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Conversion of M Serotype 24 of *Streptococcus pyogenes* to M Serotypes 5 and 18: Effect on Resistance to Phagocytosis and Adhesion to Host Cells

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In this study, we utilized recombinant strains expressing the M5 and M18 proteins and M$^-$ mutants of group A streptococci to compare the abilities of these M proteins to confer resistance to phagocytosis and to mediate adhesion to host cells. The data indicate that the M5 and M18 proteins can confer resistance to phagocytosis, that fibrinogen is required for this resistance, and that these M proteins can mediate adhesion to HEp-2 cells.

The attachment of *Streptococcus pyogenes* to host cells and the survival of the organism once it has invaded deeper tissues are two characteristics that contribute to the virulence of this organism. One surface protein that has been functionally linked to both of these characteristics is the M protein. The M6 and M24 proteins mediate streptococcal adhesion to human cells (3, 11, 22) and confer resistance to phagocytosis in whole human blood (3, 12, 13, 19). Although the M5 protein expressed in *Streptococcus sanguis* conferred resistance to phagocytosis (15), it has not been confirmed that the M5 protein expressed in group A streptococci also confers resistance or mediates adhesion. Moses et al. (10) recently showed that expression of the M18 protein in *S. pyogenes* 87-282 was required for optimal resistance to phagocytosis. However, we found that inactivation of *emm18* in 87-282 had little effect on its survival in whole human blood (9). Thus, the role of the M18 protein in resistance to phagocytosis and in adhesion is not clear.

In this study, we investigated the abilities of the M5 and M18 proteins to mediate streptococcal adhesion to host cells and to confer resistance to phagocytosis and the role of fibrinogen in this resistance. To accomplish this goal, *emm5.1* from M type 5 *S. pyogenes* strain Manfredo (M5 Manfredo) and *emm18.1* from M type 18 *S. pyogenes* strain 87-282 were amplified by PCR and ligated into the temperature-sensitive plasmid pG+Host6. The resulting plasmids, pG+M5 and pG+M18, were electroporated into M24Ω, an M-negative isogenic mutant of M type 24 *S. pyogenes* strain Vaughn (M24 Vaughn) (3). Two clones, one that expressed M5 and one that expressed M18, were selected for further study and labeled 24-5 and 24-18, respectively. To verify that these clones expressed the correct M protein, the streptococci were immobilized on microtiter plates and reacted with specific antisera to N-terminal synthetic peptides as previously described (3). The preparation of rabbit antisera to N-terminal synthetic peptides of the M5 [SM5(1–35)] and M18 [SM18(1–30)] proteins has been described (5, 6). As expected, 24-5 and 24-18 did not react with the anti-M24 peptide serum, but they did react with anti-SM5(1–35) and anti-SM18(1–30), respectively (Table 1). Neither M24Ω nor M18Ω, an M-negative isogenic mutant of 87-282 whose construction and characterization will be described elsewhere (9), reacted with the respective type-specific antisera. The levels of antibody binding to M5 Manfredo and 24-5 were similar, as were those of antibody binding to 87-282 and 24-18, indicating that the quantities of M proteins expressed on their surfaces were similar. To confirm that the levels of expression of M proteins were similar, anti-SM5(235–264) serum, which is directed against the common C repeat domain of M proteins, was reacted with the streptococci in an enzyme-linked immunosorbent assay with streptococci in suspension as the solid phase. M24 Vaughn, M5 Manfredo, 87-282, 24-5, and 24-18 had optical densities at 450 nm (OD_{450}) of ~0.6, whereas M24Ω had an OD_{450} of less than 0.02. Western blots demonstrated that the M5 proteins expressed by 24-5 and by M5 Manfredo exhibited the same electrophoretic mobility. Similarly, 24-18 and 87-282 expressed M18 proteins of the same size. The recombinant strains 24-5 and 24-18 grew in whole human blood, whereas M24Ω failed to multiply in human blood. Furthermore, 24-5 and 24-18 were opsonized by antiserum to pepsin-extracted M5 protein and antiserum to pepsin-extracted M18 protein, respectively. These data indicate that 24-5 and 24-18 express the appropriate M proteins and that their expression confers resistance to phagocytosis.

The binding of fibrinogen was also investigated because it is associated with resistance to phagocytosis (15, 24–26), increased virulence of group A streptococci (17, 18, 20), and adhesion of streptococci to virus-infected cells (16). Fibrinogen was radiolabeled with [^{3}H]formaldehyde (specific activity of 4,100 cpm/μg) as previously described (24). Fibrinogen was added to 500-μl cultures of streptococci at an OD_{530} of 0.2, and the mixtures were rotated for 30 min. The bacteria were washed three times in phosphate-buffered saline containing 0.05% Tween 20, and the amount of fibrinogen bound was determined by scintillation spectroscopy. M24 Vaughn bound 10.29 μg of fibrinogen, 24-5 bound 6.65 μg, 24-18 bound 1.56 μg, and M24Ω bound 0.08 μg. The ability of these recombinant strains to multiply in whole blood was dependent on the presence of fibrinogen (Table 2). Even 24-18, which bound dramatically less fibrinogen than 24-5 and M24 Vaughn, was dependent on the presence of fibrinogen for growth in whole blood. That this low level of fibrinogen binding to 24-18 still confers resistance to phagocytosis is not surprising, since Whitnack et al. (26) showed that as little as 1/10 of the maximal level of fibrinogen binding to M24 Vaughn can confer resistance. The data for the M5 and M24 proteins are consistent with

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TABLE 1. Surface expression of M proteins by *S. pyogenes* strains[a]

Strain	A_{450} obtained by ELISA[b] with:			
	NRS[c]	Anti-SM18(1–30)	Anti-SM5(1–35)	Anti-M24 peptide
M5 Manfredo	0.132 ± 0.022	NT[d]	1.261 ± 0.181	NT
M24 Vaughn	0.070 ± 0.006	0.131 ± 0.015	0.079 ± 0.020	3.102 ± 0.035
87-282	0.073 ± 0.014	1.678 ± 0.100	NT	NT
M24Ω	0.056 ± 0.012	0.038 ± 0.009	0.081 ± 0.023	0.032 ± 0.010
M18Ω	0.064 ± 0.012	0.095 ± 0.009	NT	NT
24-5	0.078 ± 0.014	NT	1.474 ± 0.188	0.048 ± 0.00
24-18	0.092 ± 0.017	1.825 ± 0.066	NT	0.004 ± 0.003

[a] Streptococci were immobilized on microtiter wells and reacted with a 1:1,000 dilution of the indicated antiserum, followed by reaction with a 1:2,000 dilution of peroxidase-labeled goat anti-rabbit immunoglobulin G. The substrate tetramethyl benzidine was added and the absorbance at 450 nm was recorded. The values are the means ± standard deviations for quadruplicate wells.
[b] ELISA, enzyme-linked immunosorbent assay.
[c] NRS, normal rabbit serum.
[d] NT, not tested.

previous reports indicating that the binding of fibrinogen to these M proteins can prevent opsonization and phagocytosis (15, 25).

The M5 and M18 proteins also serve as adhesins in streptococcal attachment to HEp-2 cells. M24 Vaughn, 24-5, 24-18, and 87-282 attached to HEp-2 cells in similar numbers, whereas the levels of adhesion for M24Ω and M18Ω were dramatically reduced (Fig. 1). However, the adhesion of M18Ω was higher than that of M24Ω, suggesting that 87-282 might utilize an additional adhesin. The mechanism(s) whereby these M proteins mediate adhesion is not completely understood. A fucosylated glycoprotein on HEp-2 cells (21) and the membrane cofactor protein CD46 on keratinocytes (11) have been proposed as receptors for the M6 protein. The M6 protein appears to mediate attachment to keratinocytes by interactions between its C repeat domain and CD46 (11). The C repeats of M proteins do not appear to be involved in streptococcal adhesion to HEp-2 cells, because pepsin-extracted M protein, which lacks C repeats, blocks adhesion of streptococci to HEp-2 cells (1). While M proteins mediate adhesion to certain types of host cells, other streptococcal adhesins may also be involved (2, 4, 8).

In the present study, we have shown that the M5 and M18 proteins confer resistance to phagocytosis when expressed on the surface of the M-negative type 24 strain. Moses et al. (10) recently found that the growth of strain 87-282 in whole blood was dependent on expression of the M18 protein. However, in a related study (9), we found that inactivation of *emm18* in strain 87-282 had little to no effect on growth in blood. The basis for these different findings is not clear. One possible explanation is that there is an additional survival factor that may be variably expressed in the genetic background of 87-282 (9). The fact that the binding of fibrinogen to the M18 protein in strain 87-282 is not required for growth in blood (7), while the growth of 24-18 is dependent on fibrinogen binding to the M18 protein, supports the concept that the genetic background of the host strain may influence the functional contribution of M proteins to virulence of the organism. Others have also found that surface components of group A streptococci besides M protein can contribute to survival of the organisms in blood. The hyaluronate capsule (7, 23) and M-like proteins (14) can contribute to survival of the organisms in whole blood. Taken together, these results suggest that the relative contributions of M proteins to survival of group A streptococci in blood may depend on the genetic background of the host strain and coexpression of other virulence factors.

TABLE 2. Role of M protein and fibrinogen in group A streptococcal resistance to phagocytosis

Strain	Inoculum size (CFU)	CFU of streptococci grown with human blood cells reconstituted with[a]:		
		Plasma	Serum	Serum + fibrinogen
M24 Vaughn	39	>10,000	110	>10,000
M24Ω	147	0	0	0
24-5	42	>10,000	0	>10,000
24-18	132	>10,000	376	>10,000

[a] Human blood cells from a nonimmune donor were washed and resuspended in plasma, serum, or serum plus fibrinogen. The streptococci were added to the mixtures, the mixtures were rotated for 3 h at 37°C, and the total numbers of CFU were determined by plating on blood agar.

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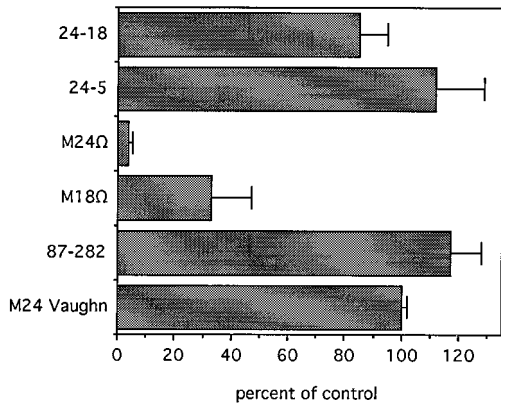

FIG. 1. Role of M proteins in streptococcal adhesion to HEp-2 tissue culture cells. The indicated streptococci were incubated with HEp-2 cells, nonadherent streptococci were removed by washing, and the adherent bacteria were stained with Giemsa and counted by light microscopy. The mean level of adhesion of M24 Vaughn was 34 bacteria per cell. Adhesion of M24 Vaughn was used as the control and set at 100%. Each experiment was done in triplicate. Means plus standard deviations are shown.

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