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Biological and Immunochemical Identity of M Protein on Group G Streptococci with M Protein on Group A Streptococci

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Previous evidence for the presence of an M or M-like protein on group G streptococci has been based on the ability of these strains to survive in human blood. In addition, cross-reactions between group A and group G streptococci have been demonstrated, but they have relied either on whole bacterial cell vaccine-induced polyclonal sera or crude protein extracts of these cells. In this study two monoclonal antibodies prepared against the purified, native group A streptococcal M6 protein demonstrated a high degree of cross-reactivity with group G streptococcal clinical isolates (9 and 19 of 22 strains examined, respectively). Ten of these strains exhibited resistance to phagocytosis when rotated in human blood. In addition, immunoblot analysis of crude mutanolysin extracts of group G streptococci with one of the M6 monoclonal antibodies illustrated a remarkable similarity in the protein pattern of these extracts as compared with those of group A streptococcal M protein. The immunoblots further demonstrated a variation in the relative molecular weights of the extracted proteins from strain to strain over a range of 57,000 to 77,000. In addition, a purified, pepsin-derived fragment (M_r, 43,000) from a group G strain was capable of eliciting rabbit antibodies that were opsonic for group G cells in a bactericidal assay. These functional and immunochemical data, in concert with DNA hybridization between group G streptococcal DNA and a group A M6 gene probe (J. R. Scott, W. M. Pulliam, S. K. Hollingshead, and V. A. Fischetti, Proc. Natl. Acad. Sci. USA 82:1822–1826, 1985), provide strong evidence for the presence of an M protein on these organisms and indicate its probable role as a virulence molecule on the surface of group G streptococci.

Group G streptococci are commonly found in humans in the lower respiratory tract, in the gastrointestinal tract, in the female genital tract, and on the skin (2). Their first isolation from human disease was in 1935 by Lancefield and Hare (26) in a case of puerperal sepsis. More recently, these streptococci have been isolated with increasing frequency from cases of acute endocarditis (5, 23), neonatal sepsis (11), arthritis (23, 31), and pharyngitis (37). The group G organisms are classified according to the Lancefield immunological grouping scheme based on reactivity with the group G-specific cell wall rhamnose determinant (6).

The M protein of group A streptococci is a fibrous coiled-coil dimer of alpha-helices extending from the cell surface of these organisms (32, 38). Because of its antiphagocytic nature, M protein is one of the primary virulence factors of this bacterium, and it is also responsible for the serological specificity of the group A streptococci (25). In addition to the group A streptococci, M or M-like proteins have also been reported on streptococci of groups B (29), C (29, 39), E (10), and G (29, 30). The presence of a group G M protein has been suggested by studies in which the cross-reaction of anti-group A M protein polyclonal sera with group G strains was demonstrated (29, 30), as well as the ability of these organisms to survive in human blood (27), a property that is indicative of the presence of M protein in group A streptococci (25). These investigations were carried out, however, either on whole group G organisms or on crude extracts from group G organisms in conjunction with polyclonal sera. Therefore, it is difficult to determine if an M protein similar to that found on the group A streptococci is actually responsible for these M-like protein phenomena.

In an attempt to provide further evidence for the presence of a group G M protein, we examined this problem both immunochemically and biologically. Results of this investigation demonstrated that monoclonal antibodies to the group A streptococcal M6 protein cross-reacted with group G streptococcal strains. In addition, crude mutanolysin extracts of group G strains showed a marked similarity to the M protein extracted from group A streptococci, particularly with reference to immunoblot pattern and size variation among group G strains. In addition, the M protein purified from group G streptococci by limited pepsin proteolysis was capable of eliciting rabbit antibodies that were opsonic for group G organisms.

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MATERIALS AND METHODS

Bacterial strains. All strains used in this study were from the Rockefeller University Culture Collection, which is housed in our laboratory.

Isolation and purification of M6 proteins. M6 protein was isolated from type 6 group A streptococci with phage lysin and purified as described previously (18). The M6 protein, as coded for in *Escherichia coli* carrying the structural gene for this molecule (ColiM6) (33), was isolated and purified as described previously (16).

Production of monoclonal antibodies. Monoclonal antibodies to M6 protein isolated from type 6 group A streptococci with phage lysin were prepared as described previously (16). For monoclonal antibodies prepared to ColiM6 this procedure was also followed, with the exception that SP/2-0 myeloma cells were used in the fusion instead of NS-1 cells.

Bacterial dot blot immunoassay. Overnight Todd-Hewitt broth cultures of group G streptococcal strains were washed

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TABLE 1. Reactivity of group G streptococcal strains with anti-group A streptococcal M6 protein monoclonal antibodies 10B6 and 10F5[a]

Strain	Isolation site	Yr	Reaction vs monoclonal antibodies[b]	
			10B6	10F5
D738	NA[c]	1973	—[d]	0.3
D758	NA	1974	—	0.1
D845	Throat	1975	—	1.1
D851	Skin	1975	0.6	1.6
D856	Throat	1975	4.0	2.3
D862	Throat	1975	7.1	2.7
D884	Throat	1975	7.2	2.5
D885	NA	1975	—	1.2
D886	NA	1975	—	1.3
D887	NA	1975	—	1.5
D890	NA	1975	6.0	3.7
D891	NA	1975	—	1.3
D892	NA	1975	6.3	3.1
D953	Urine	1976	—	1.6
D954	Blood	1976	6.3	3.0
D955	Blood	1976	—	1.7
D956	Axilla	1976	5.3	2.8
D957	Groin	1976	0.3	1.1
D959	Blood	1976	—	1.8
F405	Blood	1981	—	0.2
F656	NA	1984	2.8	2.6
F672	NA	1984	4.1	3.3

[a] Determined by bacterial dot blot immunoassay.
[b] Values are densitometer readings (peak height counts) × 10^{-5} (1 peak height count = 1.25 × 10^{-4} mV deflection on densitometer).
[c] NA, Not available.
[d] —, Values recorded on the densitometer were less than those for conjugate controls.

twice with phosphate-buffered saline (PBS; pH 7.4) containing 0.02% sodium azide. Washed cells were then suspended to an optical density at 650 nm of 0.5 (tube diameter, 18 mm), and 25-μl fractions were then attached to a nitrocellulose sheet via suction through a dot blotting manifold (Bio-Rad Laboratories, Richmond, Calif.). Nitrocellulose sheets were allowed to air dry and then were blocked for a minimum of 2 h in PBS containing 0.5% Tween (PBS-Tween). Dot blots were then processed with appropriate monoclonal antibodies and alkaline phosphatase conjugate as in the immunoblots described by Fischetti et al. (16). The intensity of immunoreactive dots was monitored with a reflectance densitometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) with a 530-nm filter interfaced with an integrator (3390A; Hewlett-Packard Co., Palo Alto, Calif.). Peak heights were adjusted with conjugate controls and ColiM6 protein standards; one peak height unit was equal to 1.25 × 10^{-4}-mV deflection on the densitometer.

Rotation of bacteria in blood and bactericidal assay. To determine the ability of the group G strains to resist phagocytosis, they were rotated in normal human blood by the method of Lancefield (24). Hyperimmune sera produced in rabbits to purified group G M protein (see below) were assayed for the presence of opsonic antibodies by the indirect bactericidal assay described by Lancefield (24) and as modified by Fischetti et al. (15). Briefly, 0.1 to 0.2 ml of specific serum or PBS was added to 0.1 ml of an appropriate dilution of virulent group G streptococci in the presence of 0.4 ml of heparinized (10 U/ml) normal human blood, which was used as a source of phagocytic cells. Cultures were capped and rotated at 37°C for 3 h, and the number of colonies in 100 μl was assayed by the pour plate technique in 0.8% sheep blood agar.

Mutanolysin extraction of group G strains. Mutanolysin extraction of group G streptococci was done by a modification of the methods described by Siegel et al. (36) and Hamada et al. (19). Streptococcal strains were grown overnight in 50 ml of Todd-Hewitt broth, washed in 0.02 M sodium phosphate buffer (pH 7.0), and suspended in this buffer to an optical density at 650 nm of 0.5 (tube diameter, 18 mm). This suspension (50 ml) was centrifuged at 8,000 × *g* and then suspended in 1 ml of phosphate buffer containing 30% raffinose. Mutanolysin (Sigma Chemical Corp., St. Louis, Mo.) was added to a final concentration of 100 μg/ml and incubated with rotation at 37°C for 15 min. The resultant protoplasts were then removed by centrifugation at 12,800 × *g* for 10 min, and the supernatants were stored at −70°C.

SDS-PAGE and immunoblots. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were done as described previously (16).

Purification of a pepsin-derived fragment of group G M protein. M protein was isolated from group G strain D886 (PepMG) by limited pepsin proteolysis by the method described by Manjula and Fischetti (28). Crude PepMG was dialyzed against 0.02 M Tris (pH 9.0) and partially separated on a 52.5-ml DEAE-Sepharose column (Pharmacia Fine Chemicals, Piscataway, N.J.) with a 210-ml linear NaCl gradient of 0 to 0.25 M. Peak fractions containing PepMG were pooled and dialyzed against 0.05 M Tris containing 0.075 M NaCl (pH 8.0). Final purification was by chromatography on a 1-ml fast protein liquid chromatographic (FPLC) column (MonoQ; Pharmacia) with a 20-ml linear NaCl gradient from 0.075 to 0.11 M. Purity was monitored at all steps by SDS-PAGE (see above).

Immunization of rabbits. New Zealand white rabbits were immunized with purified PepMG as described previously (16). Sera were screened at appropriate intervals for opsonic antibodies by the bactericidal assay described above and for antibody binding titers by enzyme-linked immunosorbent assay (ELISA; see below).

ELISA. ELISA was performed by using the Fischetti modification (14) of the method described by Engvall and Perlmann (12). The titer endpoint was the greatest dilution giving a reading of ≥1.0 at 30 min of development.

RESULTS

Cross-reactions of anti-group A streptococcal M6 protein monoclonal antibodies with group G streptococcal strains. Anti-group A M6 protein monoclonal antibodies 10A11, 10B6, 10F5, and 3B8 were reacted by dot blot immunoassay with 22 human clinical isolates of group G streptococcal strains isolated from various sites. Only those densitometric readings above those of conjugate controls of 10^5 or greater were clearly reactive on visual inspection, and thus, this value was used as the lower limit for positive reactions. By using this criterion, 10B6 was found to cross-react with 9 of the 22 strains tested, wheres 10F5 cross-reacted with 19 of these strains (Table 1). Monoclonal antibodies 10A11 and 3B8 were not reactive with any of the group G strains tested and thus, were not included in Table 1.

Comparison of group A and group G mutanolysin extracts. To demonstrate to what component of the group G streptococcal cell surface these anti-group A M protein monoclonal antibodies were reacting, crude mutanolysin extracts of the group G strains were separated by SDS-PAGE and then Western blotted. The blots illustrated in Fig. 1 were devel-

oped with monoclonal antibody 10F5. The proteins in the group G mutanolysin extracts had a multiple banding pattern characteristic of and very similar to those of the proteins in the group A streptococcal extract (Fig. 1, lane a). The group G extracts also possessed the diffuse upper bands similar to those in the group A extract, which is characteristic of the M protein bound to remnants of the cell wall (17). Monoclonal antibody 10B6 gave similar results for those strains with which it was cross-reactive, whereas monoclonal antibodies 10A11 and 3B8 did not react with any group G mutanolysin extracts.

Size variation of group G mutanolysin extracts. Like group A M protein extracts (17), a marked variation was observed in the molecular weights of the predominant proteins in the group G mutanolysin extracts (Fig. 1). The relative molecular weights of these proteins ranged from 57,000 to 77,000, as measured from the lowest major protein band in each extract.

Survival of group G strains in human blood. As a measure of their ability to resist phagocytosis, 10 group G strains were rotated in normal human blood for 3 h. Of the 10 strains tested, 6 survived and grew well when exposed to human phagocytic cells (Table 2). The remaining four strains also divided when they were rotated in human blood, but to a lesser degree than the others. All strains grew well in the nonrotated control cultures (in which they were not as exposed to blood phagocytes).

Functional aspects of purified group G M protein. As established previously (32), a fragment of the group A M protein representing the amino-terminal half of the native molecule is released from the streptococcal cell with pepsin at a suboptimal pH (5.8). Similar treatment of group G strain D886 also resulted in the release of a protein with a relative molecular weight of approximately 43,000. The molecule was purified to homogeneity (PepMG) (Fig. 2) and used to immunize rabbits. Antibodies to the PepMG molecule were found to have high binding titers, (>1:102,400) to the PepMG protein, as determined by ELISA, and opsonic activity to group G streptococci, as determined in an indirect bactericidal assay. There were 33 colonies in the inoculum. After 3 h of rotation in normal human blood, the inoculum multiplied to 1,504 CFU in the absence of immune serum, whereas only 9 CFU were recovered from the culture containing the hyperimmune anti-PepMG serum. By Western blot analysis, none of the anti-group A M protein monoclonal antibodies was reactive with the PepMG preparation, nor were they opsonic in the bactericidal system (data not shown).

TABLE 2. Survival of group G streptococcal strains in nonrotated and rotated cultures in human blood

Strain	CFU in:		
	Inoculum	Nonrotated culture	Rotated culture
D851	19	>2,500	>2,500
D856	23	>2,500	816
D862	15	>2,500	>2,500
D884	12	>2,500	1,432
D885	39	>2,500	>2,500
D886	33	>2,500	>2,500
D892	13	>2,500	>2,500
D953	22	>2,500	>2,500
D954	23	>2,500	191
D959	32	>2,500	316

DISCUSSION

By using two cross-reactive monoclonal antibodies to the M6 protein of group A streptococci as a probe, we identified an M protein-like molecule on the surface of several strains of group G streptococci. Monoclonal antibody 10B6 was found to cross-react with 9 of the 22 group G strains tested on bacterial cell dot immunoblots, whereas monoclonal antibody 10F5 cross-reacted with 19 of these strains (Table 1). Although cross-reactivity between group A and G streptococci has been observed before (29, 30), these earlier studies employed polyclonal sera raised against whole bacterial cell vaccines for reactions with crude protein extracts. Although suggestive, in those studies such molecules were not purified or characterized from the group G streptococci, and thus, a direct comparison with the group A M protein could not be made. The results of this study are in agreement with those of DNA probe experiments in which hybridiza-

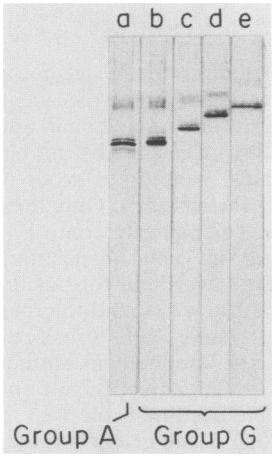

FIG. 1. SDS-PAGE and Western blot analysis of crude group A and group G streptococcal mutanolysin extracts processed with group A M6 monoclonal antibody 10F5. Lane a, group A strain D471; lanes b to e, group G strains D862, D884, D959, and D851, respectively.

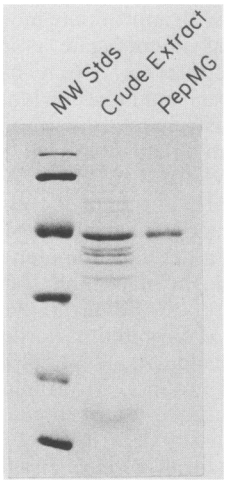

FIG. 2. SDS-PAGE of crude and purified pepsin-derived PepMG protein from group G streptococcal strain D886. Molecular weight standards (MW Stds) are phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).

tion between the group A streptococcal M6 gene and DNA from group C and G streptococci was shown (34). Additional corroboration of the hybridization results exists in cross-reactions between these group A M6 monoclonal antibodies and group C streptococcal extracts (unpublished data). The epitopes for monoclonal antibodies 10B6 and 10F5 are located in the carboxy-terminal half of the group A M6 molecule, each of which is conserved in the M proteins of over 30 different group A M serotypes (21, 22). It now appears that this region of the M protein molecule is also conserved within a second and, likely, a third streptococcal group. Monoclonal antibodies 10A11 and 3B8, which react with the variable region of the group A M6 protein (22; K. F. Jones, S. A. Khan, B. W. Erickson, S. K. Hollingshead, J. R. Scott, and V. R. Fischetti, manuscript in preparation), do not react with the group G strains (data not shown).

Further evidence for an M-like protein on the group G streptococci is the ability of several of these strains to multiply well in human blood (Table 2). In group A streptococci, this ability to evade phagocytosis and survive in the human host is due to the M protein on the surface of these organisms (25). Despite the fact that M protein appears to be present on these strains (by dot blot immunoassay), some of the group G strains did not multiply well when rotated in the blood of the donor used in this study. Similar results were obtained with other donors. In group A streptococci, protection against infection by these organisms is dependent on the presence of type-specific opsonic antibodies directed against specific determinants on the M protein (24), thus suggesting that this donor may have had antibodies that were specific for some group G strains (nonsurvivors) and not for others (survivors). Precedence for this manner of type-specific behavior in group G streptococci was presented by Lawal et al. (27), who attempted to set up a classical M serotyping scheme for group G organisms and succeeded in identifying six serotypes.

Mutanolysin, an *N*-acetyl-muramidase (19), was used to extract the cell wall antigens from the group G streptococci to compare them with similar extracts from group A organisms. When Western blots were reacted with monoclonal antibody 10F5 (Fig. 1), the group G extracts demonstrated a remarkable similarity to the multiple banding pattern of the group A M protein. This pattern was shown to be characteristic of phage lysin and mutanolysin extracts of group A M proteins, including the diffuse upper bands, which represent M protein bound to cell wall fragments containing the group A-specific carbohydrate (17). It is likely that the diffuse upper bands observed on the group G mutanolysin extracts are also representative of cell wall fragments containing complexes of both group G M protein and group G-specific carbohydrate. This suggests that the mechanism of attachment of the group G M protein to the cell wall may be similar to that for the group A M protein (17, 20). In extracts of different group G strains, the M protein also varied in relative molecular weight among strains, a property that was demonstrated for the M protein of group A streptococci by Fischetti et al. (17). Although the size variation for group G strains was not as extensive as that for group A (20,000 range versus 40,000 range), this may be a function of the number of strains studied rather than an actual difference in the size range between these organisms. Thus, the genetic mechanisms involved in generating these size variants in group A streptococci (S. K. Hollingshead, V. A. Fischetti, and J. R. Scott, Mol. Gen. Genet., in press) are also conserved within the group G organisms.

Additional evidence for the relationship between the M proteins of group A and group G streptococci is the fact that treatment of the group G streptococci with pepsin at suboptimal pH released a protein from group G strain D886 representing approximately half of the native molecule. This was apparent from the fact that immunoblot analysis of mutanolysin extracts of strain D886 (data not shown) revealed a molecule nearly twice the size of the pepsin-derived protein. In the group A M6 protein, the pepsin-sensitive site also seems to be near the center of the molecule (22).

In previous investigations, the ability of pepsin-derived fragments of group A streptococcal M protein to elicit opsonic antibodies in rabbits has been shown (3, 28, 35). The fact that a pepsin-derived fragment from group G strain D886 resulted in the stimulation of antibodies that were opsonic for group G cells in vitro suggests that the M protein of group G streptococci, as in group A streptococci, may be one of the primary virulence factors associated with these bacteria.

Protein G is an immunoglobulin G (IgG)-binding protein that is isolated from the surface of some group G streptococcal strains and has been shown to bind all subclasses of mouse IgG (1, 4, 13). The possibility that the proteins involved in this study were protein G molecules instead of M protein can be ruled out on the basis that our monoclonal antibodies 10A11 and 3B8, which are IgG1 and IgG3, respectively (Jones et al., manuscript in preparation), did not bind to the mutanolysin extracts of group G strains or the intact cells. There is also no evidence to suggest that protein G is capable of eliciting opsonic antibodies, an ability that is inherent in the PepMG that was isolated in this investigation.

In recent studies, epitopes on group A streptococcal M5, M6, and M19 proteins, which are also present on human heart sarcolemmal membrane proteins (7, 8) and human cardiac myosin (9), have been described. The significance of these cross-reactive antibodies in the pathogenesis of post-streptococcal sequelae, particularly rheumatic heart disease, is not clear. The close similarity among group A, group G, and, likely, group C M proteins, however, raises concerns regarding the little information that is currently available with respect to the sequelae that may be associated with these non-group A streptococcal infections.

The data presented in this report demonstrate that there is a close relationship between the surface M protein of groups A and G streptococci; this was determined by both immunochemical and biological methods. Although further structural and functional studies of the group G M protein need to be performed, the evidence presented here, in concert with DNA hybridization data (34), strongly suggest the presence of M protein on group G streptococci and its probable role as a virulence factor for these organisms.

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