

## STREPTOCOCCAL M PROTEIN SIZE MUTANTS OCCUR AT HIGH FREQUENCY WITHIN A SINGLE STRAIN

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Streptococcal M proteins,  $\alpha$ -helical coiled-coil dimeric molecules on the surface of the group A streptococcus (1, 2), are antiphagocytic structures considered to be the major virulence factor for this organism (3). Type-specific antibodies to the M molecule allow phagocytosis of the invading streptococci, but antigenic variation of the protein has facilitated the survival of these organisms in nature. Besides the >75 serologically different M proteins defined to date, there is an increasing number of strains that cannot be identified with the currently available sera (World Health Organization Conference, 1984, Tokyo, Japan).

In addition to antigenic variation, which is a common feature of the M protein molecule, we recently discovered (4) that the M protein also exhibited a size variation among streptococcal strains. This size variation was observed both between strains of different M types, as well as among strains within an M type. The observed variations in M protein size did not appear to occur rapidly, since, in both a single outbreak of 32 separate isolates of type 6 strains and in a strain passed in mice 192 times, no size changes were observed.

Our recent elucidation (5) of the DNA sequence of the gene encoding the M6 protein led us to suggest that the size changes in the M molecule could be generated by homologous recombination between the observed repeated regions within the gene. To determine whether this suggestion was correct, it was necessary to first obtain a series of strains derived from the same parent with M proteins of different sizes.

In this study, we explored the possibility that, although they appear to occur rarely in nature within a specific strain, mutants with altered M sizes occur frequently enough in laboratory cultures to allow us to find them. We devised a method to identify deletion mutants even when they constitute a minority of the streptococcal population, and used it to isolate three independent deletion mutants from a single streptococcal strain and one additional mutant from one of the first deletion strains. These mutants occur at a frequency of  $\sim 1$  in  $2 \times 10^9$  CFUs in a laboratory-grown culture.

### Materials and Methods

*Bacterial Strain and Growth.* Streptococcal strain D471 is M type 6 from the Rockefeller University collection. A strain resistant to 100  $\mu\text{g}/\text{ml}$  of streptomycin (Sm)<sup>1</sup> was selected

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<sup>1</sup> *Abbreviations used in this paper:* Sm, streptomycin; TH, Todd-Hewitt broth.

by serial passage in the antibiotic. All cultures were in Todd-Hewitt (TH) broth containing 100 µg/ml Sm (TH-Sm broth).

*Bactericidal Assay.* The indirect bactericidal assay was carried out as originally described by Lancefield (6), with modifications by Fischetti et al. (7).

*Antisera.* Opsonic antibodies were prepared to the complete M protein molecule produced in the *E. coli* (ColiM6) (8), which was purified and used for immunization as described (9). Opsonic antibodies to a synthetic peptide consisting of the *N*-terminal 20 amino acids of the native M6 protein (9), were prepared as described (K. F. Jones, S. K. Hollingshead, J. R. Scott, and V. A. Fischetti, manuscript in preparation). Briefly, a synthetic peptide derived from the sequence of the *N*-terminal 20 amino acids of M6 protein (9) was purified by HPLC and coupled to ovalbumin as a carrier. Rabbits were immunized with 100 µg of peptide-carrier complex in CFA and boosted with the complex in IFA. M6 mAb 10A11 and 10B6 were prepared against purified M6 protein as described (10).

*Absorption Experiments.* Streptococci were grown in TH broth for 18 h, centrifuged, washed in saline, suspended in one-tenth of the original volume, and heat killed at 56°C for 60 min. Cells were adjusted to an OD<sub>650</sub> nm of 1.0 (using an 18-mm tube). For absorption, 15 ml of the cell suspension was sedimented, resuspended in 1.0 ml of saline, transferred to a 1.5-ml microcentrifuge tube, and pelleted in a Savant high-speed centrifuge. The cells were suspended in 300 µl of antiserum and the tube was rotated at 4°C for 18 h. The cells were sedimented for 10 min and the supernatant was used for ELISA and indirect bactericidal assays.

*ELISA.* ELISAs were performed with ColiM6 protein-sensitized microtiter plates as described previously (11), and the reaction was analyzed on an ELISA-5 reader and data analyzer (Physica, Inc., New York).

*Extraction of M Protein with Phage Lysin.* Cultures were grown at 37°C for 18 h in V-bottom 96-well microtiter plates in 200 µl of TH-Sm broth. The cells were then sedimented in a Sorvall model RT600 centrifuge at 2,000 rpm for 20 min. After removing the supernatant, the cells in each well were suspended in 50 µl of 10 mM phosphate buffer, pH 6.1, containing 30% raffinose, 5 mM DTT, and a 1:100 dilution of phage lysin (4), and the plate was incubated at 37°C for 1 h. The resultant protoplasts were sedimented at 2,000 rpm for 20 min, and the supernatant, containing the digested cell wall and released M protein, was mixed with an equal volume of SDS loading buffer in a separate microtiter plate. The solution in the plate was heated intermittently in a microwave oven for 60 s at a moderate setting. 5 µl was loaded onto SDS-PAGE minigels, electrophoresed, and immunoblotted to determine the size of the M protein.

*SDS-PAGE and Western Blot.* Proteins in 5 µl samples were separated on 10% SDS-polyacrylamide minigel slabs (70 × 220 × 0.75 mm) (SDS-PAGE) and transferred to nitrocellulose as previously described (9). The Western blots were reacted with either M6 mAb 10A11 or 10B6 as described by Jones et al. (10), and the reactive bands visualized as described by Blake et al. (12).

*Isolation of Size Mutants.* An overnight culture of type 6 strain D471 in TH-Sm broth was diluted to 10–30 CFU per 100 µl. 100 µl was dispensed into V-bottom microtiter plates containing 100 µl of TH-Sm broth. The plates were incubated at 37°C overnight, at which time the plates were shaken on a rotary shaker to suspend the settled cells. A replica plate was prepared by aseptically dispensing 50 µl from the growth plate to a second microtiter plate. The replica plate was sealed and placed at –50°C until needed. The growth plate was processed for lysin extraction, SDS-PAGE, and Western blot as described above. When M protein of a smaller size was observed on the Western blot, the cells in the corresponding well of the replica plate were diluted 1:100 and plated on TH-Sm agar. After incubation at 37°C for 18 h, colonies were picked with sterile toothpicks into microtiter wells containing 200 µl of TH-Sm broth, and reincubated at 37°C overnight. At this time, replica plates were again made and the cells were extracted with phage lysin and processed for Western blots as described above.

*Pepsin Digestion.* M protein was extracted from the streptococcal surface with pepsin at pH 5.8 as previously described (13). Crude pepsin digests were used for Western blot analysis and probed with M6 mAb 10A11 (10).

*Reconstruction Experiment.* Experiments were performed on mixed streptococcal cells to determine the proportion of M protein size mutants that are required in a population for detection by Western blot analysis. Two type 6 streptococcal strains producing M proteins that differ in size by ~5 kD were used in these experiments. Streptococci were grown to an OD of 0.8, and mixed at various ratios in a total of 200  $\mu$ l in microtiter wells. The cells were then centrifuged and processed for lysin extraction and Western blot analysis for the presence of M protein as described above.

## Results

*Level of Detection of M Protein Size Mutants.* These studies are based on the assumption that M protein size mutants are present in streptococcal cultures. To reduce the number of wells to be extracted and analyzed by Western blot, we needed to know what fraction of the population the size mutant must represent in order for it to be detected. Two strains of M6 streptococci producing different size M proteins were mixed at various ratios, lysin extracted, and the M protein was analyzed by Western blot. We found that, at our level of resolution, we could detect a size change of up to 1 small M producer to 30 large M producers. In the 1:30 mixture however, the small M molecule was just detectable in our system. When a similar experiment was performed with the ratios reversed, we could not easily detect a strain producing the larger M protein even at a ratio of 1:2 (large M/small M). This is due to the fact that the protein bands from a larger M producer comigrate with the higher M protein bands produced by the smaller M producer.

*Isolation of M Protein Size Mutants from M6 Strain D471.* Microtiter wells were inoculated with ~7 CFU each of strain D471, and the cultures were incubated overnight. Western blot analysis of the M protein from 33 microtiter wells indicated that one (well D9) contained cells also producing M protein smaller than that produced by the parental strain (Fig. 1). The contents of this well were plated, colonies were picked, and the size of the M protein was examined after lysin extraction and Western blot analysis. Each colony selected contained organisms that produced M protein of a single size, either that of the parent D471 strain, or a new smaller-sized M molecule (Fig. 2). Five serial subcultures of one of the size-variant colonies, D471dD9, followed by extraction and Western blot analysis, indicated that the size of the M protein produced by this strain was stable. By this procedure, therefore, we succeeded in isolating an M6 deletion mutant.

Two separate experiments performed in the same way resulted in the isolation of two additional deletion mutants (D471dC10 and D471dE11) of approximately the same size as the D471dD9 mutant (data not shown). In one experiment, 16 CFU were inoculated per well while the other was started with 23 CFU per well. In these experiments, one size mutant was identified among each 96 wells examined. Thus, the frequency of isolation of deletion mutants from strain D471 under these conditions is ~1%. Since at least 3–5% of the CFU in the well must be mutant to be recognized by our method, the frequency of these mutants in a laboratory-grown culture is ~1 in  $2 \times 10^3$  CFUs.

*Isolation of a Second M Protein Deletion Mutant from Mutant D471dD9.* Starting with an overnight culture of the first deletion mutant, D471dD9, the same process was used (29 CFU/well) to isolate an additional mutant producing an M

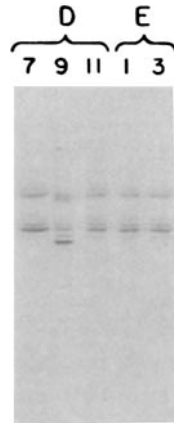


FIGURE 1. Western blot analysis of the lysin extracts of M6 streptococci in five microtiter wells, one of which contains a deletion mutant (well D9). The proteins in the lysin extracts of the streptococci grown in microtiter wells were separated by SDS-PAGE and transferred to nitrocellulose. The presence of M6 protein was identified with an M6 mAb. The multiple pattern seen is typical of M protein extracted with phage lysin, with the upper bands representing M protein still bound to cell wall fragments (4). The M protein produced by streptococci in wells D7, D9, D11, E1, and E3 are shown. Well D9 contains streptococci producing M protein both smaller and equal to that produced in the neighboring wells.

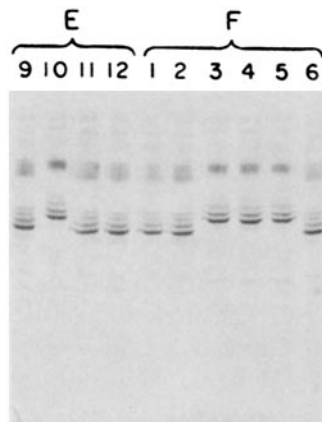


FIGURE 2. Western blot analysis of lysin extracts of M6 streptococci isolated from the contents of well D9 (Fig. 1). The streptococci in well D9 were plated, and colonies were picked into microtiter wells, grown, extracted with lysin, and processed as described in Fig. 1. The M protein produced by streptococci in wells E9 to E12 and F1 to F6 are shown.

protein smaller than the D471dD9 parent. This mutant was found in one well (A10) of 73 examined. Isolation of the cocci in microtiter well A10 indicated, as with the first isolate, that it contained streptococci that produced either the parental D471dD9 size M protein or a new smaller M molecule (D471dD9dA10) (Fig. 3). Upon subculture, this size was also found to be stable. No mutant with M protein of this size was ever observed to occur directly from strain D471, although >300 wells inoculated with at least 20 CFU/well were screened.

*Size of M Protein from Deletion Mutants.* Fig. 3 shows a Western blot comparing the M protein of the two size mutants, D471dD9 and D471dD9dA10, to the

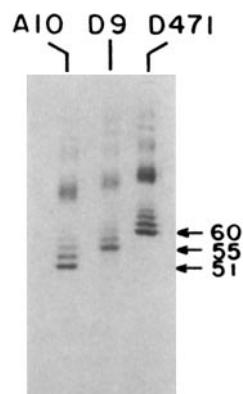


FIGURE 3. Western blot analysis of lysin extracts of M6 streptococcal strain D471 and two size-mutant derivatives of this strain, D471dD9 (D9) and D471dD9dA10 (A10). Western blots were treated with an mAb against the M6 protein as described in Materials and Methods.  $M_r \times 10^{-3}$  are shown to the right.

TABLE I  
*Survival of M6 Protein Size Variant Streptococci in Human Blood,  
and Opsonization with M6 Antiserum*

Strain	Inoculum	Antiserum	Colony count after 3 h at 37°C
D471	23	None	644
		Anti-M6*	1
D471dD9	13	None	650
		Anti-M6	0
D471dD9dA10	9	None	792
		Anti-M6	0

Streptococci (100  $\mu$ l) were added to 0.4 ml of heparinized human blood and 100  $\mu$ l of serum or broth, and the tubes were rotated for 3 h at 37°C. Colony counts were then made by plating 100  $\mu$ l samples.

\* Opsonic antibodies prepared in a rabbit immunized with purified ColiM6 protein (9).

parent strain, D471. From the major lower band in each group on SDS-PAGE, we estimate a 4,000–5,000  $M_r$  difference between the M proteins in these strains.

*Function of Smaller M Protein Molecules.* To determine whether the strains producing the smaller M molecules were able to survive in human blood as well as the parent D471 strain, a bactericidal assay was performed. All strains survived in human blood lacking type 6 antibodies and were phagocytized in the presence of type 6 opsonic antibodies prepared against the whole M6 protein (Table I).

When the three strains were used to absorb type 6 opsonic antibodies prepared against the native M6 protein, all three had the determinants to remove these antibodies (Table II). In addition, binding antibodies to the native ColiM6 protein could be absorbed to the same extent by the parent D471 strain and the two size mutants (Table III, top).

*Location of the Deletion.* Pepsin digestion of the M protein on intact streptococci was used to determine the location of the deletion in the M molecule. Since pepsin at pH 5.8 cleaves the M protein at approximately the center of this fibrillar

TABLE II  
*Indirect Bactericidal Assay Demonstrating Absorption of M6 Opsonic Antibodies with Strains D471, D471dD9, and D471dD9dA10*

Absorption*	Colony count after 3 h 37°C using inoculum 31
Control‡	>1,000
None	47
D471	900
D471dD9	>1,000
D471dD9dA10	>1,000

\* Absorption of anti-M6, an opsonic antiserum prepared in a rabbit against ColiM6 protein (9).

‡ Number of colony forming units after 3-h rotation with only blood and streptococci. Assay as described in Table I.

TABLE III  
*ELISA Titers of M6 Antisera after Absorption with Strains D471, D471dD9, and D471d9dA10*

Sera	Absorbed	Titer*
Anti-M6‡	None	12,800
	D471	800
	D471dD9	800
	D471dD9dA10	800
Anti-N terminal§	None	6,400
	D471	200
	D471dD9	200
	D471dD9dA10	400

\* Titer represents the reciprocal of the highest dilution giving an OD<sub>405</sub> of 1.0 ± 0.2.

‡ An opsonic antiserum prepared in a rabbit to the ColiM6 molecule (9).

§ Antiserum prepared against a synthetic peptide representing the N-terminal 20 amino acids of the native M6 protein.

molecule (2, 5), releasing the N-terminal half in solution, the size of the pepsin fragment should help determine in which half of the molecule the deletion was located. The size decrease of the M proteins from the mutants D471dD9 and D471dD9dA10 was reflected in the size of the fragments released by the limited pepsin digestion (Fig. 4), suggesting that the size mutants had deletions within the N-terminal half of the M protein.

Absorption experiments were performed to rule out the possibility that the size variations were the result of deletions of the N-terminal end of the molecule. An antiserum prepared against a synthetic peptide, representing the N-terminal 20 amino acids of the native M6 molecule, was absorbed with strain D471, and the size-variant strains D471dD9 and D471dD9dA10 (Table III, *bottom*). All three strains were able to absorb the N-terminal-specific antibodies from this serum. Since this sequence is not repeated elsewhere in the M6 molecule (5), the results suggest that the N-terminal end of the M protein is intact in the two size variants.

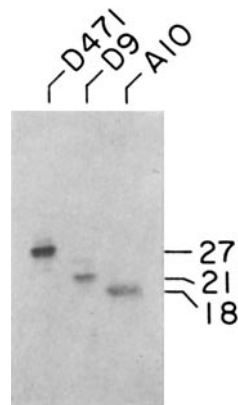


FIGURE 4. Western blot analysis of pepsin fragments of the M protein extracted from streptococcal strain D471 and two size-mutant derivatives of this strain, D471dD9 (D9) and D471dD9dA10 (A10).  $M_r$  are shown to the right. Pepsin fragments were identified with mAb 10A11 (10).

### Discussion

Pathogenic organisms often evade the immune defenses of their hosts by varying their major surface antigens, many of which are proteins. The frequency of such antigenic variation may be as high as 1 per 1,000 cells, since the changes do not result from random mutation. In the best-studied cases, trypanosome surface glycoproteins (14), pili of *Neisseria gonorrhoeae* (15), and the major surface proteins of *Borrelia* (16), rearrangement of DNA within the genome causes the changes in the protein. The mechanisms of rearrangement appear to differ among these organisms, but all involve recombination between DNA sequences exterior to the structural gene for protein and the expressed copy of the structural gene.

The group A streptococcus is also able to elude its host's immune surveillance system by varying the antigenic structure of its M protein, the major virulence factor for the organism. In addition to changes in serological type, the M molecule undergoes major shifts in molecular size (4).

In the method used here to select for size variants, we took advantage of the sensitivity of the Western blot and the specificity of our M6 mAb to identify a deletion mutant that constituted 3–5% of the cells in a mixed population. Inoculating each culture to be screened with at least 20 CFU allowed us to identify deletion mutants that occurred in M6 strain D471 at a frequency of  $\sim 1$  in  $2 \times 10^3$  CFUs. This is higher than most spontaneous mutation frequencies. We suggest that the deletions result from homologous recombination between long blocks (45 or 72 bases) of tandemly reiterated DNA within the structural gene for M6 (5). In agreement with this, we found that the deletions within the isolated mutants are located in the region of the molecule encoded by the repeat blocks: the *N*-terminal half of the molecule but not the *N*-terminal 20 amino acids. Experiments are in progress to further test this hypothesis.

The size of the pepsin fragments extracted from the deletion mutants suggests that the entire deletion is confined to this segment of the molecule. However, the size of the pepsin fragments from both deletion mutants (Fig. 4) are not exactly proportional to the size differences seen in the lysin-extracted molecule

(Fig. 3). This may be due either to the fact that the altered peptides run anomalously in SDS-PAGE, or that there has been a shift in the location of the pepsin-sensitive site caused by conformational changes induced by the deletions.

In spite of their frequent occurrence, deletion mutants are unlikely to be selected during routine laboratory manipulations designed to isolate a pure culture. There is no selective growth advantage for strains with either the wild type or smaller-sized M proteins (our unpublished data). Thus, for a deletion mutant to be randomly isolated by colony selection from a laboratory or throat culture, it would have to be present in the original culture at a high frequency ( $\geq 5\%$ ). This may explain our previous inability to observe these changes both in a mouse-passaged strain and in strains isolated from a small epidemic (4). Furthermore, based on the sensitivity of our system, in a culture started from a single CFU, only mutants arising before the third doubling would be identified. We conclude that the mutants we isolated in this study were present in the cultures used to inoculate the microtiter wells, and did not arise during growth in the wells.

No appreciable difference was observed in the ability of the deletion mutants to survive in human blood or to absorb M6 antibodies, although the M molecule had been reduced by as much as 9,000  $M_r$  from that of D471. Thus, the deletions occur in regions of the M protein molecule that do not appear to be necessary for its antiphagocytic function. This was also observed among the strains of M6 streptococci from our collection. All retained the ability to survive in human blood, although the M protein varied in  $M_r$  by almost 20,000 (4). Such results point to a functional domain near the N terminus of the M protein, which is not affected by the spontaneous size change.

The results of this study lend support to the idea that the strains exhibiting a different size M protein molecule within successive isolates from single patients (4 and our unpublished data) may actually be derived from the original infecting streptococcus, and are not the result of separate acquisitions of organisms having the same M type. Since each clinical isolate is derived from a single colony, the mutant with the variant M protein size seems to replace the original infecting strain within the in vivo population. This suggests that the mutant strain may have some selective advantage in the patient. The mechanism by which this occurs is currently under investigation.

### Summary

Streptococcal M protein, the antiphagocytic molecule on the surface of the organism, was previously found to exhibit extensive size heterogeneity between as well as within M serotypes. In this study, methods were devised to isolate M protein size mutants within a laboratory-grown culture. We were able to isolate three independent M protein deletion mutants and one additional mutant, which was derived from the first deletion mutant. We found that these deletion mutants occur at a frequency of  $\sim 1$  in  $2 \times 10^3$  CFUs in culture. Functional studies revealed that the deletion mutants were able to survive as well as the parental strain in human blood. They also had the determinants necessary to absorb opsonic antibodies as well as the parent. Pepsin digestion experiments localized the deletions within the N-terminal half of the M molecule, which is distal to the cell wall surface. This is the region of the molecule in which extensive sequence



repeats are found. This is consistent with the suggestion that the size changes may be the result of homologous recombination between the repeat regions in the gene. These results support the idea that strains showing M protein size variation within successive clinical isolates from single patients may be derived from the initial infecting organisms, and are not the result of separate unrelated acquisitions of the same serotype. This size change may be important in the survival of the streptococcus in vivo.

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