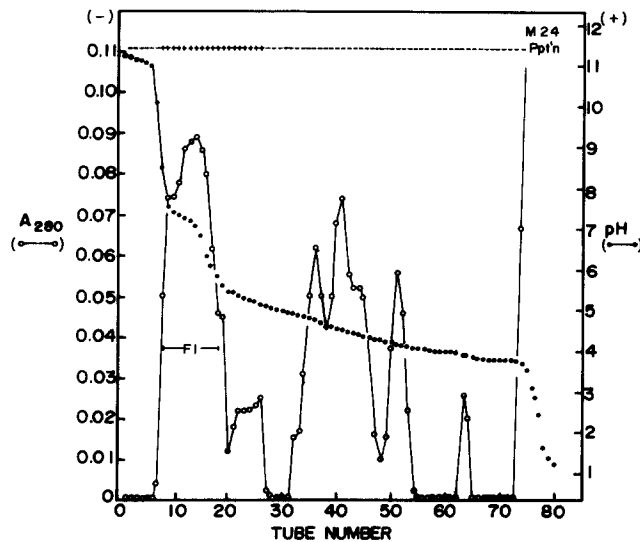


FIG. 2. Isoelectrofocusing of pep M24. 50 mg of M protein was applied to a 440 ml electrofocusing column containing 5% ampholyte solution composed of 1 part pH 3-10 to 9 parts pH 4-6 ampholines in a sucrose gradient. The tubes in the first peak were pooled as indicated (FI). Ppt'n, precipitation with type-specific M24 antiserum; +, positive reaction; -, negative reaction.

TABLE II
Purification of pep M

M protein preparation	Yield per 60-liter batch	Methyl-pentose	NTSM antigen titers*	TSM Antigens titers*	
				Ppt'n	OI
Crude pepsin extract	615	1.4	1:256	1:128	1:4
Ammonium sulfate fraction, 30% saturation	150	1.2	ND	1:256	1:16
QAE-Sephadex fraction, 0.3 M NaCl eluate	120	<0.5	ND	1:1,024	1:64
Isoelectric fraction, pH 7.15	84	<0.5	<1:2	1:1,024	1:64

* See footnote in Table I.

Previous studies in our laboratories have indicated that guinea pigs serve as a model for toxic skin reactions to M protein preparations in man (15). Guinea pigs were injected intradermally with pep M preparations obtained at various steps of purification. Five of five guinea pigs injected with the crude pepsin extract developed strong (average diameter of 14 mm of induration) delayed-type skin reactions (Table III). With increasing purification however, the frequency and degree of reactivity decreased; the electrofocused pep M was unreactive in the five guinea pigs tested.

The immunogenicity of the highly purified pep M was studied in rabbits. 100- μ g doses of purified pep M were emulsified in complete Freund's adjuvant and injected intracutaneously into rabbits. Sera obtained at 2-wk intervals demon-

TABLE III
Delayed-Type Skin Reactions in Unimmunized Adult Guinea Pigs to Intradermal Injections of Pep M24 Preparations

Pep M24 preparation	No. of skin reactions/no. of guinea pigs tested
Crude extract	5/5 (14)*
Ammonium sulfate (30% saturation)	5/5 (9)
QAE-Sephadex (0.3 M NaCl eluate)	3/5 (9)
Electrofocussed pep M24	0/5 (0)

* Numbers in parentheses indicate average diameter of induration among guinea pigs showing positive skin reactions.

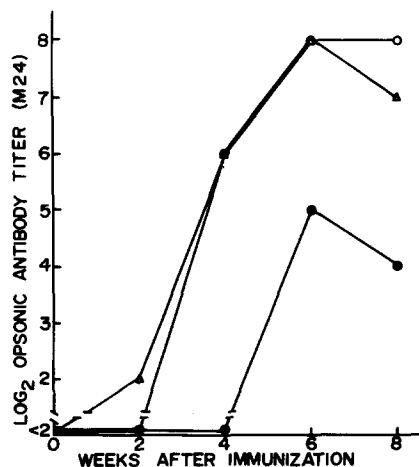


FIG. 3. Opsonic antibody response in three rabbits to a single injection of 100 μ g of purified pep M24 emulsified in complete Freund's adjuvant. O, rabbit no. 1; Δ , rabbit no. 2; \bullet , rabbit no. 3. None of the sera were able to opsonize heterologous type 30 streptococci.

strated the development of maximal type 24 opsonic antibody titers 6 wk after immunization (Fig. 3). Two of the three rabbits developed titers of 1:256 and the third showed a weaker maximum response of 1:32. Indirect bactericidal tests of the antisera were in agreement with the opsonic tests (Table IV). Immunodiffusion analysis of the antisera at 6 and 8 wk revealed a single precipitin arc of identity between a conventional crude acid extract of M24 and the highly purified pep M24 (Fig. 4), indicating that the immunizing pep M antigen lacked the contaminating precipitating antigen(s) often present in crude acid extracts of M protein (12). The lack of cross-reactive antibodies was further confirmed by the inability of the antisera to passively hemagglutinate LTA-modified erythrocytes or to fix complement using a partially purified HCl extract of type 30 M protein as antigen (Table V). These results indicated that the electrofocussed pep M24 was immunogenic and immunochemically homogeneous.

Physicochemical Properties of Pep M24. Electrophoresis in 7% polyacrylamide gels demonstrated a single Coomassie blue staining band in the electrofocussed fraction as compared to the multiple bands present in the fractions obtained during earlier steps of purification (Fig. 5). Electrophoresis in SDS

TABLE IV
Indirect Bactericidal Tests of Sera of Rabbits Immunized with pep M24

Rabbit serum*: collected at 2-wk intervals after immunization with pep M24	No. of colonies of type 24 streptococci after 3 h growth in test mixture		
	97	Inoculum 49	9
Preimmune	2,415	1,115	245
2 wk	1,515	635	65
4 wk	25	0	0
6 wk	0	0	0
8 wk	370	30	0

* The sera of the two rabbits showing the highest opsonic antibody responses (see Fig. 3) were pooled for these experiments.

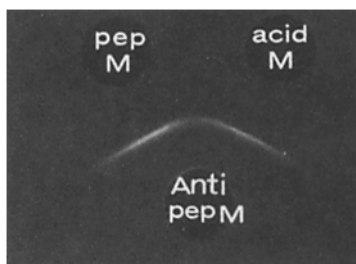


FIG. 4. Agar gel diffusion test of antiserum (anti-pep M) from rabbit immunized with purified pep M24 against the homologous immunizing antigen (pep M) and a crude HCl extract (acid M) of type 24 streptococci.

TABLE V
Presence of TSM and Absence of NTSM Antibodies in Sera of Rabbits Immunized with Purified pep M24

Rabbit serum	NTSM antibodies		TSM antibodies	
	CF titers with M30	PHA titers with LTA-RBC	Precipitin reactions*	Opsonic titers
Anti-pep M24				
Rabbit no. 1	<1:2	<1:2	++++	1:256
no. 2	<1:2	<1:2	++++	1:256
no. 3	<1:2	<1:2	++	1:32
Anti-M24 (CDC)	1:512	1:256	++++	1:128

* Capillary precipitin reactions with standard hot acid extracts prepared from type 24 streptococci.

polyacrylamide gels showed a single band of protein (Fig. 6) with a mobility consistent with an average mol wt of 33,500 daltons (Fig. 7).

Amino acid analysis demonstrated that the most abundant amino acid was alanine followed by glutamic acid, lysine, leucine and aspartic acid (Table VI). A small amount (2.7 residues per 100 amino acids) of phenylalanine was the only detectable aromatic amino acid. The absence of half-cysteine indicates that

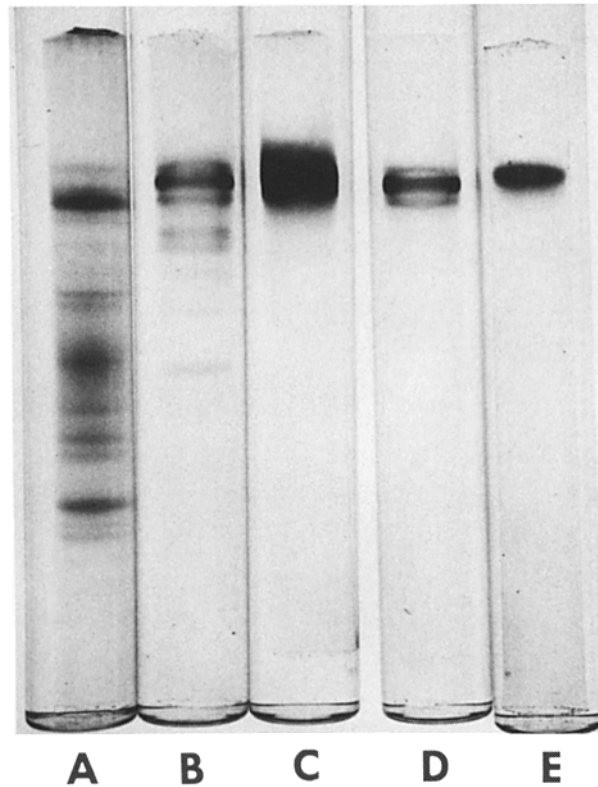


FIG. 5. Polyacrylamide gel electrophoresis of pepsin-extracted type 24 M protein at various steps of purification. (A), crude pepsin extract; (B), 60% saturated ammonium sulfate precipitate; (C), 30% saturated ammonium sulfate precipitate; (D), fraction I eluted from QAE-Sephadex A50; (E), electrofocused FI.

disulfide cross-links are probably not involved in the structure of pep M24. The pep M contains approximately 2 methionine residues per 100 amino acids. Assuming a mol wt of 33,500, each M protein molecule should contain between six and seven residues of methionine and, therefore should be susceptible to degradation into smaller specific peptides by cyanogen bromide. Preliminary studies in our laboratory (unpublished observations) have indicated that cyanogen bromide cleaves the pep M into seven peptides.

Amino Acid Sequence of the Amino Terminal Region of Pep M24. Automated Edman degradation yielded the sequence of the first 29 amino acids of the amino terminal region of pep M24 (Fig. 8). The amino terminal amino acid was found to be valine. Only one new amino acid was detected at each step of Edman degradation, confirming the homogeneity of the purified pep M24.

Discussion

It has become apparent that the purified TSM protein antigen of group A streptococci is often closely associated with nontype-specific moieties that are shared among several different serotypes of M protein (2, 3, 33, 34). Widdowson

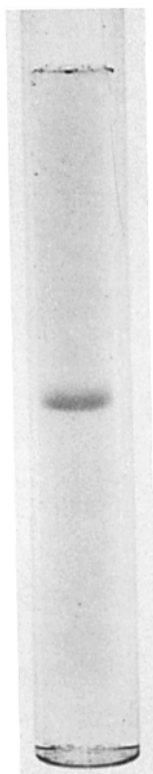


FIG. 6. Electrophoresis of 100 μ g of electrofocused pep M24 on SDS polyacrylamide.

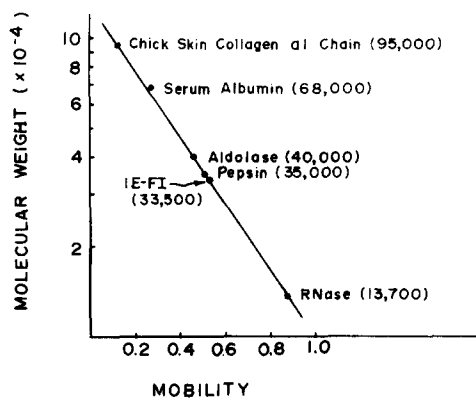


FIG. 7. Molecular weight determination of pep M24 (isoelectrofocussed FI) by electrophoresis in SDS polyacrylamide gel using proteins of known molecular weight as markers.

et al. (33) called the nontype-specific antigens "M-associated protein" or "MAP" because of the sensitivity of the antigens to trypsin digestion. In our studies we have referred to these antigens as simply NTSM because at least a portion of the antigens associated with M proteins appeared to be nonprotein in nature (21). Later we found that the nonprotein antigen is LTA (32).

body. Neither the purified alkaline-extracted M nor pep M possessed detectable NTSM antigens or nontype-specific skin reactivity in adult guinea pigs. Our previous studies have demonstrated that adult guinea pigs develop natural delayed hypersensitivity to NTSM antigens present in most streptococcal M protein preparations (15). Frederick et al. (35) recently demonstrated naturally acquired delayed hypersensitivity to LTA in guinea pigs which may account for at least a portion of the delayed skin reactivity of the crude M protein preparations.

In addition to the above differences, the two type 24 M protein preparations also differed considerably in their amino acid compositions. Whereas the most abundant amino acids in the alkaline M extract had been found to be glycine followed by aspartic acid, glutamic acid, proline, and lysine, the predominant amino acids in pep M were shown to be alanine followed by glutamic acid, lysine, leucine, and aspartic acid; the pep M had no detectable proline and only small amounts of glycine. These differences may account for the differences in the immunochemical properties of these two type 24 M protein preparations.

Our preliminary studies of the covalent structure of pep M protein indicate that valine rather than alanine (36) was the amino terminal amino acid. The appearance of only one new amino acid at each step of Edman degradation confirmed the homogeneity of the purified pep M24. The presence of an estimated six to seven methionine residues per molecule of M protein lend promise for attempts to cleave the molecule to its smallest active peptide with cyanogen bromide. The possibility that the relatively few phenylalanine residues, the only detectable aromatic amino acid, may be strategically placed in the molecule raise the prospects that chymotrypsin digestion may also result in smaller immunoreactive peptides.

These studies should provide an initial attack on the primary molecular structure of the type-specific moiety of M protein involved in protective immunity against group A streptococcal infections. Clarification of the structural features of the M protein molecule that render it serotype specific as compared to those structural features that account for serotype nonspecificity are important goals both in regard to the understanding of the pathogenesis of streptococcal infections and their sequelae and in regard to developing safe and effective vaccines against group A streptococcal infections.

Summary

M protein was extracted from type 24, group A streptococci with pepsin at pH 5.8 and was further purified by ammonium sulfate precipitation, ribonuclease digestion, ion-exchange chromatography, and isoelectric focusing. The purified pepsin extract of M (pep M) protein was shown to be free of nontype-specific immunoreactivity in (a) complement fixation tests with heterologous M antiserum, (b) skin tests in normal adult guinea pigs, and (c) passive hemagglutination tests for the presence of lipoteichoic acid sensitizing or antigenic activity. The pep M24 was highly immunogenic; two of three rabbits developed opsonic antibody titers of 1:256 and the third a titer of 1:32 6 wk after a single injection of 100- μ g doses of pep M24 emulsified in complete Freund's adjuvant. The antisera lacked nontype-specific antibodies and produced single precipitin lines in agar

gel diffusion tests against crude HCl extracts of the homologous M protein. Thus, the type-specific antigenic determinant(s) of type 24 M protein appears to be separable from immunotoxic, cross-reactive antigens without loss of immunogenicity in rabbits.

The mobility of pep M24 upon electrophoresis in 10% sodium dodecyl sulfate polyacrylamide gel was consistent with an average mol wt of 33,500 daltons. Amino acid analysis demonstrated a predominance of alanine, followed by glutamic acid, lysine, leucine, and aspartic acid. Pep M24 contained an estimated six to seven methionine residues and approximately ten phenylalanine residues per molecule. No other aromatic amino acids were detected. Automatic Edman degradation of pep M24 yielded the sequence of the first 29 amino acids (the amino terminal amino acid being valine) of the amino terminal region of the molecule. The detection of only one new amino acid at each step of Edman degradation confirmed the homogeneity of the purified pep M24.

The expert technical assistance of Bobby Curtis and Tom Marshall is deeply appreciated. We thank Mrs. Johnnie Smith for excellent secretarial assistance.

Received for publication 10 January 1977.

References

1. Stollerman, G. H. 1967. Prospects for a vaccine against group A streptococci: the problem of the immunology of M proteins. *Arthritis Rheum.* 10:245.
2. Beachey, E. H., and G. H. Stollerman. 1971. Toxic effects of streptococcal M protein on platelets and polymorphonuclear leukocytes in human blood. *J. Exp. Med.* 134:351.
3. Beachey, E. H., and G. H. Stollerman. 1973. Mediation of cytotoxic effects of streptococcal M protein by nontype-specific antibody in normal human sera. *J. Clin. Invest.* 52:2563.
4. Fox, E. N., and M. K. Wittner. 1969. New observations on the structure and antigenicity of the M proteins of the group A streptococcus. *Immunochemistry.* 6:11.
5. Cunningham, M. W., and E. H. Beachey. 1975. Immunochemical properties of streptococcal M protein purified by isoelectrofocusing. *J. Immunol.* 115:1002.
6. Fischetti, V. A., E. C. Gotschlich, G. Siviglia, and J. B. Zabriskie. 1976. Streptococcal M protein extracted by nonionic detergent. I. Properties of the antiphagocytic and type-specific molecules. *J. Exp. Med.* 144:32.
7. Russell, H., and R. R. Facklam. 1975. Guanidine extraction of streptococcal M protein. *Infect. Immun.* 12:679.
8. Vosti, K. L., and W. K. Williams. 1975. Release of type 12 M proteins by treatment of whole cells with cyanogen bromide. Abstract of the Sixth International Symposium on *Streptococcus pyogenes*. Institute of Hygiene and Epidemiology, Prague. 11.
9. Havlicek, J. 1975. M protein of type 12 *Streptococcus pyogenes*. Isolation by electrofocusing and some molecular weight-dependent properties. *Pathol. Microbiol.* 42:147.
10. Widdowson, J. P. 1975. Type specific and non-specific protein antigens of group A streptococci: immunochemical and physicochemical differences between different serotypes. Abstract of the Sixth International Symposium on *Streptococcus pyogenes*. Institute of Hygiene and Epidemiology, Prague. 12.
11. Cunningham, M. W., and E. H. Beachey. 1974. Peptic digestion of streptococcal M protein. I. Effect of digestion at suboptimal pH upon the biological and immunochemical properties of purified M protein extracts. *Infect. Immun.* 9:244.

12. Beachey, E. H., G. L. Campbell, and I. Ofek. 1974. Peptic digestion of streptococcal M protein. II. Extraction of M antigen of group A streptococci with pepsin. *Infect. Immun.* 9:891.
13. Becker, C. G. 1967. Enhancing effect of type-specific antistreptococcal antibodies on emergence of streptococci rich in M protein. *Proc. Soc. Exp. Biol. Med.* 124:331.
14. Lancefield, R. C., and G. E. Perlmann. 1952. Preparation and properties of type-specific antigen isolated from Group A, type 1 hemolytic streptococcus. *J. Exp. Med.* 96:71.
15. Beachey, E. H., H. Alberti, and G. H. Stollerman. 1969. Delayed hypersensitivity to purified streptococcal M protein in guinea pigs and in man. *J. Immunol.* 102:42.
16. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265.
17. Dubois, M., K. A. Giles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28:350.
18. Dische, Z., and L. B. Shettles. 1948. A specific color reaction of methyl pentose and a spectrophotometric micromethod for their determination. *J. Biol. Chem.* 175:595.
19. Davis, B. J. 1964. Disc electrophoresis. *Ann. N. Y. Acad. Sci.* 121:404.
20. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations in dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244:4406.
21. Beachey, E. H., I. Ofek, and A. L. Bisno. 1973. Studies of antibodies to nontype specific antigens associated with streptococcal M protein in rheumatic fever. *J. Immunol.* 111:1361.
22. Kang, A. H. 1972. Studies on the location of intermolecular cross links in collagen. *Biochemistry.* 11:1828.
23. Edman, P., and G. Begg. 1967. A protein sequenator. *Eur. J. Biochem.* 1:80.
24. Dixit, S. N., J. M. Seyer, A. O. Oronsky, C. Corbett, A. H. Kang, and J. Gross. 1975. Covalent structure of collagen: amino acid sequence of $\alpha 1$ -CB6A of chick skin collagen. *Biochemistry.* 14:1933.
25. Zimmerman, C. L., J. J. Pisano, and E. Appella. 1973. Analysis of amino acid phenylthiohydantoin by high speed liquid chromatography. *Biochem. Biophys. Res. Commun.* 55:1220.
26. Smithies, O., D. Gibson, E. M. Fanning, R. M. Goodfliesh, J. G. Gilman, and D. L. Ballantyne. 1971. Quantitative procedures for use with the Edman-Begg sequenator. Partial sequences of two unusual immunoglobulin light chains, Rzf and Sac. *Biochemistry.* 10:4912.
27. Chase, M. W. 1967. Production of antiserum. *Methods Immunol. Immunochem.* 1:197.
28. Fiedel, B. A., and R. W. Jackson. 1976. Immunogenicity of purified and carrier-complexed lipoteichoic acid. *Infect. Immun.* 13:1585.
29. Beachey, E. H., and I. Ofek. 1976. Epithelial cell binding of Group A streptococci by lipoteichoic acid on fimbriae denuded of M protein. *J. Exp. Med.* 143:759.
30. Beachey, E. H., and M. W. Cunningham. 1973. Type-specific inhibition of preopsonization versus immunoprecipitation by streptococcal M proteins. *Infect. Immun.* 8:19.
31. Swift, H. F., A. T. Wilson, and R. C. Lancefield. 1943. Typing Group A hemolytic streptococci by M precipitin reactions in capillary pipettes. *J. Exp. Med.* 78:127.
32. Ofek, I., E. H. Beachey, W. Jefferson, and G. L. Campbell. 1975. Cell membrane-binding properties of group A streptococcal lipoteichoic acid. *J. Exp. Med.* 141:990.
33. Widdowson, J. P., W. R. Maxted, and A. M. Pinney. 1971. An M-associated protein antigen (MAP) of group A streptococci. *J. Hyg.* 69:553.
34. Vosti, K. L. 1975. Characterization of a nontype specific antigen(s) associated with group A streptococcal type 12 M protein. *Infect. Immun.* 11:1300.

35. Frederick, G. T., R. A. Holmes, and F. W. Chorpensing. 1972. Naturally occurring cell-mediated immunity to purified glycerol-teichoic acid antigen in guinea pigs. *J. Immunol.* 109:1399.
36. Straus, D. C., and C. F. Lange. 1976. Characterization of group A streptococcal M-proteins purified by two methods. *Can. J. Microbiol.* 22:1072.