Brief Definitive Report

LOCATION OF VARIABLE AND CONSERVED EPITOPES AMONG THE MULTIPLE SEROTYPES OF STREPTOCOCCAL M PROTEIN

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M protein is a fibrillar molecule composed of two alpha-helical protein chains in a coiled-coil conformation extending from the surface of group A streptococci (1, 2). This molecule is the major virulence factor for the organism by virtue of its ability to render the bacterium resistant to phagocytosis in the human host (3). Based on type-specific antigenic determinants on the M molecule, approximately 65 different serological M types are currently recognized in nature. In man, resistance to streptococcal infection appears to be the result of the presence of type-specific antibodies directed to the M molecule which will neutralize its antiphagocytic properties (3).

Previous investigations, which revealed very limited immunological crossreactions between purified M proteins of different serotypes, were primarily confined to detergent-extracted (4) or pepsin-extracted (5) M protein fragments. These fragments comprise only the amino-terminal half of the M molecule (2, 6) and, thus, do not represent all of the determinants present on the native protein.

Development of a vaccine to control streptococcal infections may depend upon the identification of a common M protein epitope with the ability to evoke cross-protective antibodies against multiple group A streptococcal serotypes. Since 56 different M serotyping strains show DNA homology with a probe consisting of the gene for the M6 molecule, a common region(s) may exist within the M proteins of different serotypes (7).

In an attempt to identify the location of epitopes responsible for broad and limited crossreactivity among 56 of the currently known group A streptococcal M serotypes, we have used monoclonal antibodies generated against phage lysin–extracted M6 protein, which comprises nearly the complete M molecule (8). Our results localize a common (evolutionarily conserved) crossreactive epitope near

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the middle of the M molecule and an epitope of limited crossreactivity (evolutionarily divergent) in the amino-terminal half.

Materials and Methods

Bacterial Strains. Group A streptococcal strains from The Rockefeller University Culture Collection are the 56 representative strains used for serological differentiation of M types 1 through 67 in this laboratory (7) (strains D471 [type 6] and D472 [type 50] were used instead of the usual typing strains for these two M types).

Plasmids. pJRS42.13 encodes the entire structural gene for group A streptococcal M6 protein (8). pJRS42.55 was constructed using HindIII to delete DNA encoding the carboxy-terminal third (approximately) of the M6 protein (7).

M6 Protein Preparations

Isolation with Phage Lysin (LysM6). M6 protein was isolated from streptococcal strain D471 using group C streptococcal phage-associated lysin and purified as described (9 and V. A. Fischetti, K. F. Jones, and B. N. Manjula, manuscript in preparation).

Isolation from E. Coli (ColiM6). M6 protein was purified from *Escherichia coli* containing pJRS42.13, as described by Fischetti et al. (6).

Isolation with Pepsin (PepM6). M6 protein was isolated by limited pepsin extraction from strain D471 essentially by the method of Manjula and Fischetti (10). Crude PepM6 was then dialyzed vs. 0.01 M Tris, pH 8.0, and applied to a 1 ml FPLC Mono Q column (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in the same buffer. PepM6 was eluted by a 20 ml linear salt gradient from 0 to 0.06 M NaCl in 0.01 M Tris, pH 8.0, at 2 ml/min. Pooled peak fractions were assayed both by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot to determine purity. Amino acid sequence analysis of the PepM6 fragment verified that it was from the amino-terminal half of the M6 molecule (6).

Production and Purification of Monoclonal Antibodies. LysM6 monoclonal antibodies were prepared from culture supernatants as described (6).

SDS-PAGE and Immunoblot Analysis. Separation of proteins on 12% SDS polyacrylamide gels and electrotransfer onto nitrocellulose membranes was as described (6). Immunoblots were exposed to the appropriate monoclonal antibodies at an approximate concentration of 0.6 μg/ml and processed as described (6).

Colony Blots. Rabbit blood broth cultures of serotyping strains were streaked onto proteose peptone agar plates. After an 18–24 h incubation at 37°C, colonies were overlaid with a nitrocellulose circle (0.45 μm; Schleicher and Schuell, Inc., Keene, NH) and allowed to adsorb for 5 min. The circle was removed from the agar surface, washed twice for 1 h each in 0.5% Tween 20 in 0.01 M phosphate-buffered saline (PBS), pH 7.4, and processed as immunoblots (6) using monoclonals at ~1.5 μg/ml.

Whole Bacterial Cell, Enzyme-linked Immunosorbent Assay (ELISA). 100 μl of poly-L-lysine (100 μg/ml) in 0.01 M PBS, pH 7.2, was added to each well of a 96-well microtiter plate (Immulon II; Dynatech Laboratories, Inc., Alexandria, VA) for 1 h at room temperature. After excess poly-L-lysine was removed by suction, 50 μl of PBS-washed streptococcal cells (5×10^9 colony-forming units/ml in PBS) were added concurrently with 50 μl of 0.2% glutaraldehyde in PBS. After incubation at room temperature for 20 min, plates were centrifuged at 1,500 *g* for 20 min at 4°C. Wells were emptied by inversion and excess fluid removed by inverting plates onto blotting paper. After one wash with PBS, plates were incubated for 1 h at room temperature with 100 μl of 0.1 M lysine in PBS. Wells were again emptied, as above, washed once in PBS, and incubated 18 h at 4°C with 100 μl of 2% bovine serum albumin (BSA) in 0.01 M Tris, pH 7.5, with 0.02% sodium azide to block unbound sites. Monoclonal antibodies at various concentrations (100 μl in BSA) were incubated at 37°C for 3 h. Wells were emptied and washed three times with 0.1% BSA followed by an 18 h incubation at room temperature with 100 μl of goat anti–mouse IgG alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO). After three BSA washes, as described above, 200 μl of *p*-nitrophenyl phosphate (Sigma Chemical Co.)

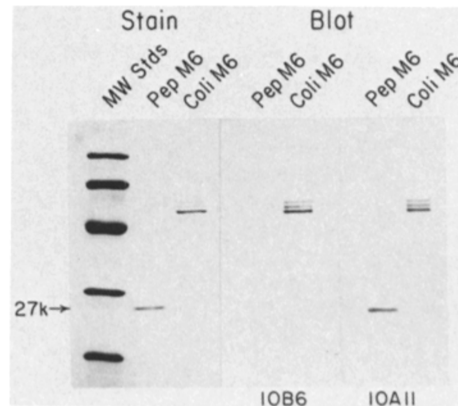

FIGURE 1. SDS-PAGE and immunoblot analyses of the entire M6 molecule (ColiM6) and the amino-terminal M protein fragment (PepM6). M6 proteins were separated on 12% SDS polyacrylamide gels and either stained with Coomassie Blue or electroblotted onto nitrocellulose. Immunoblots were processed with monoclonal antibodies 10A11 and 10B6 and visualized as described in Materials and Methods. Molecular weight standards (*MW stds*) are phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,100).

was added at 1 mg/ml in 10% diethanolamine, pH 9.8, containing 1 mM magnesium chloride. After 30 min at 37°C, plates were read at 405 nm in a Titertek Multiskan (Flow Laboratories, Inc., McLean, VA).

Whole streptococcal cells were treated with pepsin after the 0.1 M lysine step. Wells were washed twice with 0.1 M phosphate buffer, pH 5.8, and then incubated 1 h at 37°C with 100 μl of 200 μg/ml pepsin (Worthington Biochemical Corp., Freehold, NJ) in the same buffer. Digestion was stopped with 50 μl of 1 M Trizma base, the wells were washed with PBS, blocked with BSA, and processed as above.

Results

Localization of the Epitopes of Two Monoclonal Antibodies Directed Against the M6 Molecule. SDS-PAGE and immunoblotting techniques were used to locate the binding sites of monoclonal antibodies prepared against LysM6 protein. Monoclonal antibodies 10A11/B11 (10A11) and 10B6/G6 (10B6) were selected for further studies on the basis of their immunoblot reactivity to the pepsin-extracted fragment of type 6 M protein, PepM6, and to the entire M molecule isolated from *E. coli* carrying the M6 protein gene, ColiM6, as illustrated in Fig. 1. Antibody 10A11 reacts with both the ColiM6 and PepM6 molecules. This localizes its epitope in the amino-terminal half of the M molecule, distal to the cell surface. On the other hand, monoclonal 10B6 also reacts strongly with ColiM6, but does not react with PepM6. However, by both colony blot and immunoblot analyses, 10B6 reacts with the truncated M6 molecule encoded by pJRS42.55, which is deleted for the carboxy-terminal third (approximately) of the native molecule (data not shown). This places the epitope for the 10B6 antibody on the carboxy-terminal side of the pepsin cleavage site, near the center of the fibrous M6 molecule. ELISA of pepsin-digested whole streptococcal cells (data not shown) revealed the nearly complete loss of 10A11 reactivity, but no significant loss in reactivity for 10B6. This suggests that the 10A11 epitope is not repeated in the carboxy half of the M6 molecule.

Crossreactions Among Group A Streptococcal M Serotypes. To determine if

TABLE I

Reactions of M6 monoclonal antibodies 10A11 and 10B6 with Group A Streptococcal M Typing Strains, as Determined by Colony Blots

10A11			10B6		
Strain	M type	Reactivity*	Strain	M type	Reactivity
T5B/126/3	5	++	B930/61/3	3	+
D471	6	+++	T5B/126/3	5	+++
T14/46/3	14	+	D471	6	++
C105/41/5	46	++	T12/126/2	12	+
C744/RB4/6/3	47	+	T14/46/3	14	+++
A953/87/1	54	+	T23/102/RB5	23	+
			C98/115/2	24	++
			D23	29	+++
			D24/126/1	30	+++
			J137/69/2	31	+++
			C121/39/3	32	++
			C119/83/1	36	++
			C143/25/8	40	++
			C101/103/1	41	++
			C126/170/1	43	+++
			C105/41/5	46	+++
			C744/RB4/6/3	47	++
			A871/106/1	52	+++
			A952/94/1	53	++
			A953/87/1	54	++
			A963	56	++

* Reactions were scaled from + to +++ (+++ represents the most intense reaction, using that between 10A11 and M6 strain D471 as the standard); reactions were termed negative when they displayed no discernable reaction above conjugate control.

epitopes similar to those present on the M6 protein are present on M protein molecules of other streptococcal M serotypes, we reacted colony blots of group A streptococcal serotyping strains with 10A11 and 10B6. Table I reveals that 10A11 crossreacted with five non-M6 serotypes, only two of which exhibited strong reactions. In contrast, 10B6 demonstrated a much higher degree of crossreactivity, recognizing similar epitopes within 20 different M serotypes. These crossreactions were further verified to be directed specifically to the M molecule by immunoblot analysis of phage lysin–extracted M proteins from each crossreacting strain (V. A. Fischetti, K. F. Jones, and J. R. Scott, manuscript in preparation).

Discussion

Although hyperimmune rabbit sera prepared against whole streptococci often exhibit limited crossreactivity with strains of different M serotypes (4, 11–13), these antibodies rarely afford cross-protection against strains of heterologous M serotype. Vaccines prepared from purified, pepsin-extracted M protein (PepM) fragments (representing the amino-terminal half of the molecule) (5), or synthetic peptides therefrom (14), have recently been observed to exhibit limited cross-reactivity and/or cross-protection for only a small number of group A serotypes.

In this investigation, monoclonal antibodies to LysM6, the protein comprising nearly the complete M6 molecule, demonstrated more extensive crossreactivity among different group A streptococcal M serotypes. On colony blots, antibodies 10A11 and 10B6 crossreacted with 5 and 20, respectively, of the 56 different M serotypes examined. The specificities of the limited crossreactions of 10A11 closely parallel those observed by Fischetti (4) in whose study detergent-extracted

M6 protein (an amino-terminal fragment analogous to the pepsin-derived molecule [6, 15]) reacted with a limited number of unabsorbed hyperimmune rabbit typing sera (prepared against whole streptococcal cells) in a radioimmunoassay. This suggests that the 10A11-reactive epitope may be one of the major antigenic determinants within the amino-terminal region of the M molecule. Also, all the 10A11-reactive M types are also reactive with 10B6. Since these M types share two epitopes, they may be more closely related evolutionarily than those with only one epitope in common.

Immunoblot analysis of the ColiM6 and PepM6 molecules with monoclonals 10A11 and 10B6 (Fig. 1) shows that a conserved crossreactive epitope is located near the middle of the M molecule and a less common crossreactive epitope resides in the amino-terminal half. Since the amino-terminal region of the M protein is distal to the streptococcal cell surface (6), by virtue of its ~600 Å fibrous structure (2), it may be more exposed to the environment. Thus, this region may be more accessible to the host's immune surveillance system. One would then expect this region of the molecule to evolve faster in response to immunological selection and therefore display greater antigenic variability between serotypes than a more cell-proximal region of the molecule. This expectation appears to have been borne out by amino acid sequence comparisons of M proteins (16) as well as by the current finding that monoclonal 10A11 (which has an amino-terminal epitope) reacts with fewer serologically different M molecules than does 10B6, whose epitope is more centrally located. Perhaps the latter epitope is more conserved among serologically different M proteins, because of its closer proximity to the cell surface, and is thus less likely to encounter vigorous immunological pressures.

We are currently investigating whether there is a sequence conserved among all M types, which would be consistent with DNA hybridization results (7). The strong binding of monoclonal 10B6 to intact cells (detected by colony blots) indicates that when the M molecule is in its native state on the cell surface, this crossreactive site is not sequestered from antibody attachment despite its central location on the molecule. Taking advantage of the accessibility to the immune system of such conserved determinants may enable us to devise methods to stimulate a cross-protective antibody response to a large number of streptococcal M serotypes.

Summary

In studies primarily confined to the amino-terminal region of the fibrillar group A streptococcal M protein, only limited immunological crossreactions have been observed among M serotypes. In this investigation, two monoclonal antibodies generated against nearly the entire M6 molecule (LysM6) were used to determine the extent of crossreactions among M serotyping strains and to localize their epitopes on the M molecule. Colony blot and immunoblot analyses revealed that an epitope responsible for crossreactions among 5 of the 56 strains of different M serotypes tested is located in the amino-terminal half of the molecule, distal to the cell surface. In contrast, a more common crossreactive epitope, reacting with 20 of the 56 strains, is located near the middle of the M molecule. These studies also reveal that the more conserved determinant, located more proximally to the cell surface, is accessible to the immune system, even on the

whole organism, and, thus, may be useful in devising means to protect against infections by multiple group A streptococcal M serotypes.

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