THE IMPORTANCE OF THE LOCATION OF ANTIBODY BINDING ON THE M6 PROTEIN FOR OPSONIZATION AND PHAGOCYTOSIS OF GROUP A M6 STREPTOCOCCI

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The M protein of group A streptococci is a fibrous dimer of α helices arranged in a coiled coil extending ~50 nm from the surface of these organisms (1, 2). Due to its profound antiphagocytic effect, this molecule serves as the primary virulence factor for these streptococci (3) which are cleared from the infected host only after type-specific opsonic antibodies are produced to the M protein (4). mAbs to the native M6 protein and DNA hybridization studies on the M6 gene indicate that the NH_2-terminal region of this molecule is variable among M serotypes, whereas the carboxy-terminal half is conserved (5–8). This suggests that type specificity may reside in the NH_2-terminal half of the M protein, which is distal to the cell surface (9). While a number of studies on a variety of M protein fragments derived from the NH_2-terminal half of the molecule suggest the importance of this region in the production of opsonic antibodies (10–14), no systematic approach concerning the binding site and function of specific antibodies to the complete M molecule has yet been reported.

To help delineate the location of opsonogenic determinants on the M6 protein, mAbs raised against the native M6 molecule were examined for their ability to opsonize type 6 streptococci. Four of these monoclonals that had defined epitopes (reference 6; Jones, K. F., S. K. Hollingshead, J. R. Scott, and V. A. Fischetti, manuscript in preparation) were examined for their IgG subclasses and ability to fix human complement. In addition, antisera against synthetic peptides representing various areas of the native M6 protein were examined for their opsonic capabilities. These studies revealed that opsonization of streptococci is a function of the location of the antibody-binding site on the M molecule.

Materials and Methods

Purified M6 Proteins. M6 protein extracted from streptococcal cells by group C streptococcal phage-associated lysin (LysM6) was purified as described (15). M6 protein (ColiM6) was extracted and purified from an *Escherichia coli* strain expressing the structural gene for M6 protein (9).

Production of mAbs. mAbs against either LysM6 or ColiM6 were produced and protein A purified from culture supernatants as described (5, 6).

Bacterial Strains. Group A streptococcal clinical isolates representing 56 M serotypes were from The Rockefeller University culture collection and were listed previously (6).

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Synthesis of Peptides. Peptides were synthesized by the method of Barany and Merrifield (16) and were based on the sequence of the native M6 protein gene (17). A cysteine residue was placed at the COOH terminus for linkage to a carrier molecule. Synthesized peptides were purified by reverse-phase HPLC on a Brownlee C8 column (Brownlee Labs, Santa Clara, CA) using an acetonitrile–0.05% trifluoroacetic acid buffer gradient. The composition of purified peptides was verified by quantitative amino acid analysis (below). The position on the M6 molecule and the amino acid sequences for synthesized peptides are listed in Table III and illustrated in Fig. 1.

Analytical Methods. For amino acid analysis, peptides hydrolyzed in 6 N HCl or 6 N HCl/0.1% phenol at a Picotag Work Station (Waters Associates, Milford, MA) were derivatized with ethanol, triethylamine, water, and phenylisothiocyanate (7:1:1:1) and analyzed with a model 840 Data Module using a Novapak C18 column (Waters Associates). Amino acid sequence analysis was performed by automated Edman degradation in a gas phase sequencer (model 470A; Applied Biosystems, Inc., Foster City, CA). The PTH amino acids were identified by HPLC on a C18 column using either a 1084B analyzer (Hewlett-Packard Co., Rockville, MD) or a PTH analyzer (model 120A; Applied Biosystems, Inc.). All analytical procedures were performed by The Rockefeller University Central Biotechnology Facility.

Coupling of Synthetic Peptide to Carrier Protein. Peptides were coupled to the carrier protein OVA through the COOH-terminal cysteine of the peptide. The heterobifunctional reagent 3-(2-pyridyldithio)-propionic acid *N*-hydroxysuccinimide ester (Pharmacia Fine Chemicals, Piscataway, NJ) was used for thiolation of OVA (Worthington Biochemicals/CooperBiomedical, Inc., Malvern, PA) according to the manufacturers instructions. In general, 5.0 mg of purified peptide in 100 μl 0.01 M PBS, pH 7.5, was coupled to 8.0 mg of functionalized OVA in 1.7 ml PBS. All solutions were degassed with nitrogen. The mixture was allowed to mix at room temperature for 2 h and then overnight at 4°C. The solution was dialyzed against distilled water and stored frozen in aliquots at −70°C.

Rabbit Immunization. New Zealand white rabbits were bled and immunized intradermally with 200–400 μg of peptide-coupled OVA emulsified in CFA at multiple sites. Animals were boosted intramuscularly 30, 71, and 98 d after the first immunization with 100–200 μg of the respective peptide in IFA. All rabbits were bled 3 wk after the first immunization and 7–10 d after each booster immunization. All sera were filter sterilized and stored at 4°C.

Affinity Purification of Synthetic Peptide–specific Antibodies. To isolate synthetic peptide–specific antibodies from the polyclonal rabbit sera, synthetic peptides were linked to a solid matrix either by free amino groups or by the terminal cysteine. Peptides were linked by free amino groups to a glutardialdehyde-activated affinity adsorbent (Boehringer Mannheim Biochemicals, Indianapolis, IN) following the manufacturers instructions. Briefly, 3–5 mg of peptide was dissolved in 2.5 ml of 0.9% NaCl, added to 0.5 g of the affinity adsorbent, and rotated 4 h at room temperature in a 1-cm-diam column. The peptide-coupled matrix was then washed sequentially with 1.5% NaCl, 0.3 M ethanolamine (pH 7.0), 0.9% NaCl, 0.5 M propionic acid, 0.9% NaCl, and 0.01 M PBS (pH 7.4).

Coupling of synthetic peptides to thiopropyl-Sepharose 6B (Pharmacia Fine Chemicals) via cysteine was performed by a modification of the method of Carlsson et al. (18). Briefly, 3 mg of peptide was suspended in 1 ml of 0.14 M 2-ME and 1 mM disodium EDTA in 0.2 M ammonium bicarbonate, pH 8.5, in a tube flushed with nitrogen. This suspension was then rotated for 4 h at room temperature and the 2-ME was subsequently removed by six successive lyophilizations. The peptide was then dissolved in degassed 0.1 M Tris, pH 7.5, containing 6 M guanidine-HCl, 0.5 M NaCl, and 1 mM EDTA. The peptide solution was then added to 0.5 g of thiopropyl-Sepharose 6B (in a 1-cm-diam column) that had been washed successively with the Tris/NaCl/EDTA buffer (without guanidine) and then with the same buffer with guanidine. The column was sealed under nitrogen and rotated overnight at room temperature. Unbound peptide was eluted with the Tris/NaCl/EDTA buffer (without guanidine) and the column was reequilibrated with the same buffer.

Anti–synthetic peptide rabbit serum (3 ml) was adsorbed to the appropriate peptide-

linked glutardialdehyde or thiopropyl-Sepharose affinity column by rotating the mixture for 4 h at 37°C. After washing the resin extensively with PBS, adsorbed antibodies were eluted with 0.1 M glycine, pH 2.5, neutralized immediately, dialyzed against PBS, and concentrated to their original volume in a Centricon-30 (Amicon Corp., Danvers, MA).

Bacterial Dot Blot Immunoassay. Cross-reactivity of mAbs with various group A streptococcal M serotypes was investigated with the bacterial dot blot immunoassay as described (6). mAbs were adjusted to a concentration of 10 μg/ml. Affinity-purified anti-peptide antibodies were adjusted to a dilution that would give an ELISA reading of 1.0 at 405 nm after 60 min at 37°C.

Bactericidal Assay. Purified mAbs and anti-peptide sera were assayed for their ability to opsonize group A, M type 6 strain D471 streptococcal cells by the indirect bactericidal assay as described (4).

ELISA. The ELISA was performed as described (19). Titers are expressed as the dilution giving a reading of ≥1.0 at 405 nm after a 30-min incubation at 37°C. mAbs were diluted from a 1 mg/ml stock.

Complement Fixation Assay. This assay was a modification of the method as outlined by Hudson and Hay (20). All dilutions were made in 0.03 M veronal buffered saline containing 2.5 mM $MgCl_2$, 0.75 mM $CaCl_2$, and 0.1% gelatin. The reaction mixture consisted of 50 μl of antigen (dilutions of ColiM6 or of an OD_{650} = 1.0 suspension of M6 cells), 50 μl of mAb, and 50 μl of human complement in V-bottomed microtiter plates (Nunc Products, Roskilde, Denmark). Plates were incubated overnight at 4°C and then 30 μl of SRBC coated with anti-SRBC antibodies were added, shaken, incubated at 37°C for 30 min, shaken, and incubated an additional 10 min. Plates were then placed in an ice bath for 5 min, centrifuged at 500 *g* for 10 min, and 100 μl of the supernatant was transferred to a flat-bottomed plate (Immulon II; Dynatech Laboratories, Inc., Chantilly, VA) and read in an ELISA reader (Physica, Inc., New York, NY) at 405 nm to determine the amount of cell lysis.

mAb Isotypes. The method for isotyping followed the ELISA technique described (19) with reagents added in the following order: plates were coated with anti-mouse isotype-specific sera (Miles Laboratories, Inc., Naperville, IL) at a 1:1,000 dilution, followed by incubation with the mAb to be screened, then anti-mouse IgG alkaline phosphatase conjugate, and finally the *p*-nitrophenyl phosphate substrate.

Results

Opsonization of M6 Streptococci by mAbs. The bactericidal assay was used to identify those mAbs prepared against the native M6 protein (derived from two different hybridoma fusions) that allow for the phagocytosis of M6 streptococci. Of 19 mAbs screened, only one (mAb 3B8) was capable of completely opsonizing the M6 organisms (Table I). While some of the other mAbs, particularly 9F10, showed some opsonic activity, they only did so with 1 mg per assay, while 3B8 required only 250 μg to completely opsonize these cells. The epitopes for three of the nonopsonic mAbs were localized previously; the epitopes for mAb 10A11 were located within the "B" repeat region of the M6 protein at residues 134–139, 159–164, and 184–189, and the epitopes for mAbs 10B6 and 10F5 were both within residues 275–289 near the center of this fibrillar molecule (Fig. 1; reference 6). The epitopes for opsonic mAb 3B8 were at residues 41–46, 55–60, and 69–74 within the "A" repeat region (Fig. 1; Jones, K. F., S. K. Hollingshead, J. R. Scott, and V. A. Fischetti, manuscript in preparation).

Cross-reactions of mAbs with other M Serotypes. mAbs 10A11, 10B6, and 10F5 were previously shown to cross-react with 10, 30, and 30 different group A M serotypes, respectively, in bacterial dot blot immunoassays, indicating that despite their location on the M6 molecule, the immunodeterminants for these mAbs

TABLE I

Bactericidal Assay of Anti-M6 mAbs vs. M Type 6 Streptococcal Strain D471

Antibody	Inoculated	Stationary	Rotated	Antibody	Inoculated	Stationary	Rotated
10B6	48	>2,500	>2,500	10A11	48	>2,500	>2,500
3B8	30	>2,500	0*	10F5	30	>2,500	>2,500
14B11	30	>2,500	>2,500	14G12	30	>2,500	>2,500
15B4	30	>2,500	>2,500	9F10	24	>2,500	533
10H1	24	1,940	1,162	12G4	24	>2,500	1,858
14B10	24	>2,500	1,784	15E1	24	>2,500	1,236
2H4	24	>2,500	>2,500	4G8	24	>2,500	>2,500
5G10	24	>2,500	>2,500	11E1	24	>2,500	>2,500
12B5	24	>2,500	>2,500	13F4	24	>2,500	>2,500
15G1	24	>2,500	>2,500				

Bactericidal assay was performed according to the method of Lancefield as follows: 0.1 ml of log phase bacteria was inoculated into tubes containing 1 mg of appropriate antibody in 0.1–0.2 ml PBS and 0.4 ml of heparinized normal human blood; after 3 h of incubation at 37°C, bacterial growth or phagocytosis in stationary or rotated tubes was assayed by plating 0.1 ml of each culture in 0.8% sheep blood agar. Counts are expressed as CFU.

* Contained 0.25 mg of antibody.

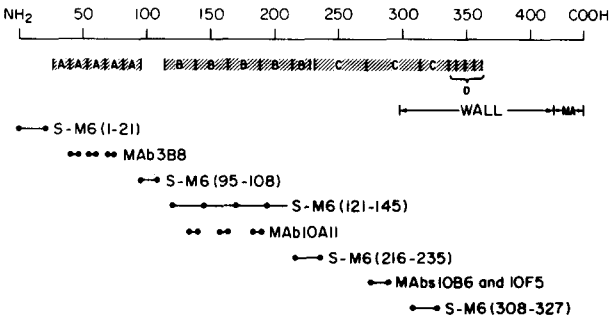

FIGURE 1. Position of mAb epitopes and synthetic peptides on the amino acid sequence of the M6 protein (17). Shaded areas with *A*, *B*, *C*, and *D* represent amino acid sequence repeats within the M6 molecule, as described (2). Those mAbs with more than one epitope position on this chart (mAbs 3B8 and 10A11) and the synthetic peptide with repeated sequences (S-M6[121–145]) are represented by multiple sets of dots and lines (see Tables II and III for actual amino acid residue positions). *WALL* is the COOH-terminal region of the molecule buried within the cell wall and *MA* is the membrane anchor region at residues 417–441 (Pancholi, V., and V. A. Fischetti, manuscript submitted for publication).

were accessible on the whole organism (6). Of the 56 M serotypes tested by the dot blot method, opsonic mAb 3B8 demonstrated type specificity by reacting only with the homologous M6 strain (data not shown).

ELISA Titers of Anti-M6 mAbs. mAbs were assayed for their binding titers to the ColiM6 protein by ELISA. All four mAbs demonstrated high titers to the native M6 molecule with 10B6 showing an exceptionally high binding capacity relative to the others (Table II).

IgG Subclass and Complement Fixation by Anti-M6 mAbs. Even though all four mAbs were capable of binding to whole streptococcal cells, only one (3B8) had the ability to initiate phagocytosis (Table I). To help explain the reason for this observation, mAbs were further characterized with respect to their IgG subclass and capacity to consume human complement in the presence of ColiM6 or M6 streptococcal cells (Table II). Despite their inability to opsonize type 6 streptococci, mAbs 10B6 (IgG2a) and 10F5 (IgG2b) were able to effectively fix complement, although mAb 10F5 was slightly less effective when bound to ColiM6.

TABLE II

Epitope Position, IgG Subclass, ELISA Binding Titer, Complement Fixation, and Opsonic Activity of Anti-M6 mAbs

Antibody	M6 protein epitope position	IgG subclass	ELISA titer*	C′ fixation‡		Opsonic activity§
				ColiM6	M6 cells	
3B8	41–46 55–60 69–74	IgG3	2,500	25–50	25–50	+
10A11	134–139 159–164 184–189	IgG1	3,200	—‖	—‖	–
10B6	275–289	IgG2a	179,800	12.5–25	25–50	–
10F5	275–289	IgG2b	11,700	50–100	25–50	–

* ELISA binding titers are expressed as the reciprocal of the dilution that gives an absorbance of ≥1.0 at 405 nm after 30 min of incubation at 37°C. All mAbs were adjusted to a concentration of 1 mg/ml before dilution.

‡ Human complement fixation by mAbs reacted with either ColiM6 or M6 streptococcal strain D471 cells. Values are expressed as the amount of mAb (μg/ml) needed for 50% lysis of indicator SRBCs when complexed with optimal concentrations of ColiM6 (usually 50 μg/ml) or M6 cells (usually a 1:8 or 1:16 dilution of the original stock suspension) relative to complement controls.

§ See Table I.

‖ No complement fixed at 400 μg/ml.

mAb 10A11, an IgG1, did not fix complement, even at the relatively high antibody concentrations used. mAb 3B8 (IgG3) was capable of fixing human complement when bound to either ColiM6 or M6 cells. Very little variation was observed in the degree to which 10B6, 10F5, or 3B8 were able to fix complement, despite the fact that 10B6 and 10F5 are of the subclasses regarded to be the most efficient at complement fixation (21).

Analysis of Anti-synthetic Peptide Antibodies. To investigate areas of the M molecule not included by the epitopes of the mAbs, peptides representing various regions of the M6 protein (Fig. 1) were synthesized. Sera obtained from rabbits immunized with OVA-coupled synthetic peptides were assayed for their ability to bind to the M6 protein (ELISA) and opsonize type 6 cells in bactericidal assays. The data compiled in Table III shows that all peptides produced antibodies with high binding titers to the purified M6 protein, although antisera to S-M6(95–108) and S-M6(121–145) were somewhat less reactive. The bactericidal data demonstrates that only the amino-terminal peptide S-M6(1–21) was capable of eliciting antibodies opsonic for the type 6 streptococci. Although antibodies to S-M6(95–108) showed marginal activity in bactericidal assays, they were not nearly as efficient as those prepared against the amino terminus. Affinity-purified antibodies were analyzed by bacterial dot blot immunoassay to determine their reactivity to intact streptococcal cells. All antibodies, except those prepared against S-M6(308–327) were able to bind the streptococcal-bound M6 protein.

Discussion

The current study shows three mAbs, each able to fix complement and bind to M protein on the cell surface (6) and, therefore, potentially capable of opsonizing M6 streptococci, yet only one (mAb 3B8) did so. The major difference

TABLE III
Characterization of Anti-M6 Synthetic Peptide Sera

Peptide	Sequence*	Anti-M6 ELISA titer‡	Bactericidal§		
			Inoculum	Stationary	Rotated
S-M6(1–21)‖	RVFPRGTVENPDKARELLNKYC	25,600	42	2,021	57
S-M6(95–108)	NKGLTKKLSEAEEEC	2,560	24	>2,500	748
S-M6(121–145) [146–170] [171–195] [196–211P]	GTLKKTLDETVKDKIAKEQESKETIC	3,200	22	2,186	1,720
S-M6(216–235)	SKQDIGALKQELAKKDEGNKC	51,200	26	>2,500	1,619
S-M6(308–327)	NSKLAALEKLNKELEESKKLC	25,600	26	>2,500	1,786

* Sequence of peptides is the single-letter amino acid code.
‡ ELISA titers are expressed as the reciprocal of the dilution of serum giving an OD_{405} of ≥1.0 after a 30-min incubation.
§ Bactericidal assay was performed as in Table I. Values are expressed as CFU and were averaged from at least four different experiments.
‖ Numbers on peptides denote their amino acid position on the M6 molecule (17). Numbers in brackets represent repeats of the original sequence with "P" denoting a partial sequence repeat.

observed between these mAbs was the location of their epitopes, with those for the type-specific 3B8 antibody positioned within the NH_2-terminal 1/6 of the M molecule (Jones, K. F., S. K. Hollingshead, J. R. Scott, and V. A. Fischetti, manuscript in preparation), while those for 10B6 and 10F5 were more centrally located within this fibrous protein (Fig. 1; reference 6). Since mAb 10A11 did not fix complement, it is not certain whether it was not opsonic because of this or because of the location of its determinants on the molecule.

To study regions not covered by the mAbs, antisera raised against synthetic peptides representing various areas of the M6 protein (Fig. 1) were also examined for their opsonizing capacities in this study. Of the six peptides tested, only the amino terminus, S-M6(1–21), was capable of eliciting opsonic antibodies. This was true despite the fact that the other peptides were capable of producing antibodies with high binding titers to the native M6 protein and which had the ability (except S-M6[308–327]) to bind to the M6 protein as presented on the intact streptococcal cell. Anti-S-M6(308–327) antibodies did not bind to whole cells because this region of the M molecule is located within the cell wall (Pancholi, V., and V. A. Fischetti, manuscript submitted for publication). Peptide S-M6(95–108) may represent the fringe area of the NH_2-terminal opsonogenic region of the M6 protein, in that antibodies to this peptide showed a marginal capacity for opsonization relative to the other peptides, but this was not significant when compared to the amino terminus.

These studies are in agreement with previous observations that despite extensive serological cross-reactions between M serotypes, protection against group A streptococcal infection relies on type-specific opsonic antibodies produced by the infected host (4), which react with type-specific determinants on the M protein molecule (22). In addition, while type-specific opsonic antibodies were shown to result in rabbits hyperimmunized with M protein or M^+ streptococcal cells, the ability of these sera to opsonize heterologous M serotypes was rarely seen (23–26). In these earlier studies, however, the location of these opsonogenic determinants was unknown. More recent immunochemical analyses have indicated that regions of the fibrillar M protein that are proximal to the cell surface

(COOH terminus) contain immunodeterminants more conserved among M serotypes than those distally located (NH_2 terminus) (5–7), and that the NH_2-terminal 25% of the molecule is hypervariable (2, 7, 27). Thus, as shown here, the NH_2-terminal region was likely to contain the type-specific opsonogenic determinants of this molecule.

Only a few opsonogenic determinants on different M proteins have been specifically identified thus far and the majority of these were type specific. Synthetic peptides representing overlapping areas of the first 35 amino acids of PepM5 showed that residues 14–26 could elicit type-specific opsonic antibodies and that at least one of these epitopes was located at residues 14–20 (28). Overlapping synthetic peptides of only the NH_2-terminal 31 residues of PepM6, from a strain other than the one used in this study, showed results similar to those presented here in that the peptide representing the first 21 residues was able to stimulate type-specific opsonic antibodies (13). While there is some evidence that opsonogenic epitopes may be present in regions of the PepM24 molecule outside of the amino-terminal region, the location of these epitopes has not yet been defined (29). A more recent study showed that a synthetic peptide from a conserved area (residues 164–197) of the PepM5 protein was capable of producing antibodies that were marginally effective in promoting phagocytosis of M5, M6, and M19 streptococci (30). This finding is not compatible with our current data and may be a matter of the stringency used to define an opsonic antibody.

The opsonogenic epitope of mAb 3B8, while not in the NH_2-terminal 21 amino acids found with synthetic peptide studies (13, 28, this study), is located at residue 41–46 (and repeated at 55–60 and 69–74) within the amino-terminal "A" repeat region of the M6 protein. This region has been shown to be involved in the generation of size variants of the M protein by homologous recombination (31) as well as a region involved with antigenic diversity in the M molecule (Jones, K. F., S. K., Hollingshead, J. R. Scott, and V. A. Fischetti, . manuscript in preparation). Whether or not such recombination events could produce new opsonogenic epitopes in this area will require further investigation.

Although it is clear that the NH_2-terminal region of the M molecule plays an important role in the generation of type-specific opsonic antibodies, it is uncertain why antibodies with similar characteristics (binding titer, surface exposed epitopes, and complement-fixing capacity), but located more proximal to the cell would not allow for phagocytosis. It is difficult to predict whether steric effects imposed by the binding of the antibody in this location on the M molecule inhibit the interaction of antibody-bound C3b with its receptor on the phagocyte. Recent evidence suggests that fibrinogen present in human plasma reduces the ability of cross-reactive antibodies to bind to group A streptococci (32). However, in a more recent study, Horstmann et al. (33) have shown that M protein specifically binds Factor H, a control protein of the alternative complement pathway. Factor H thus prevents the deposition of C3b on the surface of the streptococcus, preventing phagocytosis via the C3 receptors on the phagocytic cells. Perhaps those complement-fixing antibodies bound to the central region of the M molecule are also under the control of Factor H and, thus, are unable to effectively bind C3, whereas, those antibodies bound to the NH_2-terminal

region bypass Factor H control. Conversely, antibodies bound within the NH_2-terminal region of the M protein allow for the deposition of C3b at this end of the M molecule, which may not occur by the nonimmune mechanisms of the alternative pathway (34). Thus, C3b molecules not accessible to the regulation of the M protein–bound Factor H would be free to interact with phagocytic receptors. This hypothesis is currently being tested.

Summary

One of 19 mAbs against the native group A streptococcal M6 protein proved opsonic for type 6 organisms in a bactericidal assay. The opsonic and three nonopsonic antibodies were selected for isotype and complement fixation studies based on previous knowledge of their epitope site on the M6 molecule. While mAb 3B8 (IgG3), whose epitope is in the NH_2-terminal hypervariable region of the molecule (distal from the cell), and mAbs 10B6 (IgG2a) and 10F5 (IgG2b), both located in the conserved central region of the molecule, all fix complement, 10A11 (IgG1) did not. Only mAb 3B8 was opsonic despite the fact that mAbs 10B6 and 10F5 both exhibited similar complement-fixing capacity, binding titer, and surface exposure of epitopes. Analysis of antibodies raised against synthetic peptides representing various regions of the M6 protein showed that only the amino-terminal peptide (residues 1–21) was capable of eliciting opsonic antibodies, despite the fact that peptides from other areas produced antibodies with high-binding titers to the native M6 protein and also with the ability to bind to intact streptococcal cells. These results not only support the observed type specificity of opsonic antibodies, but also clearly point to the importance of the location of antibody binding on the M molecule relative to the actual functional capacity of the antibody with respect to the opsonization and phagocytosis of M6 streptococci. These results may underscore the recently observed role of complement Factor H in the antiphagocytic activity of the M protein.

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