IMMUNOCHEMICAL LOCALIZATION AND AMINO ACID SEQUENCES OF CROSSREACTIVE EPITOPES WITHIN THE GROUP A STREPTOCOCCAL M6 PROTEIN

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M protein is a fibrous, predominantly α-helical, coiled-coil molecule extending from the cell surface of group A streptococci (1, 2). Through its ability to allow this organism to escape phagocytosis by the host's defenses, M protein acts as the primary virulence factor for these streptococci. Protection from streptococcal infection is due primarily to opsonization by type-specific antibodies directed against the M protein (3).

Previously, serological crossreactions among purified M proteins of various serotypes proved limited (4, 5), with restricted crossprotection in phagocytic assays (5). These studies were confined, however, to fragments of the M protein that represent approximately the amino terminal half of the native molecule, distal to the streptococcal cell surface (6). Similar limited crossreactivity was seen (7–9) with peptides synthesized on the basis of amino acid sequence determinations within these amino terminal fragments. Opsonic antibodies in these studies, however, were type-specific. Recently, a study (10) using mAbs directed against the entire M6 protein suggested that the amino terminal region of the M protein molecule was variable with respect to antigenic epitopes shared among M serotypes. Conversely, more extensive crossreactions may be reserved for regions more proximal to the cell surface (i. e., the carboxy terminal half of the molecule). The mAb directed toward the amino terminal half of the M6 protein (10A11) crossreacted with 5 different M serotyping strains, whereas the mAb directed toward the carboxy terminal half (10B6) did so with 20 of these serotyping strains. Through the use of DNA probes, the location of the conserved epitope was found (2) to be located more centrally in the native molecule, and just carboxy terminal to the pepsin cleavage site, which divides the molecule approximately in half. As a further definition of the antigenic structure of the M molecule, Manjula et al. (11) identified regions within the amino terminal half of the M5 and M6 proteins that crossreact, and they defined a hypervariable antigenic domain located in the amino terminal quarter of these proteins. In a more recent study using DNA probes of the M6 gene to identify constant and variable regions of M proteins in other serotypes, it was found that the carboxy terminal region of the M protein was conserved among strains of several different

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serotypes (12). The DNA sequence of the M6 molecule revealed the presence of three distinct blocks of extensive sequence repeats. Two repeat blocks were in the amino terminal half of the molecule (regions A and B) and one was carboxy terminal to the pepsin cleavage site (region C) (13).

In this study, to determine the extent of crossreactivity of mAbs 10A11 and 10B6 and that of a newly isolated anti–M6 protein mAb, 10F5, we used clinical isolates of group A streptococci representative of 58 M serotypes that had not been passaged in the laboratory. To specifically define the epitopes on the M6 molecule for these crossreactive antibodies, purified M6 protein was selectively cleaved with pepsin and staphylococcal V8 protease. Resultant peptides were separated by HPLC and selected mAb-reactive peptides were subjected to amino acid sequence analysis. Alignment of these amino acid sequences with the complete DNA sequence for the M6 protein gene (13), in concert with selective inhibition studies with synthetic peptides, revealed the location and amino acid sequences of these crossreactive epitopes on the M6 protein.

Materials and Methods

Isolation and Purification of M Protein from Escherichia coli (ColiM6). M6 protein was purified from *E. coli* containing plasmid pJRS42.13 bearing the structural gene for type 6 M protein (*emm*6; references 13, 14), as described by Fischetti et al (6).

Production and Purification of mAbs. mAbs 10A11 and 10B6 were prepared against phage lysin-extracted M6 protein and purified from culture supernatants as previously described (6). mAb 10F5 was prepared against ColiM6 protein (above) using techniques applied previously (6), with the exception that BALB/c × SJL/J (F_1 cross) were used for the immunization and that SP2/O myeloma cells were used for the fusion. mAbs 10A11 and 10B6 have been previously characterized (10) as having a variable epitope in the amino half of the M6 protein and a conserved epitope near the center of this molecule, respectively.

Bacterial Strains. Clinical isolates of group A streptococci, representing the various M serotypes, were obtained from The Rockefeller University Culture Collection housed in this laboratory. Strains selected were not passaged in the laboratory other than lyophilization to preserve cultures. M serotypes and their respective strains were as follows: M1, D710; M2, D444; M3, D922; M4, D896; M5, B788; M6, D471; M8, D784; M9, D339; M11, A658; M12, A735; M13, D742; M14, D469; M15, D424; M17, 1GL12; M18, 1RP268; M19, 1GL205; M22, F312; M23, 1RP14; M24, 22RS72; M25, D316; M26, 11RS100; M27, A910; M28, F416; M29, D470; M30, 1GL120; M31, F376; M32, 10RS101; M33, A984; M36, A457; M37, D466; M38, 2RSC3; M39, D869; M40, D733; M41, D463; M42, 1RS79; M43, C506; M44, C848; M46, A837; M47, C716; M48, D493; M49, D938; M50, A203; M51, A291; M52, A889; M53, D948; M54, D432; M55, D442; M56, D633; M57, D306; M58, D998; M59, D465; M60, D398; M61, D812; M62, A956; M63, A459; M65, A793; M66, A794; M67, D795.

Bacterial Dot Blot Immunoassay. mAbs 10A11, 10B6, and 10F5 were assayed for their ability to react with the various representative M serotype strains using the bacterial dot blot immunoassay. Briefly, overnight Todd-Hewitt broth cultures of group A streptococcal strains were washed twice with PBS, pH 7.4, containing 0.02% sodium azide. Washed cells were then suspended to an OD of 0.5 at 650 nm (18-mm tube) and 25-μl aliquots were attached to a nitrocellulose sheet via suction through a dot-blotting manifold (Bio-Rad Laboratories, Richmond, CA). Nitrocellulose sheets were allowed to air dry and were blocked for a minimum of 2 h in PBS containing 0.5% Tween (PBS/Tween). Dot blots were then processed with appropriate mAbs and alkaline phosphatase conjugate as per immunoblots described by Fischetti et al. (6). Intensity of immunoreactive dots was monitored by a reflectance densitometer with a 530 nm filter (Gilford Instrument Laboratories, Inc., Oberlin, OH) interfaced with a 3390A integrator (Hewlett-Packard

Co., Rockville, MD). Peak heights were adjusted with conjugate controls and ColiM6 protein standards.

Enzymatic Cleavage of M Protein with Pepsin. ~100 nmoles of ColiM6 (5.8 mg) lyophilized from distilled water were dissolved in 0.98 ml of 100 mM sodium phosphate buffer, pH 5.8. Pepsin (Worthington Biochemical Corp., Freehold, NJ) was added at a 1:20 (enzyme/substrate) ratio from a 2 mg/ml stock and the mixture was rotated at 37°C for 2 h. After this incubation, the digest was centrifuged at 12,800 *g* for 10 min and the supernatant was frozen in aliquots at −70°C.

Enzymatic Cleavage with Bacterial Protease. ColiM6 was cleaved at glutamic acid residues by staphylococcal strain V8 protease (Worthington Biochemical Corp.), according to the method of Drapeau (15). Cleavage was at an enzyme/substrate ratio of 1:100 in 50 mM ammonium bicarbonate/2 mM EDTA for 12 h at room temperature. The reaction mixture was then lyophilized in aliquots and stored at −70°C.

Blocking of Lysine Residues. Lysine residues were blocked by dihydroxypropylation of the ε-amino group of these residues according to the method of Acharya et al. (16).

Blocking of Arginine Residues. Arginine residues were blocked by treatment with 1,2-cyclohexanedione according to the method of Patthy and Smith (17).

HPLC Separation of Peptides. HPLC separations of peptides resulting from enzymatic digestion of ColiM6 protein were performed on a Waters HPLC system (Waters Associates, Milford, MA). Linear acetonitrile gradients were run in 0.05% TFA at 1 ml/min on either a Partisil PXS 10/25 ODS-2 (C18) column (Whatman Inc., Clifton, NJ) or an Ultrapore RPSC (C3) column (Altex Scientific, Inc., Berkeley, CA). Elution of peptides was monitored at 220 nm. Column fractions corresponding to reactive peaks were pooled and taken to near dryness in a Speedvac (Savant Instruments, Inc., Hicksville, NY).

Molecular Size Determination of Isolated Peptides. Relative molecular weights of peptides isolated by HPLC were determined by 15% SDS-PAGE (18), followed by immunoblotting on nitrocellulose (6) with their respective reactive mAbs. Based on the migration of molecular weight standards (phosphorylase b, 94,000; BSA, 67,000; OVA, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; α-lactalbumin, 14,400), the apparent molecular weights of the peptides of interest detectable by this method were: N-PD15, ~9,500; N-PD22, ~9,200; N-PD29, ~10,000 (data not shown).

ELISA. ELISA of the peptides separated by HPLC was performed as follows: Aliquots (10–50 μl) were taken from fractions representing absorbance peaks on the HPLC chromatograph and were dried onto 96-well plates (Immulon II; Dynatech Laboratories, Inc., Alexandria, VA) overnight at 37°C under vacuum. Carbonate buffer (0.05 M $Na_2CO_3/NaHCO_3$, pH 10, 100 μl) was added to each well and incubated at 37°C for 2 h. Plates were then washed and processed with appropriate antibodies and alkaline phosphatase conjugate according to Fischetti's modification (19) of the method of Engvall and Perlmann (20). After the final wash, immunoreactivity was visualized with the addition of 200 μl of 1 mg/ml *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) in 10% diethanolamine containing 1 mM $MgCl_2$, pH 10. Plates were read in an Elida-5 ELISA plate reader (Physica, Inc., New York) at 405 nm.

ELISA of crude enzymatic digests or selectively blocked peptides was performed by diluting the respective preparations in carbonate buffer, adding 100 μl per well on a 96-well plate, and incubating them overnight at 4°C. Plates were then washed and processed as above.

Competition ELISA. For competition ELISA, wells were coated with 100 μl of 100 ng/ml solution of ColiM6 in PBS, pH 7.4, 18 h at 37°C. Optimal mAb dilutions were determined by titration vs. ColiM6. Competing peptides were mixed with the optimal antibody dilution in PBS containing 0.05% Brij 35, pH 7.4, (PBS/Brij) at decreasing molar excesses relative to the ColiM6, starting with 2000× molar excess. Peptide-mAb mixtures were rotated 18 h at 4°C and 100 μl of each were added per well of a ColiM6 protein–sensitized, PBS/Brij-washed plate and incubated for 3 h at 37°C. Plates were then washed and processed as above and percent inhibition was determined.

Amino Acid Sequence Analysis. Amino acid sequence analyses were performed on 5–10 nmol peptide samples by an automated Edman degradation method at The Rockefeller

TABLE I
Group A Streptococcal M Serotypes Reactive with M6 Protein mAbs 10A11, 10B6, and 10F5, as Determined by Bacterial Dot Blot Immunoassay

Monoclonal antibody		
10A11	10B6‡	10F5‡
M5,* M6, M14	M1, M3, M4	M1, M2, M3
M27, M29, M32	M5, M6, M12	M4, M5, M6
M37, M53, M57	M14, M18, M22	M8, M12, M14
M58	M23, M24, M26	M17, M18, M19
	M27, M29, M30	M23, M26, M27
	M32, M36, M38	M28, M29, M30
	M41, M43, M44	M32, M33, M37
	M46, M48, M50	M38, M39, M43
	M52, M53, M55	M44, M48, M53
	M56, M57, M58	M54, M55, M56
	M62	M57

Strains listed are those above the lower limit for positive reactions. A densitometer reading of 10^5 peak height units (1 peak height unit = 1.25×10^{-4} mV) above conjugate control values (just visible to the naked eye) was taken as the cutoff point.

* M serotypes of clinically isolated strains, as determined by M type-specific antiserum.

‡ Underlined M serotypes (below mAbs 10B6 and 10F5) were positive for their respective mAbs alone, whereas those M serotypes not underlined were positive for both 10B6 and 10F5.

University Protein Sequencing Facility on a gas phase sequence analyzer (Applied Biosystems, Inc., Foster City, CA).

Synthesis of Peptides. Synthetic peptides were prepared according to the solid-phase method of Barany and Merrifield (21) and were purified by HPLC. Sequences were verified by amino acid sequence analysis (see above).

Results

Crossreactions of mAbs with M Serotypes. The reactivity of mAbs 10A11, 10B6, and 10F5 with nonpassaged clinical isolates representing each of 58 M serotypes was monitored using the bacterial dot blot immunoassay. Table I shows the crossreactions determined by reading the processed dot blots with a densitometer; values $>10^5$ densitometric units were considered positive. Using this criterion, mAb 10A11 was crossreactive with 9 different non-M6 serotypes, 10B6 with 30, and 10F5 with 30. However, the same 30 strains were not recognized by both mAbs 10B6 and 10F5. Together, these two antibodies recognized 39 of the 58 M serotypes examined (67%).

Isolation of Reactive, Pepsin-derived Peptides. The strategy used for the isolation of reactive peptides to mAbs recognizing variable (10A11) and conserved (10B6 and 10F5) epitopes was to cleave the ColiM6 protein with pepsin to separate these epitopes into different M protein fragments. After this cleavage, the peptides were separated by reverse-phase HPLC on a C3 column (Fig. 1*A*). Elution fractions containing peptide peaks were subsequently dried onto microtiter plates and processed by ELISA with the 10A11, 10B6, and 10F5 mAbs.

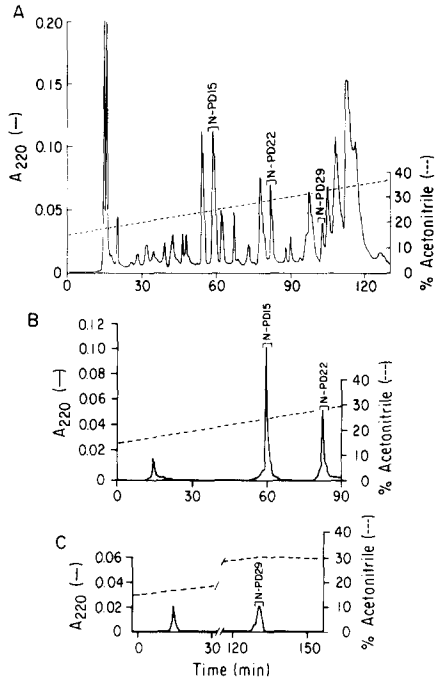

FIGURE 1. Reverse-phase HPLC of a pepsin digest of ColiM6 protein on a C3 column. (*A*) Separation of the crude pepsin digest; 15–40% linear acetonitrile (ACN) gradient at 1 ml/min (150 min). (*B*) Rechromatography of pepsin fragments N-PD15 and N-PD22; gradient as in (*A*). (*C*) Rechromatography of pepsin fragment N-PD29; 15–30% linear ACN gradient at 1 ml/min (130 min), isocratic at 30% (30 min).

Several peptide peaks were reactive with these antibodies, but three native (N)[1] peptides (N-PD15, N-PD22, and N-PD29) were selected for further purification and study due to their relative reactivity and isolated position on the chromatogram with respect to other peaks. The three peptides were repurified by pooling fractions corresponding to the reactive peaks and rechromatographing on the C3 HPLC column (Fig. 1 *B* and *C*). The ELISA readings on the purified peptides (Table II) show that peptide N-PD29 reacted with mAb 10A11, while N-PD15 and N-PD22 reacted with mAbs 10B6 and 10F5.

Amino Acid Sequence Analysis and Location of Reactive Pepsin-derived Peptides. Peptides N-PD15, N-PD22, and N-PD29 were subjected to partial amino acid sequence analysis with the resultant sequences presented in Fig. 2. Based on the observed sequence, peptides were positioned within the M6 molecule by alignment with the amino acid sequence predicted from the DNA sequence for *emm*6, described by Hollingshead et al. (13). Fig. 3 shows the alignment of the $10A11^+$ N-PD29 peptide in the amino terminal half of the native M molecule, as expected (10). $10B6^+$ and $10F5^+$ peptides N-PD15 and N-PD22 overlap in the carboxy terminal half of the protein.

Identification of the 10A11 Epitope. On the basis of the amino acid sequence of peptide N-PD29 isolated from the ColiM6 protein and its position on the M6

[1] *Abbreviations used in this paper:* ACN, acetonitrile; CHD, 1,2-cyclohexanedione; DHP, dihydroxypropylation; N, native; S, synthetic.

TABLE II

Reactivity of Purified Peptides Isolated from Native M6 Protein with mAbs 10A11, 10B6, and 10F5, as Determined by ELISA

Peptide	Monoclonal antibody		
	10A11	10B6	10F5
N-PD15	<0.100*	>2.0	>2.0
N-PD22	<0.100	>2.0	0.602
N-PD29	1.696	<0.100	<0.100
N-V8-1	<0.100	>2.0	>2.0
N-V8-1/DHP‡	ND	0.120	1.129
N-V8-1/CHD§	ND	0.214	1.305

* Absorbance readings at 405 nm after 30 min incubation in substrate.
‡ Dihydroxypropylated N-V8-1 peptide.
§ N-V8-1 peptide treated with 1,2-cyclohexanedione.

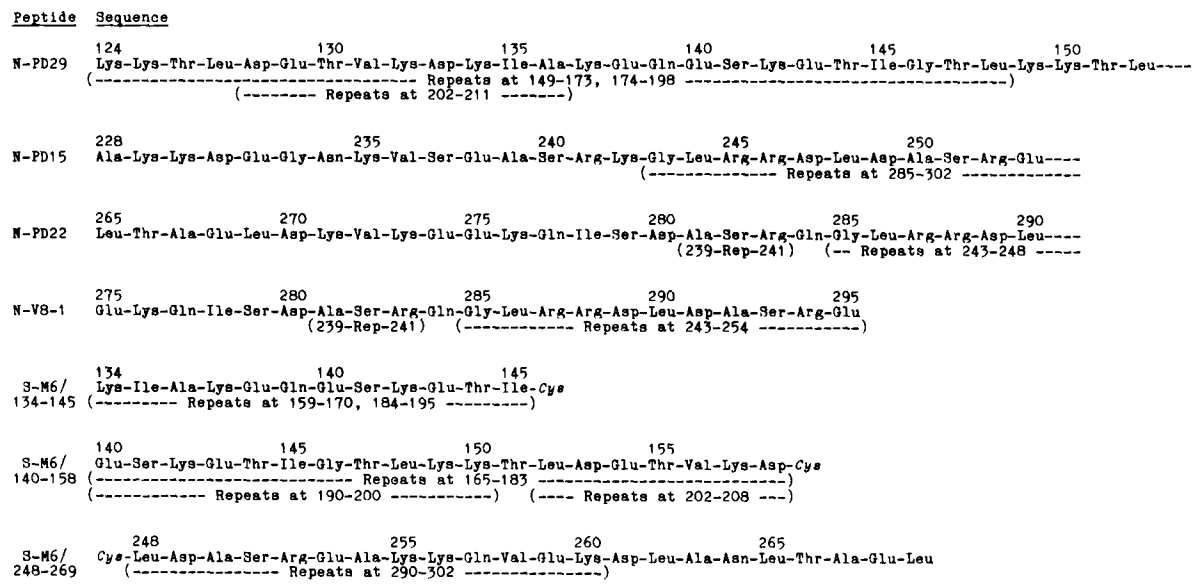

FIGURE 2. Amino acid sequences of reactive peptides isolated from the native M6 protein and synthetic peptides. Synthetic peptides were prepared with cysteines (*Cys*) for use in coupling to a carrier protein. *PD*, peptide derived from pepsin digestion of ColiM6; *V8*, peptide derived from V8 protease digestion of ColiM6.

molecule, two peptides were synthesized to aid in locating the epitope site for mAb 10A11. Fig. 2 lists the amino acid sequence of these synthetic peptides and Fig. 3 lists their position relative to the native peptide and the entire M6 molecule. Both native and synthetic peptides were used in competition ELISA's to compete for the ability of these mAbs to react with the entire M6 protein.

Fig. 4*A* illustrates the competitive inhibition of the binding of monoclonal 10A11 to ColiM6 by peptide N-PD29 and synthetic (S) peptides S-M6/134–145 and S-M6/140–158. As expected, peptide N-PD29 inhibited nearly 100% of this reaction. In addition, peptide S-M6/134–145 also inhibited this reaction, narrowing the epitope site for this antibody to residues 134–145, which are repeated

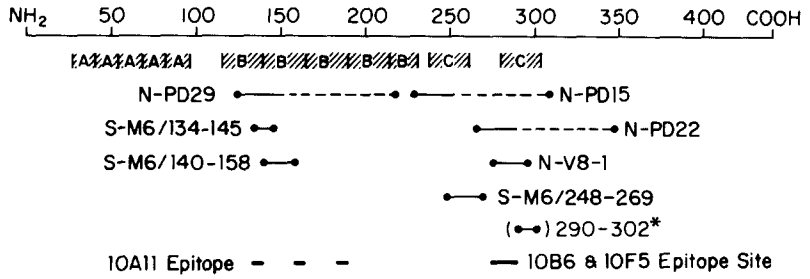

FIGURE 3. Position of native (*N*) and synthesized (*S*) peptides within entire M6 molecule. Amino acid sequence of M6 protein determined from the nucleotide sequence of the M6 protein gene. *A*, *B*, and *C* represent regions of sequence homology within the M6 sequence. (●——●) Sequence verified by amino acid sequence determination; (●---●) inferred from DNA sequence. *PD*, peptide derived from pepsin digestion of ColiM6; *V8*, peptide derived from V8 protease digestion of ColiM6. Asterisk indicates that residues 290–302 are an amino acid sequence repeat of residues 248–260 located in synthetic peptide S-M6/248–269.

at positions 159–170 and 184–195 within the M6 molecule. On the other hand, peptide S-M6/140–158, which overlaps S-M6/134–145 at residues 140–145, did not inhibit this reaction, thus defining the 10A11 epitope as the hexapeptide sequence comprising residues 134–139 (Lys-Ile-Ala-Lys-Glu-Gln). This sequence is repeated at residues 159–164 and 184–189 within the B repeat region of the M6 molecule (13).

Identification of the 10B6 Epitope: Isolation of Reactive, V8 Protease-cleaved Peptides. Since pepsin-derived, 10B6-reactive peptides N-PD15 and N-PD22 overlap, we attempted to find a smaller, reactive peptide in the region they share. We found several glutamic acid residues within the regions covered by these peptides. Therefore, staphylococcal V8 protease was selected to cleave ColiM6 at these amino acids. The peptides in the V8 protease digest of ColiM6 were separated on a C3 reverse-phase HPLC column (Fig. 5). Only one peak reacted with mAb 10B6 and none reacted with mAb 10A11. The $10B6^+$ fractions were pooled and rechromatographed on a C18 reverse-phase HPLC column (Fig. 5, inset). Only one of the resultant peaks was reactive with 10B6 on ELISA (Table II) and was designated N-V8-1. This peptide was sequenced in its entirety (Fig. 2) and found to correspond to the 21 residues at positions 275–295 in the native M6 molecule (Fig. 3).

Fig. 4*B* illustrates the competitive inhibition of 10B6 binding to ColiM6 by peptides N-PD15, N-PD22, and N-V8-1 and S-M6/248–269. Peptides N-PD15, N-PD22, and N-V8-1 inhibit the reaction, as expected. This placed the epitope site for mAb 10B6 in the 21-residue span of peptide N-V8-1, 275–295. The first 13 amino acids of synthetic peptide S-M6/248–269 repeat at residues 290–302. This sequence repeat overlaps peptide N-V8-1 at residues 290–295. Since this synthetic peptide and, by inference, its sequence repeat did not appreciably inhibit the reaction of mAb 10B6 with N-V8-1, the 10B6 epitope was thereby narrowed to residues 275–289. A portion of this sequence (281–289) is repeated at residues 239–247, with an amino acid substitution (lysine for glutamine) at residue 242, which may represent a repeat of this epitope site (Fig. 2).

Dihydroxypropylation (DHP) of Peptide N-V8-1. To further define the position of the 10B6 epitope within the 15 residue span (275–289), the ϵ-amino group of

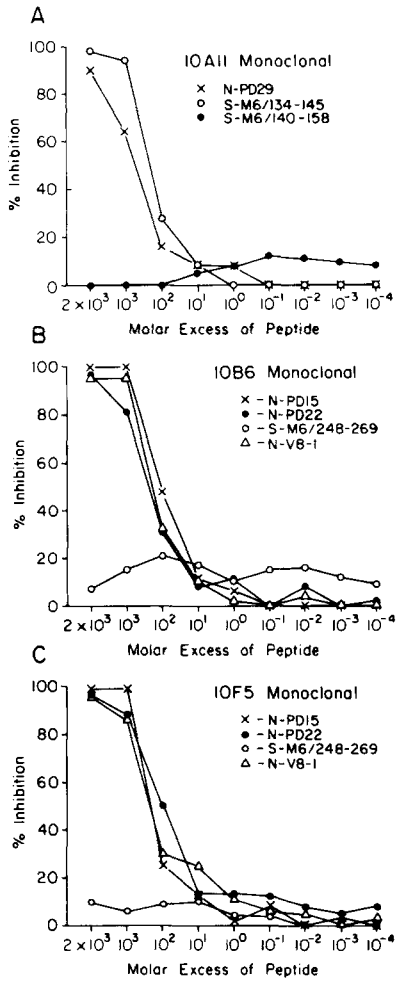

FIGURE 4. Competition ELISAs with N and S peptides vs. ColiM6. Percent inhibition of binding of mAbs 10A11 (*A*), 10B6 (*B*), and 10F5 (*C*) to ColiM6 protein by decreasing molar excesses of the various peptides.

the sole lysine in the N-V8-1 peptide, located at residue 276, was blocked by dihydroxypropylation. This treatment almost totally removed the ability of peptide N-V8-1 to react with mAb 10B6 (Table II), suggesting that the amino terminal end of this peptide was involved in antibody binding.

Treatment of Peptide N-V8-1 with 1,2-Cyclohexanedione (CHD). To delineate the binding site of mAb 10B6 still further, the arginines in peptide N-V8-1 at residues 283, 287, 288, and 294 were blocked with 1,2-cyclohexanedione. As was the case for DHP treatment of this peptide, CHD treatment almost entirely eliminated reactivity with the 10B6 mAb (Table II), thus suggesting that these arginine residues may also be part of the 10B6 epitope.

Identification of the 10F5 Epitope. Since mAb 10F5 showed similar reactivity to the pepsin-derived peptides N-PD15 and N-PD22 as mAb 10B6, a similar approach to the definition of its epitope on the M6 protein was taken. mAb

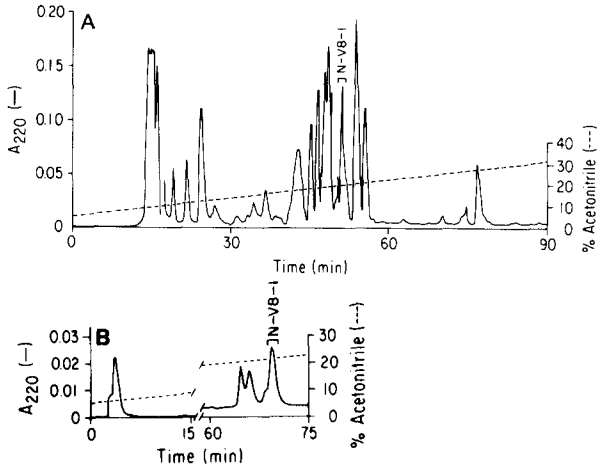

FIGURE 5. (*A*) Reverse-phase HPLC of V8 protease digest of ColiM6 protein. Separation of crude V8 protease digest; 5–40% linear ACN gradient at 1 ml/min (120 min) on a C3 column. (*B*) Rechromatography of N-V8-1 peptide; 5–40% linear ACN gradient at 1 ml/min (150 min) on a C18 column.

10F5 reacted strongly with the V8 protease-derived peptide N-V8-1 (Table II), indicating that its epitope was also located somewhere within this 21 residue peptide. Competition ELISA with native peptides N-PD15, N-PD22, and N-V8-1 and synthetic peptide S-M6/248–269 (Fig. 4*C*) showed similar results for mAb 10F5 as for 10B6 (Fig. 4*B*). The lack of competition by peptide S-M6/248–269 narrowed the epitope site for mAb 10F5 to the sequence of 15 residues (275–289) that also contains the 10B6 epitope. As was the case for 10B6, the 10F5 epitope may be repeated at residues 239–247.

Blocking experiments on peptide N-V8-1, including DHP treatment of the lysine residue at position 276 and CHD treatment of the arginines at positions 283, 287, 288, and 294 (Table II), showed some diminution of the reactivity of mAb 10F5 for this peptide, although not to the extent as that experienced for mAb 10B6.

Discussion

The immunological diversity of the group A streptococci is shown by the immunospecificity of the large number of M protein serotypes within these organisms (3). Crossreactions seen among M serotypes have been limited due to the fact that previous researchers confined their studies to the *N*-terminal half of the molecule (4, 5). One mAb prepared against the entire M6 protein, 10A11, which showed limited crossreactivity, also bound to the amino terminal half of this molecule (10), distal to the cell surface (6). Another mAb generated against this protein, 10B6, showed greater crossreactivity with other M serotypes than had previously been experienced, and bound a region near the center of this molecule (10), more proximal to the cell surface.

In the current study, mAbs 10A11 and 10B6, as well as an additional mAb, 10F5, were reacted with clinical strains representing 58 M serotypes that had not been passaged in the laboratory. mAb 10A11 crossreacted with nine non-

M6 strains, an increase of four compared with crossreactions cited in the previous study (10), in which laboratory passaged strains were used. mAbs 10B6 and 10F5 proved to be highly crossreactive, each binding to 30 non-M6 strains, an increase of 10 for mAb 10B6, where clinical isolates were used. This difference may indicate either that laboratory passage reduces the amount of M protein present on the surface of these strains or that the bacterial dot blot immunoassay is more sensitive than the colony blot immunoassay used previously (10).

The site for the 10A11 epitope, in the relatively variable amino terminal half of the M6 molecule, was determined to be the six-amino-acid sequence comprising residues 134–139 on the M6 protein (Lys-Ile-Ala-Lys-Glu-Gln). This epitope is repeated twice at residues 159–164 and 184–189, within the B repeat region of the molecule (Fig. 3) (13). The 10A11 mAb crossreacted with nine non–type M6 strains including the M5 typing strain T5B/126/3 (10) and the M5 nonpassaged strain, B788. Manjula et al. (22) have sequenced the entire pepM5 fragment of strain B788. Amino acid residues 154–159 of this pepM5 sequence, in an α-helical region of the molecule (23), are identical to residues 134–139, 159–164, and 184–189 of ColiM6, making up the 10A11 epitopes, and are likely responsible for the crossreactivity seen between these two serotypes with this antibody.

Both mAbs 10B6 and 10F5 had their epitope sites identified as the 15-amino-acid residue sequence 275–289 (Glu-Lys-Gln-Ile-Ser-Asp-Ala-Ser-Arg-Gln-Gly-Leu-Arg-Arg-Asp), overlapping the C repeat region in the carboxy terminal half of the molecule (Fig. 3). This sequence is partially repeated at residues 239–247, with an amino acid substitution at residue 242, suggesting that one or both of these epitopes may be repeated. The fact that DHP of the sole lysine located at residue 276 of the N-V8-1 peptide blocked this peptide's ability to react with monoclonal 10B6, suggests that this lysine and the amino terminal region of this peptide are involved in this determinant. However, the possibility that blocking the ϵ-amino group of the lysine resulted in a conformational change in the peptide, thus altering an epitope at another site within the peptide, cannot be ruled out. The same is also true with regard to the blocking of the arginines at residues 283, 287, 288, and 294 in the N-V8-1 peptide. Since blocking these lysine and arginine residues had a much less profound effect on the binding of 10F5, these residues may be at the fringes of the 10F5 epitope, which may be centered between them (i. e., residues 277–282). The differential effect of arginine and lysine blocking on binding of 10B6 and 10F5 makes it apparent that these two mAbs bind to two distinct sites within the same 15-amino-acid sequence. This is borne out by the differences in the M serotypes with which each mAb crossreacts. Despite the fact that mAbs 10B6 and 10F5 each recognize 30 different M serotypes of streptococci, they do not recognize the same strains in all cases. Combined, 39 of the 58 group A streptococci M serotypes examined may be identified with these two monoclonals.

Chou-Fasman analysis of the ColiM6 amino acid sequence (V. A. Fischetti, K. F. Jones, S. K. Hollingshead, and J. R. Scott, manuscript in preparation) reveals that the epitopes for mAbs 10A11, 10B6, and 10F5 are in predominantly α-helical regions of the M6 protein. The sequence for the 10A11 epitope (residues 134–139) repeats at residues 159–164 and 184–189, which are also contained

within a large α-helical region and thus, are likely to serve as additional epitopes for this antibody.

The epitope site for 10B6 and 10F5, however, contains an additional feature, in that the region encompassing residues 282–285 within the 15 residue site shows some β turn potential due to the helix breaking potential of the glycine at position 285. This possible disruption of the helix may be partly responsible for the immunogenicity of this particular site on the molecule, by better exposing this area on the surface of the M protein fiber.

mAbs 10B6 and 10F5 each crossreacted with 30 different M serotypes, only two of which, type 5 and type 24, have had their M proteins sequenced to any degree (7, 22, 24). These sequences, however, are incomplete with respect to the entire M molecule, since they deal only with amino terminal fragments derived from pepsin digestion, and therefore no sequences corresponding to the 10B6 and 10F5 epitopes are currently available.

This study has shown that the epitopes for mAbs 10B6 and 10F5 are located centrally within the M6 protein, yet are still accessible to antibody binding on intact streptococcal cells. In spite of this accessibility and the fact that 10B6 and 10F5 are of IgG subclasses that should fix complement (IgG2a and IgG2b, respectively), these mAbs, as well as 10A11 (IgG1), were not opsonic in phagocytic assays (K. F. Jones, S. A. Khan, B. W. Erickson, S. K. Hollingshead, J. R. Scott, and V. A. Fischetti, manuscript in preparation). Whether the lack of function of these antibodies is due to the location of their epitopes on the molecule or their complement fixing capabilities is currently under investigation. Epitopes located more proximal to the cell wall may be shared by a greater number of group A streptococcal M serotypes, as suggested by DNA hybridization experiments (12). However, whether these epitopes are also accessible to antibody binding on the cell surface or capable of eliciting opsonic antibodies must await further studies. It is hoped that the identification of these and other epitopes shared by many of the group A streptococcal M serotypes may form the basis for synthetic vaccines that can elicit crossprotective antibodies against multiple M serotype infection or that can be used as reagents for the identification of M protein-containing streptococci.

Summary

mAbs 10A11, 10B6, and 10F5, raised against the native group A streptococcal M6 protein, were examined for their crossreactivity with non–laboratory passaged clinical isolates, representing 58 M serotypes, by bacterial dot blot immunoassay. mAb 10A11 crossreacted with 9, mAb 10B6 with 30, and mAb 10F5 with 30 different non-M6 serotypes. To identify the epitopes for these antibodies, the native M6 protein was cleaved with pepsin or staphylococcal V8 protease. Resultant peptides were purified by HPLC, examined for binding to crossreactive mAbs in ELISA, and reactive peptides were subjected to amino acid sequence analysis. Peptides were aligned with the amino acid sequence of the entire M6 protein predicted by the DNA sequence of the M6 gene. Competitive inhibition studies using peptides synthesized on the basis of peptide and DNA sequences, in concert with selective blocking of amino acid residues, allowed for the further identification and placement of these crossreactive epitopes within the M6

molecule. The 10A11 epitope was located within the six amino acid residues at position 134–139, which repeat at positions 159–164 and 184–189 within the variable amino terminal half of the native molecule. The conserved 10B6 and 10F5 epitopes were positioned within a 15-amino-acid span at position 275–289, with the possibility that either epitope could have been repeated at residues 239–247. Chemical modification of amino acids within this sequence aided in the differentiation of these two epitopes. Such studies should aid in the recognition of a sequence(s) common to a greater number of M serotypes, which may be useful for future vaccine development or group A streptococcal identification.

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