SIZE VARIATION OF THE M PROTEIN IN GROUP A STREPTOCOCCI

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Streptococcal M protein, the fibrillar molecule on the surface of the group A streptococcus, is considered to be one of the major factors responsible for the ability of this human pathogen to cause disease (1). Though type-specific opsonic antibodies directed towards the M molecule enable the infected host to clear the invading streptococcus (1, 2), the ability of this organism to vary its antigenic structure has probably been the major factor responsible for its survival. To date, over 75 serologically different M proteins have been identified in nature along with an increasing number of nontypable strains isolated from infected individuals (World Health Organization Conference, 1984, Tokyo, Japan).

Based on structural and physicochemical studies, it appears that the M protein is composed of two predominantly alpha-helical protein chains assembled in a coiled-coil (3–5) and extending nearly 600 Å from the cell surface (5). Partial sequence analysis of three different M molecules has revealed that these sequences are essentially different, but have a common placement of hydrophobic residues (4, 6, 7) that is responsible for maintaining the coiled-coil structure (4, 5) common to those M molecules studied. Though many of these studies have been useful in understanding the structure and immunochemistry of the M molecule, they have been primarily confined to an amino-terminal fragment of the protein comprising nearly half the molecule (pepsin-extracted M protein) (4, 6, 7).

Several different procedures for obtaining M protein from whole streptococci or their isolated cell walls have been used, including extraction with acid (8), alkali (9), pepsin (10, 11), and nonionic detergent (12). In all cases, however, the resulting product was probably a fragment of the native molecule. Despite some attempts to isolate the M protein from the streptococcus with cell wall–solubilizing enzymes (13–16), the complexity of the resulting digest has offered little information about the nature of the native molecule as it exists on the streptococcus. To avoid the destructive properties of certain extraction techniques, M protein was purified from the secreted products of streptococcal L forms and protoplasts of type 12 streptococci (17). The molecular weight of the M protein monomer from these two sources was 58,000. In more recent studies, Scott and Fischetti (18) cloned the gene for M6 in *Escherichia coli*, and Fischetti et al. (19)

described the M molecule found in the periplasm of this new host. When the M6 molecule is isolated from the *E. coli* periplasm, the monomers have an apparent molecular weight of 55,000–58,000 (18, 19).

To investigate in detail the native form of the M protein on the streptococcus, we used both a monoclonal antibody to the M6 molecule (which is crossreactive with 20 different M types) (20) and monospecific antibody to the *N*-acetylglucosamine determinant of the group A cell wall carbohydrate. With these reagents we examined the M protein from various group A serotypes by immunoblot analysis after solubilization of the streptococcal cell wall with muralytic enzymes. The results revealed extensive size variation of the M molecule, both between and within M types.

Materials and Methods

Bacterial Strains

Except where noted, all strains were from the Rockefeller University collection. Streptococcal strains containing M protein crossreactive with monoclonal antibody 10B6 (20) are listed in Table I along with their respective M type. Streptococcal strains designated GL were derived from an epidemic of scarlet fever in young adult males at the Great Lakes Naval Training Station. Patients were admitted between February 26 and May 2, 1946. Throat cultures were taken weekly after admission and for several months thereafter. All strains isolated were M typed with available rabbit typing sera (21). Type 6 strains isolated from a hospital epidemic of pharyngitis were supplied by Dr. Richard Facklam of the Centers for Disease Control, Atlanta, GA. *E. coli* K12 strain C600NR(pJRS42.13) containing *emm6*, the structural gene for the M6 protein obtained from streptococcal strain D471 (18), was used for the isolation of M6 protein (19).

Passage of D471 in the Laboratory

A streptomycin-resistant mutant of M6 streptococcal strain D471 was grown overnight at 37°C in Todd-Hewitt broth (Difco Laboratories, Inc., Detroit, MI) supplemented with 0.2% yeast extract. To reduce the chance of contamination, streptomycin was added to the medium (10 mg/ml) after the seventh passage. The streptococcal chains were sonicated until microscopic examination revealed that single cells predominated. The culture was then diluted and plated to obtain a viable count. By comparing this with the count obtained in a Petroff-Hauser chamber, we determined that at least 50% of the cells were viable after sonication. These cells were diluted either 10^{-4} or 10^{-6} into the same medium and grown again overnight. The process was repeated 11 times and the number of generations of growth was calculated from the viable counts obtained at each step.

Antibodies

Monoclonal antibodies were prepared against purified lysin–extracted M6 protein as described (20) and group A–specific antiserum was prepared in rabbits as described (21). Monospecific antibodies to the group A–specific *N*-acetyl-D-glucosamine (GlcNAc)[1] determinant (which may also react with the GlcNAc moiety of the peptidoglycan) were prepared as follows: Group A streptococcal strain J17A4 was grown in 2 liters of Todd-Hewitt broth, centrifuged, and washed twice in 100 mM ammonium bicarbonate buffer, pH 8.0, and suspended in 10.0 ml of this buffer. The cells were heat killed at 56°C for 45 min, washed once in the ammonium bicarbonate buffer, and suspended in 5 ml of this buffer. Trypsin was added at 100 μg/ml final concentration and incubated for 30 min at 37°C to remove surface proteins. The cells were then washed twice in saline and once in 100 mM ammonium bicarbonate containing 100 μg/ml soybean trypsin inhibitor. After two washes in phosphate-buffered saline, pH 7.0 (PBS), hyperimmune group A–specific

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[1] *Abbreviations used in this paper:* Cm, chloramphenicol; GlcNAc, *N*-acetyl-D-glucosamine; PBS, phosphate-buffered saline; PED buffer, phosphate buffer containing EDTA and dithiothreitol; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

antiserum (prepared against whole group A streptococci [21]) was added to the cell pellet (three times the packed cell volume) and the mixture was allowed to rotate at 4°C for 4 h. The cells were washed three times in PBS, once in 3% NaCl, then twice in PBS. To remove the bound GlcNAc-specific antibodies, the washed cells were suspended in 5.0 ml 3% GlcNAc hapten in PBS and mixed at 4°C for 1 h. After sedimenting the cells at 6,000 g, 1 mg/ml bovine serum albumin was added to the supernatant and the mixture was filtered through a 0.22-μm-pore membrane (Millipore/Continental Water Systems, Bedford, MA) and dialyzed against PBS containing 0.02% sodium azide.

SDS-PAGE and Immunoblot Analyses

Proteins were separated on 12% sodium dodecyl sulfate–polyacrylamide gel slabs (SDS-PAGE) and transferred to nitrocellulose as previously described (19). The nitrocellulose immunoblots were reacted with either 10B6, a monoclonal antibody to the lysin-extracted M6 molecule (19, 20), or GlcNAc-specific antibodies, and the reactive bands were visualized as described by Blake et al. (22).

Bactericidal Assay

The indirect bactericidal assay was carried out as originally described by Lancefield (2) with modifications by Fischetti et al. (12).

Enzyme Digestions

Extraction of M protein with phage lysin. Crude group C phage–associated lysin was prepared as described (23). Streptococci were diluted 1:100 from an overnight culture into 50 ml of Todd-Hewitt broth and incubated at 37°C until they reached 0.5 OD at 650 nm (determined in an 18 mm tube). The cells were sedimented, suspended in 1.0 ml 50 mM phosphate buffer, pH 6.1, containing 5 mM EDTA and 5 mM dithiothreitol (PED buffer), and transferred to a 1.5 ml microcentrifuge tube. After sedimentation, the cells were washed once in the PED buffer and resuspended in 1.0 ml of 30% raffinose in the PED buffer. Phage lysin was added to the cell suspension at a final concentration of 360 lytic units per milliliter (23) and incubated for 45 min at 37°C. The resulting protoplasts were sedimented at 8,000 g for 10 min, an aliquot of the supernatant was added to the SDS-PAGE loading buffer, and the tube was placed in a boiling water bath for 3 min.

Identical lysin extraction experiments were performed with type 6 streptococci that had been digested with trypsin. For these studies, streptococci were grown as described, sedimented and washed twice in 100 mM ammonium bicarbonate, pH 8.0, and suspended in 10 ml of the same buffer. Trypsin was added to a concentration of 100 μg/ml and the mixture was incubated for 15 min at 37°C. The cells were then washed twice in PED buffer containing 100 μg/ml soybean trypsin inhibitor and 50 μg/ml chloramphenicol (Cm), and once in PED buffer containing the Cm alone. The cells were then suspended in 1.0 ml PED buffer containing 30% raffinose and 50 μg/ml Cm and digested with lysin as described above.

Mutanolysin. Further digestion of the streptococcal extract after digestion with phage lysin was performed with mutanolysin, an N-acetyl muramidase (kindly supplied by Dr. S. Kotani, Osaka University, Japan). After centrifugation to remove the protoplasts, 100 μl mutanolysin (1.0 mg/ml in 50 mM phosphate, pH 6.1) were added to 1.0 ml of the lysin digest and the mixture incubated at 37°C for 1 h. At this time, an aliquot of the digest was added to the SDS gel loading buffer and boiled for 3 min.

Iodination of the streptococcal surface proteins. An overnight culture of streptococci was diluted 1:100 into 10 ml of Todd-Hewitt broth and incubated at 37°C until it reached an 0.5 OD at 650 nm. The cells were centrifuged, washed twice in 50 mM phosphate buffer, pH 7.0, and resuspended in 100 μl of the same buffer. [125]I (10 μl at 1.0 mCi/10 μl sp act) was added to the cell suspension along with one Iodobead (Pierce Chemical Co., Rockford, IL). The mixture was allowed to stand at ambient temperature for 15 min, when the reaction was stopped by transferring the cells to a clean tube. The cells, freed from the residual ^{125}I by several washes in phosphate buffer, were added to streptococci that had been prepared for lysin extraction (see above). The mixture of labeled and

unlabeled cells was lysin extracted as described above and processed for a standard immunoblot using M6 monoclonal antibody (see above). After development of the immunoreactive bands on nitrocellulose, the membrane was exposed to x-ray film (Kodak BB-1).

Results

Appearance of the M Protein Extracted With Phage Lysin.   Streptococcal group C phage–associated lysin (lysin), an N-acetyl-muramyl-L-alanine amidase (24), was used to solubilize the streptococcal cell wall and release the M protein. In the presence of 30% raffinose, the protoplasts remain mostly intact, which reduces contamination with cytoplasmic and membrane fractions (25). When the proteins in a crude lysin extract of M6 streptococci (strain D471) were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with a monoclonal antibody to the M6 protein, multiple bands were observed (Fig. 1A). Retreatment of the extract with lysin did not change the observed pattern. A control lane containing lysin alone showed no reaction. A sample of purified M6 protein produced in *E. coli* C600NR(pJRS42.13) was placed on the gel for comparison (Fig. 1A). The results indicate that the M protein in a fresh lysin digest of streptococci is larger than the protein from the *E. coli* periplasm and that the lysin-extracted strepto-
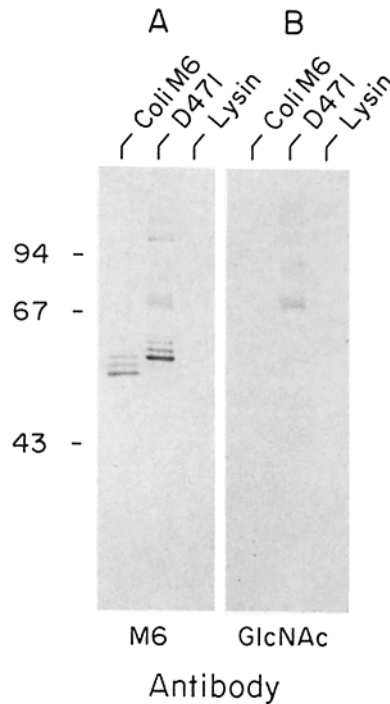

FIGURE 1.   Immunoblot of a lysin extract of type 6 streptococcal cells (D471) compared with purified M6 protein isolated from *E. coli* carrying the M6 genome (*ColiM6*). (*A*) The proteins in a 12% SDS gel were transferred to nitrocellulose and reacted with M6 monoclonal antibody 10B6. (*B*) A duplicate section of the gel was blotted and reacted with GlcNAc-specific antibody and developed as described in Materials and Methods. A sample of lysin (*Lysin*) was added to the gels as a control. Molecular weight standards are: 94,000, phosphorylase *b*; 67,000, bovine serum albumin; 43,000, ovalbumin.

coccal preparation contains additional anti-M6–reactive bands significantly larger than any found in the *E. coli* preparation.

To determine whether the apparently larger size and multiple banding pattern in the lysin digest resulted from the presence of streptococcal cell wall components still bound to the M protein, we reacted a duplicate blot with antibodies to the GlcNAc determinant of the streptococcal group A carbohydrate. The upper broad bands of the D471 lysin digest (Fig. 1*B*) also reacted strongly with this antibody, indicating that these molecules contain both M6 protein and cell wall components. All GlcNAc-reactive bands were also reactive with anti-M antibody. However, some of these doubly reactive bands exhibited weak anti-M reactivity. In some experiments, certain lower molecular weight-M–reactive bands also reacted with the GlcNAc antibodies, but this reaction was consistently weak. The patterns of the lysin digest observed in Fig. 1, *A* and *B* were quite reproducible from experiment to experiment, even with different lots of lysin.

In a separate experiment (data not shown), when the cells were digested with trypsin (which removes the M protein from the streptococcal surface) before lysin extraction, no immunoreactive molecules were visible on the immunoblot developed with either the M6 or GlcNAc antibodies. This indicates that the position of the GlcNAc-reactive bands in the SDS gel is dependent on their association with protein from the cell surface.

Extrinsic Labeling of the Surface Molecules.   The multiple bands in the lysin extract may be derived in part from contamination with cytoplasmic and/or membrane fractions that may contain incomplete M protein molecules not yet inserted into the cell wall. To determine whether all bands of the lysin-extracted M protein are derived from the cell surface, ^{125}I was used to label the streptococcal surface proteins having available tyrosine groups. After extracting the labeled cells with lysin, the proteins were separated by SDS-PAGE and examined by immunoblot with the M6 monoclonal antibody. The immunoblot was then exposed to x-ray film to determine the position of the radiolabeled proteins. The protein bands that reacted with the M6 antibody were also labeled strongly with [125]I (Fig. 2), indicating that all the M protein bands in the lysin extract are derived from the cell surface. Some of the more weakly labeled proteins did not react with the M6 antibody.

Size Variation of the M Protein from Different M Types.   Monoclonal antibody 10B6, an antibody prepared with purified lysin–extracted M6 protein (20), is reactive with an epitope located near the center of the fibrous M protein molecule and crossreacts with 20 serological M protein types (20). To take advantage of this property, we prepared lysin extracts from streptococci of each of these different serotypes and analyzed them by immunoblot. Fig. 3*A*, showing three M types, is representative of the results. Nearly all the M types analyzed exhibited M proteins of different apparent molecular size (Table I). Molecular weight estimates ranged from 80,000 for the M30 protein to 41,000 for the M40 molecule. When an identical blot (Fig. 3*B*) was reacted with the GlcNAc-specific antibodies, only the diffuse upper bands reacted, as seen also with the M6 extract (Fig. 1). In each strain, a strongly labeled upper band (Fig. 3*A*, arrows) that reacts with the M6 antibody did not seem to react with the GlcNAc antibody.
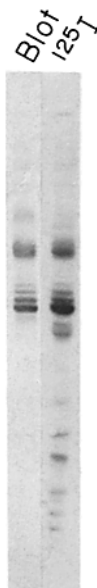
FIGURE 2.   Autoradiograph and immunoblot analysis of a lysin extract of extrinsically labeled type 6 streptococci. Type 6 streptococci were extrinsically labeled with ^{125}I in the presence of Iodobeads. The labeled cells were added to unlabeled type 6 streptococci and the mixture digested with lysin. The proteins in the digest were separated by SDS-PAGE, blotted onto nitrocellulose, and reacted with M6 monoclonal antibody 10B6. After processing for detection of the immunoreactive bands (*Blot*), the dried nitrocellulose was exposed to x-ray film to detect the radioactive bands (*[125]I*).

These bands are within the size range expected of dimeric forms of the lower anti-M–reactive monomer bands.

In the extracts from each strain, the bands reactive with GlcNAc-specific antibodies had sizes proportional to those of the lower anti-M–reactive bands (Fig. 3B). This is consistent with the interpretation that the upper anti-GlcNAc–reactive bands contain a defined cell wall fragment in addition to the M protein, and that the latter differs in apparent molecular weight from strain to strain.

Size Variation of the M Protein from Different Strains of the Same M Type. Since the apparent size of the M molecule differed among M serotypes, we wished to determine whether a similar variation occurred among strains of the same M type. Lysin-extracted M protein of type 6 strains from different clinical isolates over a period of almost 40 yr also varied in apparent size (Fig. 4) with nearly the same range as among M proteins from different serotypes (Table II). To confirm that these different strains were actually M type 6 and not merely strains containing M protein crossreactive with our M6 typing serum, we tested them in the bactericidal assay. In this assay, only type-specific antibodies effectively interfere with the antiphagocytic property of the M molecule (1). All type 6 strains used in these studies survived in normal human blood (because of the M protein) and were opsonized with antibodies prepared against purified M protein isolated from M6 strain D471 (data not shown).

Frequency of the Molecular Size Change of the M Molecule. We next examined whether the variation in apparent molecular size of the M protein from different
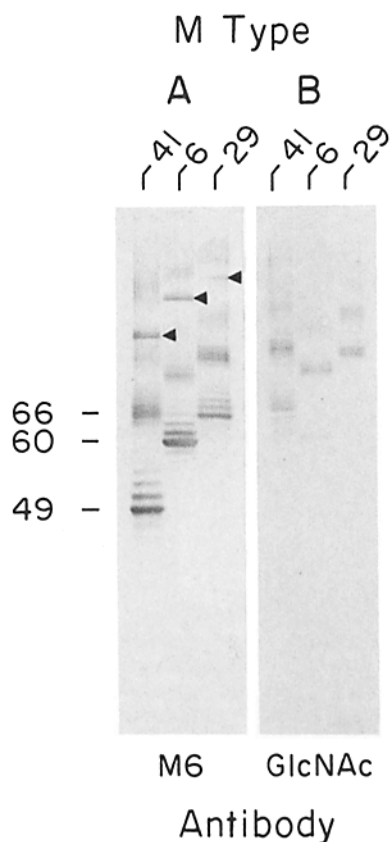
FIGURE 3.   Immunoblot analysis of lysin extracts of streptococcal M serotypes 41, 6, and 29. (A) Lysin extracts of three representative serotypes (see Table I) crossreactive with M6 monoclonal 10B6 were processed as described in Fig. 1. (B) A duplicate section of the gel was blotted and reacted with GlcNAc-specific antibody. Molecular weight markers identify the major anti-M–reactive bands for each M type in A. Arrows mark upper bands reactive with the M6 but not the GlcNAc antibody.

strains is generated rapidly or by gradual evolution within a large population. Three studies were performed to look for rapid change. First, lysin extracts of 32 type 6 strains isolated from different individuals over a 2-mo period in a single hospital epidemic were examined by immunoblot analysis. The M6 proteins isolated from all the strains were indistinguishable in size and did not exhibit the variations observed among the M6 proteins from random clinical isolates (Fig. 5). This suggests that a single M6 clone may have been responsible for the hospital epidemic during the 2-mo period. In a second study, the lysin extract of a type 6 streptococcal strain S43, passaged in mice 192 times (S43/192) over a period of >30 yr, to select for organisms that retain their M protein content (26), was compared with lysin extracts of intermediate mouse passages of this strain. In all extracts, the M protein banding patterns were essentially indistinguishable from one another (Fig. 6). Third, we passed the M6 strain D471 serially in the laboratory, separating the streptococcal chains so that each passage was started from a known number of single viable cells (see Materials and Methods).

TABLE I

Migration in SDS-PAGE of M Protein Extracted With Phage Lysin from Different M Types

M Type	Strain	Migration*	Molecular weight
30	D124/126/1	0.66	80,000
46	C105/41/5	0.79	72,000
31	J137/69/2	0.81	70,000
3	B930/61/3	0.87	67,000
29	D28	0.89	66,000
12	T12/126/2	0.92	64,000
14	T14/46/3	0.94	63,500
47	C744/RB4/6/3	0.95	63,000
23	T23/102/RB5	0.97	62,000
5	T5B/126/3	0.98	61,000
24	C98/115/2	0.99	60,500
6	D471	1.00	60,000
54	A952/94/1	1.00	60,000
43	C126/70/1	1.14	53,000
52	A871/106/1	1.17	52,000
53	A952/94/1	1.17	52,000
56	A963	1.17	52,000
36	C119/83/1	1.19	51,000
32	C121/39/3	1.22	50,000
41	C101/103/1	1.25	49,000
40	C143/25/8	1.41	41,000

Measurements were made to the lowest molecular weight anti-M protein–reactive band as determined after immunoblot analysis with crossreactive monoclonal antibody 10B6 (see Fig. 3A).

* Migration was calculated relative to M6 strain D471.

No change in size of the M6 protein was detectable after 156 generations of growth in the laboratory (data not shown).

Since the 10B6 monoclonal antibody crossreacted with the M30 protein, we used this antibody to examine the strains isolated during a major streptococcal scarlet fever epidemic that occurred at the Great Lakes Naval Training Station in the mid-1940s, in which M types 17, 19, and 30 predominated. Fig. 7A is an immunoblot of the lysin-extracted M protein of M30 streptococci isolated from a single patient (GL219) over a 7 wk period. The banding pattern of lysin-extracted M protein in all these strains appears identical. In contrast, sequential M30 isolates from a second patient (GL130, Fig. 7B) revealed two shifts in apparent size. A major shift was observed between the first and second isolate (3/18 and 4/15), and a minor size shift between the penultimate and last isolate (4/22 and 4/29).

Mutanolysin and Lysozyme Digestion. We were concerned that the marked variation observed in the molecular size of the M molecule, within as well as between M types, might be the result of different sized peptidoglycan and/or other cell wall fragments bound to the M molecule. If this were true, muralytic enzymes should alter the apparent size of the anti-M–reactive bands. No effect was observed when the lysin digests were treated with lysozyme, a muramidase (data not shown). Although treatment of the lysin digests of three different M6
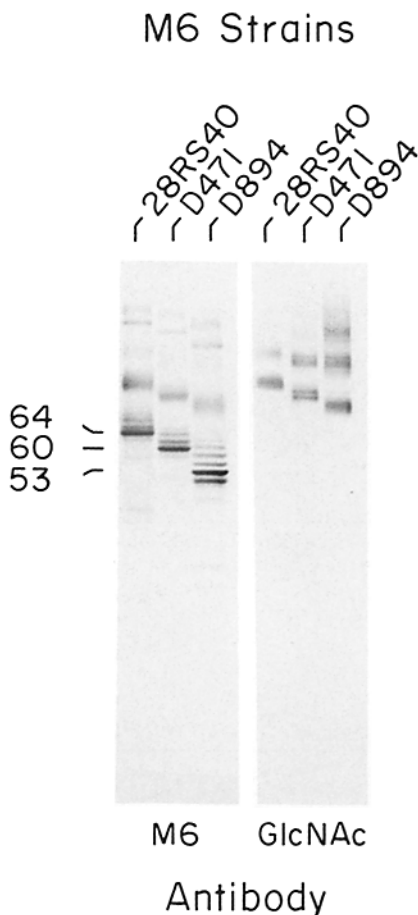
FIGURE 4. Immunoblot analysis of lysin extracts of three different type 6 streptococcal strains. Lysin extracts of three representative type 6 streptococcal strains from different clinical isolates (see Table II) were processed as described in Fig. 1. A duplicate section of the gel was blotted and reacted with GlcNAc-specific antibody. Molecular weight markers identify the major anti-M–reactive bands (M6) for each strain.

strains with mutanolysin (also a muramidase), simplified the pattern of the lower anti-M6–reactive bands (Fig. 8; compare with Fig. 4), in no case did a major M6-reactive band exhibit a shift in molecular size. Thus, these major lower anti-M6–reactive bands probably represent those M molecules least contaminated with peptidoglycan fragments (Fig. 8). This effect was particularly apparent with strains D471 and D894 (Fig. 8).

Discussion

Extrinsic ^{125}I labeling of type 6 streptococci revealed that the M protein was the major surface protein (with an accessible tyrosine), since, after extraction with phage lysin, nearly all of the ^{125}I-labeled bands were also reactive with the M6 monoclonal antibody. The weakly labeled bands that did not react with the

TABLE II

Migration in SDS-PAGE of M Protein Extracted from Various Type 6
Streptococcal Strains With Phage Lysin

Strain	Year isolated	Migration*	Molecular weight
A590	1962	0.79	72,000
20RP58	1952	0.87	67,000
9RP124	1952	0.87	67,000
35RS37	1942	0.91	65,000
28RS40	1942	0.93	64,000
F365	1979	0.93	64,000
3GL156	1946	0.95	63,000
10RP44	1950	0.96	62,000
1RP178	1955	0.96	62,000
D471	1971	1.00	60,000
F382	1980	1.03	58,000
1RP34	1948	1.10	55,000
D894	1975	1.15	53,000

Measurements were made to the lowest molecular weight anti-M pro-
tein–reactive band as determined after immunoblot analysis with cross-
reactive monoclonal antibody 10B6 (see Fig. 4).
* Migration was calculated relative to M6 strain D471.
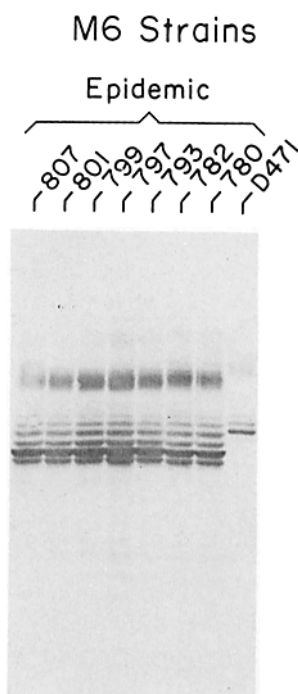
M6 Strains

FIGURE 5.   Immunoblot analysis of lysin extracts of type 6 streptococcal strains isolated in a
hospital epidemic. Lysin extracts of seven representative type 6 streptococcal strains, of 32
strains isolated from different individuals in a hospital epidemic, were processed as described
in Fig. 1 and reacted with M6 monoclonal antibody 10B6. For reference purposes, a lysin
extract of type 6 strain D471 is included on the immunoblot.
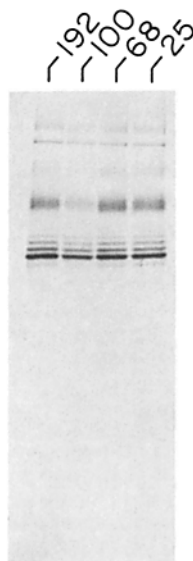
Mouse Passage

FIGURE 6.   Immunoblot analysis of lysin extracts of a single type 6 streptococcal strain after mouse passages. Type 6 streptococcal strain S43 has been sequentially passed in mice 192 times over the past 30 years. Lysin extracts of strains from intermediate mouse passages (25, 68, and 100) are compared with passage 192. Extracts were processed as described in Fig. 1 and reacted with M6 monoclonal antibody 10B6.

monoclonal M6 antibody may be either degraded products of the M protein, lacking this epitope, or other streptococcal antigens such as T and R (27, 28).

Although nothing has been published about the mode of attachment of the M protein to the streptococcal cell wall, it seems likely that the association is covalent. We find that boiling streptococci in 1% SDS does not release detectable (by immunoblot) M protein into the supernatant (unpublished results), indicating that a covalent attachment may be involved. Using nitrous acid to remove the carbohydrate from streptococcal cell walls, Swanson et al. (29) found by electron microscopy that the M protein still remained attached to the cell, suggesting that M protein may be attached through the peptidoglycan moiety and not the group carbohydrate fraction. Several lines of evidence in the current study support these conclusions: (a) After lysin extraction and immunoblot analysis, some of the protein bands reactive with the anti-M monoclonal antibody also react with antibodies to GlcNAc. Since no GlcNAc-reactive bands were present on immunoblots of lysin-extracted cells that had been previously trypsin treated to remove the M protein, this implies that the bands that react with both types of antibodies contain a complex of M protein attached to cell wall fragments. Because this association resists boiling in SDS, it is likely to be a covalent complex. (b) Mutanolysin, a muramidase, cleaves some remaining peptidoglycan fragments from the lysin-extracted M protein, resulting in a more simplified pattern of M protein bands by immunoblot. Some of the anti-M–reactive bands in the lysin extract are either removed or reduced by the mutanolysin. These presumably
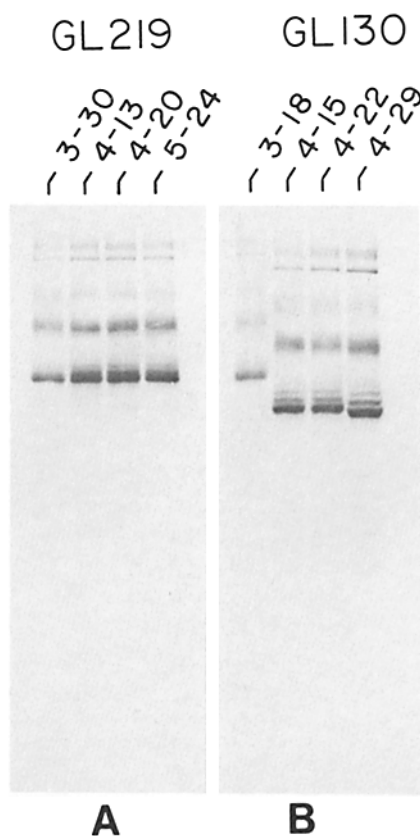
GL219   GL130

A   B

FIGURE 7. Immunoblot analysis of lysin extracts of type 30 strains isolated from two individuals during a scarlet fever epidemic. Lysin extracts of M30 streptococci isolated at different times in 1940 from two patients, (A) GL219 and (B) GL130, were processed as described in Fig. 1 and reacted with M6 monoclonal antibody 10B6.

shift in position to increase the intensity of preexisting M-reactive bands. (c) Because of the specificity of the lysin (an amidase) and mutanolysin (a muramidase), if the M protein were bound to the group carbohydrate moiety of the cell wall, anti-GlcNac reactivity would be expected with all the M-reactive bands. Since little or no anti-GlcNAc reactivity is associated with the lower anti-M–reactive bands, the cell wall attachment of the M fibrillae is not likely to be through the group carbohydrate moiety, but may be associated with the peptidoglycan fraction. The weak reactivity sometimes observed between the anti-GlcNAc antibody and certain lower anti-M–reactive bands may be due to the weak specificity of this antibody with the GlcNAc moiety of the peptidoglycan. (d) The larger anti-M– and anti-GlcNAc–reactive bands are absent from the E. coli preparation, suggesting that they are specific to the streptococcus and not part of the M6 gene product. We are currently investigating the reason that the M6 gene product, even in E. coli, appears as multiple closely spaced bands on SDS gels.

The presence of cell wall fragments on the M protein extracted from the streptococcus with lytic enzymes may explain why the streptococcal M6 molecule
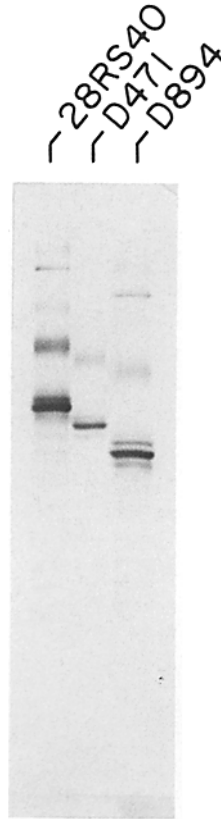
M6 Strains

FIGURE 8.   Immunoblot analysis of lysin extracts of three type 6 streptococcal strains after digestion with mutanolysin. The lysin extracts of three different type 6 streptococcal strains (see Fig. 4) were treated with mutanolysin, the digest processed as described in Fig. 1 and reacted with M6 monoclonal antibody 10B6.

appears slightly larger than the M6 protein isolated from the *E. coli* containing the M6 gene. This larger M protein size is only found in fresh lysin extracts of the streptococcal cell wall; after purification, the lysin-extracted M6 protein appears to be slightly smaller than that isolated from *E. coli* (18, and manuscript in preparation). Since the amino acid sequence of the amino-terminal region of the purified lysin–extracted M6 protein is the same as that of M6 from *E. coli* (19), the small mobility change that occurs during purification must be due to alterations in parts of the molecule other than the amino terminus. Studies are in progress to determine the nature of the alterations responsible for the observed shift in gel mobility.

It is uncertain why the bands that are reactive with both the anti-M and anti-GlcNAc antibodies are not completely sensitive to further degradation with any of the three muralytic enzymes used (lysozyme, lysin, and mutanolysin) (compare Fig. 8 with Fig. 4). These bands may represent a highly crosslinked or modified fragment of the peptidoglycan which, in the presence of the group carbohydrate

moiety, sterically blocks these enzymes from approaching the cleavage site on the peptidoglycan. Cell wall conformation and carbohydrate substitution of the peptidoglycan may play an important role in the activity of these enzymes, since lysozyme (a muramidase) is unable to solubilize the cell wall of the group A streptococci (30), while mutanolysin (also a muramidase) (31) is active on these cells.

The extensive size heterogeneity of the M molecule observed in the lysin extracts of different streptococcal strains cannot be accounted for by the presence of varying amounts of cell wall fragments bound to the M protein. As mentioned earlier, the major lower M-reactive bands on immunoblots do not react with GlcNAc antibody and thus do not appear to contain group carbohydrate. In addition, treatment of the lysin digest with mutanolysin does not alter the molecular size of these anti-M6–reactive bands on the immunoblot. Furthermore, amino acid analysis of purified preparations of lysin-extracted M6 protein (which represents the lower anti-M–reactive bands) does not reveal the presence of amino sugars at a sensitivity that would detect one residue per mole (unpublished data and manuscript in preparation). It is likely that these lower anti-M–reactive molecules have little if any cell wall contaminants and are close to the native size of the M protein monomer for that strain.

In addition to group A streptococci, other pathogenic organisms escape the immune surveillance of the host system by varying their surface antigens (32, 33). In one of the best-studied of these cases, the gonococcal pilus protein, the genome of the pathogen contains many regions of partial DNA homology to the structural gene for the pilus antigen (34, 35). Presumably, homologous recombination is important for the rearrangement of the DNA within the genome that leads to expression of the altered protein. In some gonococcal strains studied, this type of genetic rearrangement occurs at a readily detectable level (36). Within a single gonococcal colony (which represents about 20 generations of growth), as many as 5% of the cells may have pilin subunits of a different size (36).

Not only do the streptococcal M proteins show antigenic variation in nature; even within a serological M type, we have now detected size variations. Our data indicate that, at least in the few strains examined, size change does not occur rapidly; a strain passed in mice 192 times over a period of 30 years showed no detectable change in the M protein, and one passed for 156 generations in the laboratory also did not vary. This is in agreement with our inability to detect additional regions of DNA homology when the M6 gene was used to probe the streptococcal DNA under conditions that would permit 23% (37) or 50% (unpublished results) base pair mismatch. Either the genetic mechanism for variation in the M protein is different from that for antigenic variation in the gonococcal pilus or, in the few strains we studied, recombination is less common than in the gonococcal strains being investigated.

In a limited epidemic, no change in M type or molecular size could be detected during a 2 mo period within 32 individual clinical isolates. Furthermore, in a larger epidemic in the mid-1940s at the Great Lakes Naval Station, a patient (GL219), followed for 7 wk, retained streptococci with the same M type and molecular size. However, group A steptococci isolated from a second patient

(GL130) in this epidemic showed two changes in the size of M30 during a 6 wk period. The interpretation of the last result is unclear, because the M30 streptococci in this epidemic did not have M protein of a single size. When random M30 strains from other patients were examined for their M protein size after lysin extraction, variation was observed between strains. Thus, it is possible that patient GL130 was reinfected with a second and third clone of M30 streptococci. However, it is also possible that the same M30 strain did change in this patient during the 6 wk period examined. Although the existence of many different M types and many sizes of M protein within a given type indicates extensive variability in this protein, the process of genetic change does not appear to be rapid.

It is unusual for evolutionary divergence of related proteins to exhibit a change in molecular weight as broad as 40,000. Most related proteins tend to be conserved in size, and often in sequence, not just among organisms of different species and genera, but even among organisms of different families (38). Many proteins of this type are enzymes, which presumably have conformational restraints that restrict extensive size variations. However, even non-enzyme proteins such as contractile system proteins and globins are also extremely conserved in size and sequence among species (38). The size variation observed among the M molecules is unusual for related proteins.

It has been suggested previously that the antiphagocytic property of the M protein may be the result of the alignment of negatively charged amino acids in a regular array along the outer surface of the M protein fibrillae (39). In a recent analysis of the complete amino acid sequence of PepM5 (7), the pepsin fragment of the M5 molecule, a preponderance of acidic amino acids was found in external positions within the amino-terminal region (40), which is distal to the cell surface (5, 19). If both the charge domain in the amino-terminal region and the alpha-helical coiled-coil structure are conserved within M molecules, the length of the M protein fibrillae may not be critical for its antiphagocytic function (within certain broad limits). For this reason, many different sizes of M protein may persist in nature. DNA sequence analyses in progress will determine whether there are preferred sites in the molecule for insertions and deletions, whether preferred sequences are inserted, and whether single base changes are as common as insertions and deletions in the gene for the M protein.

Summary

In addition to the type-specific antigenic variation that is a well-known characteristic for the group A streptococcal M protein, we have now found that the M molecules vary with respect to their molecular size, both between M types and within an M type. By the use of an M6 monoclonal antibody, which crossreacts with 20 different M protein types, and antibodies to the N-acetyl glucosamine determinant of the cell wall, we have been able to identify the M protein molecules released from the streptococcal cell wall with muralytic enzymes, particularly group C phage–associated lysin. Immunoblot analysis of the cell extract identified M protein molecules bound to various cell wall fragments, suggesting a peptidoglycan linkage for the M molecule. M protein extracted from 20 different streptococcal serotypes revealed size variations from 41,000

to 80,000 in molecular weight. This extreme variation is unusual for related proteins. Similar size variations in the M molecule were also found in random clinical isolates of type 6 streptococci. No size change was seen in M6 protein isolated from: (a) strains within a limited epidemic, (b) a strain passaged in mice 192 times, and (c) a strain passaged in the laboratory for 156 generations, suggesting that the observed variation is not a rapid process. The results indicate that, within the broad limits observed in this study, the size of the M protein may not be critical to the antiphagocytic activity of the molecule.

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