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# Hyaluronate Capsule and Surface M Protein in Resistance to Opsonization of Group A Streptococci

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**The major virulence determinant of group A streptococci is the ability to resist opsonization and phagocytic ingestion. The present studies were performed to compare the mechanisms of resistance to opsonization of type 18 and type 24 streptococci and to determine the relative roles of M protein-fibrinogen interaction and the hyaluronate capsule in preventing phagocytic ingestion and killing. By use of parent strains and acapsular transposon mutants in the presence and absence of fibrinogen, we show that type 18 and type 24 streptococci rely on somewhat different mechanisms for resistance to opsonization. Type 24 streptococci bound fibrinogen avidly to their surfaces, and encapsulated organisms were completely resistant to opsonization only in the presence of fibrinogen. In contrast, type 18 streptococci bound 10-fold less fibrinogen than type 24 streptococci and were fully resistant to phagocytosis only when they expressed capsule. The general structural characteristics of the amino-terminal halves of type 18 and type 24 M proteins differed in that type 18 M protein contained only one complete B repeat, whereas type 24 M protein contained five complete B repeats, a structural difference which could potentially be related to the differences in fibrinogen binding between the two serotypes. Immunofluorescence assays of complement deposition were used in combination with $^{125}$I-C3 binding assays to show that encapsulated type 24 streptococci were fully resistant to opsonization by C3 only in the presence of plasma. Encapsulated and unencapsulated type 18 streptococci were equally opsonized by C3 in either plasma or serum, yet only encapsulated organisms resisted phagocytic killing in blood. The results of this study indicate that opsonization by C3 does not necessarily lead to phagocytic ingestion and that the hyaluronate capsule and M proteins are variably important in resistance of different group A streptococci to opsonization and phagocytic killing.**

Group A streptococcal infections are responsible for a variety of clinical syndromes, ranging from uncomplicated pharyngitis to life-threatening invasive disease. A major correlate of virulence of these organisms is their ability to resist phagocytosis in the nonimmune host (19). For many years, it was believed that the M protein on the surface of group A streptococci was the primary determinant of resistance to phagocytosis and virulence (20). Some strains of streptococci also express a hyaluronic acid capsule in varying amounts, which by steric interactions, repulsion from a net negative charge, or other mechanisms could interfere with phagocytic ingestion (36). Early studies attempted to dissociate the antiopsonic effects of M protein and capsule (10, 18, 27), with conflicting results. Some studies utilized naturally occurring capsule-negative variants, which also may have expressed smaller amounts of M protein (10). Alternatively, encapsulated strains were decapsulated by treatment with hyaluronidase preparations that may not have quantitatively removed the capsule or that may have been contaminated with proteases (18, 27).

During the recent resurgence of rheumatic fever and life-threatening group A streptococcal infections, many of the streptococcal isolates recovered from patients and their contacts have been mucoid (17), a colony phenotype associated with the production of large hyaluronate capsules. This finding stimulated a renewed interest in the role of the capsule in virulence. Recent studies by Wessels et al. (33) showed that an acapsular transposon mutant of type 18 streptococcus no longer resisted phagocytosis in vitro and was significantly less virulent in vivo than the encapsulated parent. This finding contrasted with earlier studies by Whitnack and Beachey (34, 35), who showed that encapsulated type 24 streptococci were fully resistant to phagocytosis only in the presence of fibrinogen (34), which binds specifically to M protein (35) and blocks activation of the alternate complement pathway (34).

The present studies were undertaken to more clearly define the roles of the hyaluronate capsule and the surface M protein in preventing opsonization and phagocytosis of group A streptococci. We used encapsulated parent strains and unencapsulated transposon mutants of type 18 and type 24 streptococci in the presence and absence of fibrinogen to show that the two serotypes use different mechanisms to avoid phagocytic ingestion and killing. The results of qualitative and quantitative complement binding assays indicated that deposition of C3 on the surface of encapsulated streptococci was necessary but not always sufficient to promote phagocytic killing. Our results suggest that different group A streptococci may have evolved different mechanisms of survival in the nonimmune host.

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## MATERIALS AND METHODS

**Opsonization assays.** In vitro opsonization assays were performed with whole, heparinized (10 U/ml) blood (2) or blood that was reconstituted with one part fresh serum containing heparin and one part cells that had been washed two

TABLE 1. Effect of fibrinogen on the association of type 24 and type 18 streptococci with PMNs

| Blood reconstituted with: | Final concn of fibrinogen (μM) | % PMNs with associated streptococci | |
|---|---|---|---|
| | | Type 24 | Type 18 |
| Plasma | | 4 | 8 |
| Serum | 0 | 56 | 2 |
| | 0.01 | 40 | 4 |
| | 0.1 | 8 | 2 |
| | 1.0 | 2 | 2 |
| Plasma + M antibody | | 98 | 96 |

times in Hanks' balanced salt solution. A standard inoculum of streptococci (2) grown to the log phase was added to 0.4 ml of blood which was rotated end over end for 30 min at 37°C. At the end of the rotation, smears were made on glass slides and stained with Wright's stain. Opsonization was quantitated by counting 50 neutrophils per slide and calculating the percent neutrophils with associated streptococci (phagocytic index) (2). Phagocytic killing of the organisms in blood was determined by a similar assay (20), except that fewer streptococci were added to the mixture, which was rotated at 37°C for 3 h. At the end of the rotation, 0.1-ml aliquots were added to melted sheep blood agar and pour plates were made to quantitate viable organisms. All in vitro opsonization assays were performed at least three different times, and the results presented are representative of all experiments.

**Acapsular transposon mutants of type 18 and type 24 streptococci.** The acapsular transposon mutants of type 18 (strain 87-282) and 24 (Vaughn) streptococci, TX72 and 24-72, respectively, have been described previously (31, 32). The acapsular mutants have been shown to express quantities of M protein similar to those of the parent strains (31, 32).

**Fibrinogen binding assays.** Human fibrinogen (grade L; KABI Diagnostics, Stockholm, Sweden) was dissolved in and dialyzed against phosphate-buffered saline (PBS) prior to use. Fibrinogen was radiolabeled by reductive N-methylation with [$^3$H]formaldehyde, as described previously (35). Assays of the binding of $^3$H-fibrinogen to type 18 and type 24 streptococci were performed as described before (35). Nonspecific binding was determined with a concentration of 200 nM $^3$H-fibrinogen and a 50-fold excess of unlabeled fibrinogen. Precipitation of fibrinogen prevented the use of the higher concentrations which would be necessary to evaluate nonspecific binding at the physiologic levels of $^3$H-fibrinogen used in these assays.

The binding of fibrinogen to pepsin extracts of M24 and M18 was determined by dot blot assays on nitrocellulose paper. A constant amount of M protein (100 μg) was added to each well of the dot blot apparatus and washed with PBS, and twofold decreasing concentrations of fibrinogen (highest concentration, 10 μM) were added. Bound fibrinogen was detected by incubating the nitrocellulose paper in peroxidase-conjugated goat anti-human fibrinogen followed by horseradish peroxidase substrate.

**Pepsin extraction and purification of M proteins.** M protein fragments were purified from limited pepsin digests (pep M) of intact type 18 and 24 streptococci, as described previously (2). The pep M24 was judged to be homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Although the pep M18 fraction was not homogeneous by SDS-PAGE, the major band (molecular mass, ~27 kDa) in the preparation reacted with rabbit antiserum raised against the recombinant M18, as determined by immunoblot analysis (5).

**Cloning and sequencing of the *emm18* gene.** The putative *emm18* gene was cloned by PCR techniques essentially as described by Podbielski et al. (26). Oligonucleotide primers copying the conserved 5′ noncoding region (MP-2) and the 3′ end of the structural gene (MP-1) were synthesized to contain 5′ *Eco*RI and *Pst*I sites, respectively. The PCR, purification of the PCR product, and ligation into the expression plasmid pKK223-3 were accomplished as described previously (5). Transformed *Escherichia coli* (JM105) organisms were screened for expression of type 18 M protein by colony blot assays using opsonic rabbit antiserum evoked by pep M18 (2). The *emm18* gene was sequenced by the dideoxynucleotide chain termination method (28).

**C3 binding assays.** Purified human complement component C3 (12) (kindly provided by Michael Pangburn, University of Texas, Tyler) was radiolabeled with $^{125}$I (Amersham Corp., Arlington Heights, Ill.) by use of *N*-chloro-benzenesulfonamide-derivatized polystyrene beads (Iodobeads; Pierce Chemical Co.) to a specific activity of $5 \times 10^5$ cpm/μg. Binding of $^{125}$I-C3 to streptococci was measured in 30% human serum or plasma as described previously (22). Higher concentrations of serum or plasma resulted in a large increase in nonspecific binding of C3 (i.e., binding in the presence of EDTA) and only a small increase in specific binding. The ratio of $^{125}$I-C3 to unlabeled C3 in the reaction mixtures was approximately 1:180. Briefly, streptococci ($4 \times 10^7$ CFU) were incubated with 30% serum or plasma containing $^{125}$I-C3 for 30 min at 37°C with end-over-end rotation. Control tubes to measure nonspecific binding contained 10 mM EDTA in addition to the other components. After incubation, unbound C3 was removed by centrifugation of the assay mixture through a bed of 15% sucrose. Specific binding was calculated by subtracting radioactivity bound in the presence of EDTA from total binding.

**Indirect immunofluorescence of C3 binding to streptococci.** Patterns of C3 binding to the surface of type 18 and type 24 streptococci were determined by immunofluorescence microscopy. Bacteria were incubated in either fresh serum or plasma at 37°C for 20 min and washed in PBS. The organisms were then incubated with fluorescein-labeled goat anti-human C3 (1:1,000; Cappel, West Chester, Pa.) at 37°C for 15 min. Negative controls were prepared with second antibody only. After washing in PBS, a drop was spread on a glass slide, lightly heat fixed, and mounted with 1 drop of 1% Gelvatol (Monsanto, Springfield, Mass.; pH 7.0) and a coverslip. Photomicrographs were made with a Zeiss Axiophot fluorescence microscope.

**Electron microscopy.** Electron microscopy was performed with type 18 and 24 streptococci that had been incubated in fibrinogen (10 μM) or PBS. The organisms were fixed in 2.5% glutaraldehyde–0.1% sodium cacodylate at pH 7.2 and postfixed in osmium tetroxide, dehydrated in alcohol, and embedded in Spurr's medium for transmission electron microscopy (1).

**Nucleotide sequence accession number.** The *emm18* gene sequence has been deposited in GenBank under the accession number U29585.

## RESULTS

**Comparison of the effects of fibrinogen on the phagocytic index of type 18 and type 24 streptococci.** Because previous studies had shown that encapsulated type 18 streptococci were resistant to phagocytosis in the absence of fibrinogen (33), we performed in vitro opsonization assays with blood reconstituted with either serum or plasma and quantitated the percentage of polymorphonuclear leukocytes (PMNs) associated with encapsulated type 18 and type 24 streptococci (phagocytic index) (Table 1). This assay does not distinguish between bacteria internalized in neutrophils and those attached but not ingested. Therefore, the results should be considered an index of total association of neutrophils with bacteria rather than a measure of phagocytic killing per se. Type 18 streptococci were not associated with PMNs after rotation in blood reconstituted with either plasma or serum (Table 1). However, significant association of type 24 streptococci with PMNs occurred in test mixtures containing serum but not in mixtures containing plasma. The ability of type 24 streptococci to resist association with PMNs increased with increasing concentrations of fibrinogen added to serum (Table 1). These data indicate that type 24, but not type 18, streptococci depend upon the presence of fibrinogen for complete resistance to association with PMNs in nonimmune blood. In the presence of type-specific M antibodies, both serotypes showed maximum phagocytic indices (Table 1).

**Comparison of the roles of hyaluronate capsule in preventing phagocytosis of type 18 and type 24 streptococci.** To determine the role of capsule in resistance to phagocytosis, in vitro opsonization assays were performed with encapsulated parent and unencapsulated transposon mutants of type 18 and type 24 streptococci. In vitro opsonization assays again showed that the type 24 parent strain had a low phagocytic index only in plasma, not in serum (Table 2). The unencapsulated type 24 strain, 24-72, was associated with PMNs in blood containing either plasma or serum. The type 18 parent strain had a low

TABLE 2. Association with PMNs of encapsulated parent and unencapsulated transposon mutants of type 18 and type 24 streptococci in whole blood containing plasma or serum

| Rotation mixture | % PMNs with associated streptococci: | | | |
|---|---|---|---|---|
| | Type 24 | Strain 24-72 | Type 18 | Strain TX72 |
| Blood cells + plasma | 2 | 44 | 2 | 62 |
| Blood cells + serum | 58 | 66 | 4 | 64 |

TABLE 3. Survival of encapsulated and unencapsulated type 18 and type 24 streptococci in human blood containing either plasma or serum

| Rotation mixture | No. of CFU surviving 3-h rotation | | | |
|---|---|---|---|---|
| | Type 24 (21 CFU)[a] | Strain 24-72 (48 CFU) | Type 18 (58 CFU) | Strain TX72 (28 CFU) |
| Blood cells + plasma | >10,000 | 5,920 | >10,000 | 3,120 |
| Blood cells + serum | 1,460 | 480 | >10,000 | 3,400 |
| Cell-free control[b] | >10,000 | >10,000 | >10,000 | >10,000 |

[a] Values in parentheses indicate sizes of inocula.
[b] Organisms were grown in plasma alone for 3 h at 37°C; this served as a control for maximum growth.

phagocytic index in blood containing either plasma or serum (Table 2). However, the unencapsulated type 18 mutant, TX72, was associated with PMNs in blood containing either plasma or serum.

The results of in vitro opsonization assays were confirmed by use of growth in blood as an indication of resistance to phagocytic killing (Table 3). In mixtures containing plasma, the encapsulated strains of type 24 and type 18 streptococci grew maximally. In mixtures containing serum, type 24 streptococci showed a significant reduction in growth, but the growth of type 18 was maximal. Unencapsulated type 24 streptococci (strain 24-72) were moderately resistant to phagocytosis in blood containing plasma and even less resistant in blood containing serum (Table 3). Unencapsulated type 18 streptococci also were only moderately resistant to phagocytosis in blood containing either plasma or serum. Previous studies using a slightly different assay found impaired resistance to phagocytosis in whole blood of both unencapsulated type 18 and 24 streptococci (31, 32). The net growth of both encapsulated and unencapsulated streptococci was somewhat greater in the current studies, which utilized conditions promoting optimal growth of the bacteria. These data suggest that type 24 streptococci are dependent upon fibrinogen binding to M protein and upon expression of capsule for optimal growth in blood. In contrast, type 18 streptococci are dependent primarily upon expression of capsule, and the presence or absence of fibrinogen appears to have little effect upon growth or resistance to phagocytosis.

**Fibrinogen binding to type 18 and type 24 streptococci.** The results reported above suggested either that type 18 and type 24 streptococci bound different amounts of fibrinogen or that the surface-bound fibrinogen resulted in functionally different effects on complement activation and deposition. To answer this question, we first performed transmission electron microscopy with type 18 and type 24 streptococci that had been preincubated with fibrinogen or buffer alone (Fig. 1). Both untreated organisms showed the characteristic surface fibrillae (Fig. 1A and C). Type 24 streptococci that had been incubated with fibrinogen showed a very heavy, electron-dense coat of surface-bound fibrinogen (Fig. 1B). Fibrinogen-treated type 18 streptococci showed much less deposition of electron-dense material, and the surface of the organisms partially retained the characteristic fibrillar structure (Fig. 1D).

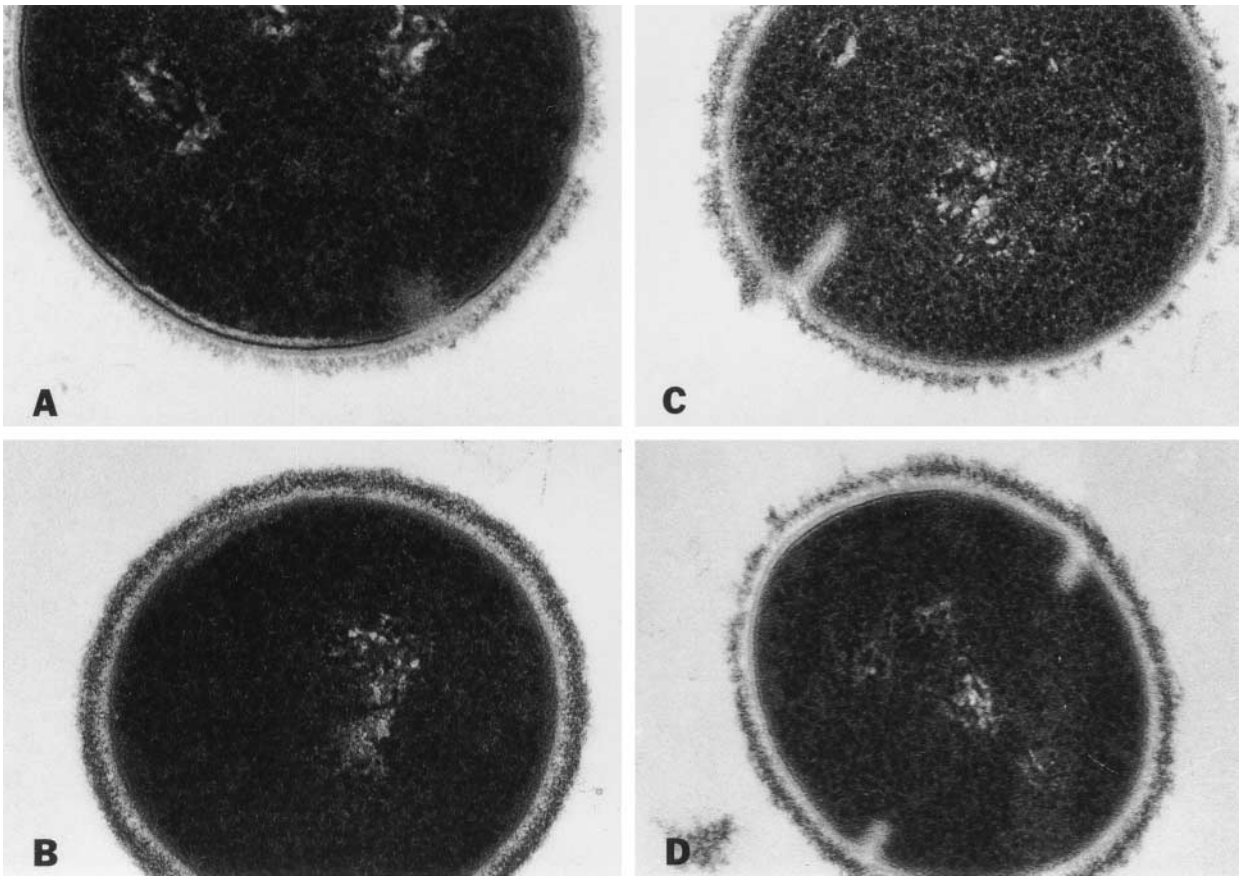


FIG. 1. Transmission electron micrographs of type 24 (A and B) and type 18 (C and D) streptococci incubated in PBS (A and C) or 10 μM fibrinogen (B and D).

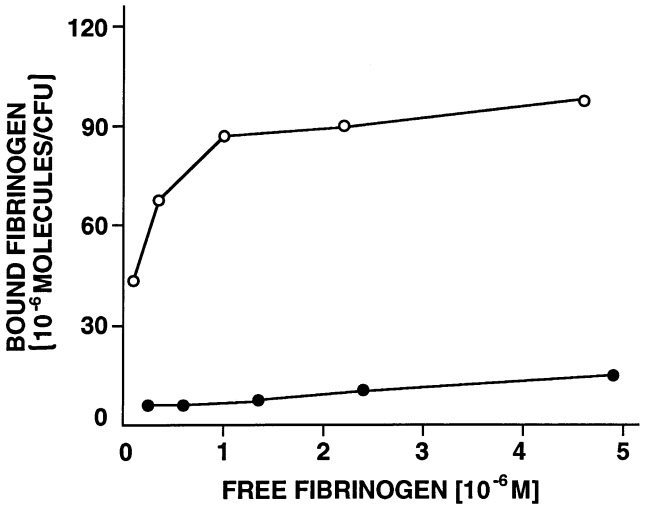


FIG. 2. Binding of $^{3}$H-fibrinogen to type 18 (●) and type 24 (○) streptococci.

Quantitative assays of $^{3}$H-fibrinogen binding were performed with type 18 and type 24 streptococci grown to the mid-log phase (Fig. 2). Binding was concentration dependent, and type 24 streptococci bound ~10-fold more fibrinogen than did type 18 streptococci (Fig. 2). Nonspecific binding of $^{3}$H-fibrinogen at a concentration of 200 nM was estimated in the presence of a 50-fold excess of unlabeled fibrinogen and found to be 3.2% for type 24 streptococci and 5.2% for type 18 streptococci. Thus, the data shown (Fig. 2) represent the total fibrinogen bound to each serotype.

To determine whether the differences in fibrinogen binding to intact type 18 and type 24 streptococci were due to differences in M protein affinity for fibrinogen, purified pepsin-extracted M proteins were used in dot blot assays of fibrinogen binding. Although the assay is only semiquantitative, pep M24 appeared to bind much more fibrinogen than did pep M18 (Fig. 3).

**Comparison of *emm18* and *emm24* structures.** To determine whether there were any obvious structural differences between M18 and M24 that may account for differences in fibrinogen binding, we sequenced the *emm18* gene and compared the deduced protein structure with the known structure of type 24 M protein (23). The predicted M18 was 443 amino acids in length and had a calculated molecular mass of 49.3 kDa and a signal peptide with an assumed length of 42 amino acids that was highly homologous to those of other M proteins (11). To confirm that the cloned gene actually encoded the M18 protein, we synthesized a peptide copying the amino-terminal 30 amino acids and immunized three rabbits with 300 μg of the peptide covalently linked to keyhole limpet hemocyanin (6). Rabbit anti-SM18(1-30) opsonized type 18 streptococci in opsonophagocytosis assays (data not shown), indicating that *emm18* encodes a protein that meets the functional definition of an M protein.

The primary structure of M18 (Fig. 4) showed the 7-residue periodicity in the placement of nonpolar amino acids that is characteristic of M proteins (25). Interestingly, M18 contains five degenerate A repeats that are related to those of type 5 M protein (7). M18 also contains 1.3 B repeats that are also structurally related to the B repeats of M5 (7). A comparison of the sequences of the amino-terminal halves of M18 and M24 revealed no significant homology. Although the two M proteins each contain related C repeats in the middle of the molecules, the most significant difference in general structural characteristics between M18 and M24 is the number of B repeats. M24 contains five complete B repeats (23) compared with only one complete B repeat in M18.

**Quantitative and qualitative differences in C3 binding to type 18 and type 24 streptococci.** We performed experiments to determine the quantity and the pattern of deposition of C3 on encapsulated and unencapsulated type 18 and type 24 streptococci in either serum or plasma. Encapsulated type 18 streptococci bound a moderate amount of C3 (9,250 ± 2,000 molecules per CFU) in serum and 35% less in plasma (Table 4). The unencapsulated type 18 strain TX72 bound slightly less C3 than the type 18 parent strain did, although the relative amounts of C3 bound in serum and plasma were similar. The most striking difference between C3 deposition in serum and that in plasma was observed with encapsulated type 24 streptococci, which bound 25,700 ± 9,100 molecules of C3 per CFU in serum but 86% less in plasma. In contrast, C3 deposition on the unencapsulated type 24 strain in plasma was reduced only 34% compared with that in serum. The increased susceptibility of type 24 streptococci to phagocytosis in serum relative to that in plasma was reflected in increased C3 binding in serum, but this effect was largely abrogated by loss of the hyaluronic acid capsule. In contrast, type 18 streptococci, which appeared similarly susceptible to phagocytosis in serum or plasma, bound similar amounts of C3 in serum and plasma, and C3 binding was not substantially altered by loss of capsule expression. Similar results were obtained when these experiments were performed independently in another laboratory by slightly different methods (3) (data not shown).

Indirect immunofluorescence assays were performed to assess the patterns of C3 deposition on both serotypes in plasma and serum (Fig. 5). As previously shown by Whitnack and Beachey (34), type 24 streptococci incubated in serum bound C3 in a smooth, circumferential pattern (Fig. 5A). When the streptococci were incubated in plasma, very little C3 was deposited on type 24 streptococci (Fig. 5B). Type 18 streptococci showed the same pattern of C3 deposition in both serum (Fig. 5C) and plasma (Fig. 5D), in which some C3 was deposited circumferentially and some only at cross walls. In agreement with the quantitative C3 binding studies, the patterns of C3 deposition on unencapsulated type 24 streptococci (strain 24-72) were similar in plasma and serum (Fig. 5E and F). Unencapsulated type 18 streptococci showed a pattern of C3 deposition similar to that of the parent organism, and again there was no difference in the patterns in serum or plasma (Fig. 5G and H). These observations are consistent with the quantitative C3 binding assays described above (Table 4).

## DISCUSSION

The results of the present study indicate that different serotypes of group A streptococci may utilize different strategies to

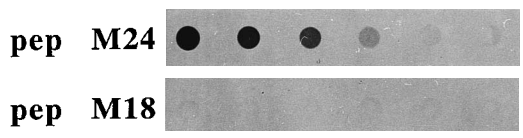


FIG. 3. Dot blot analysis of fibrinogen binding to purified pepsin extracts of type 24 (pep M24) and type 18 (pep M18) streptococcal M proteins. One hundred micrograms of pep M protein was applied to each well and then incubated with twofold decreasing concentrations of fibrinogen (highest concentration, 10 μM). Peroxidase-conjugated goat anti-human fibrinogen followed by substrate was used to develop the binding reactions.

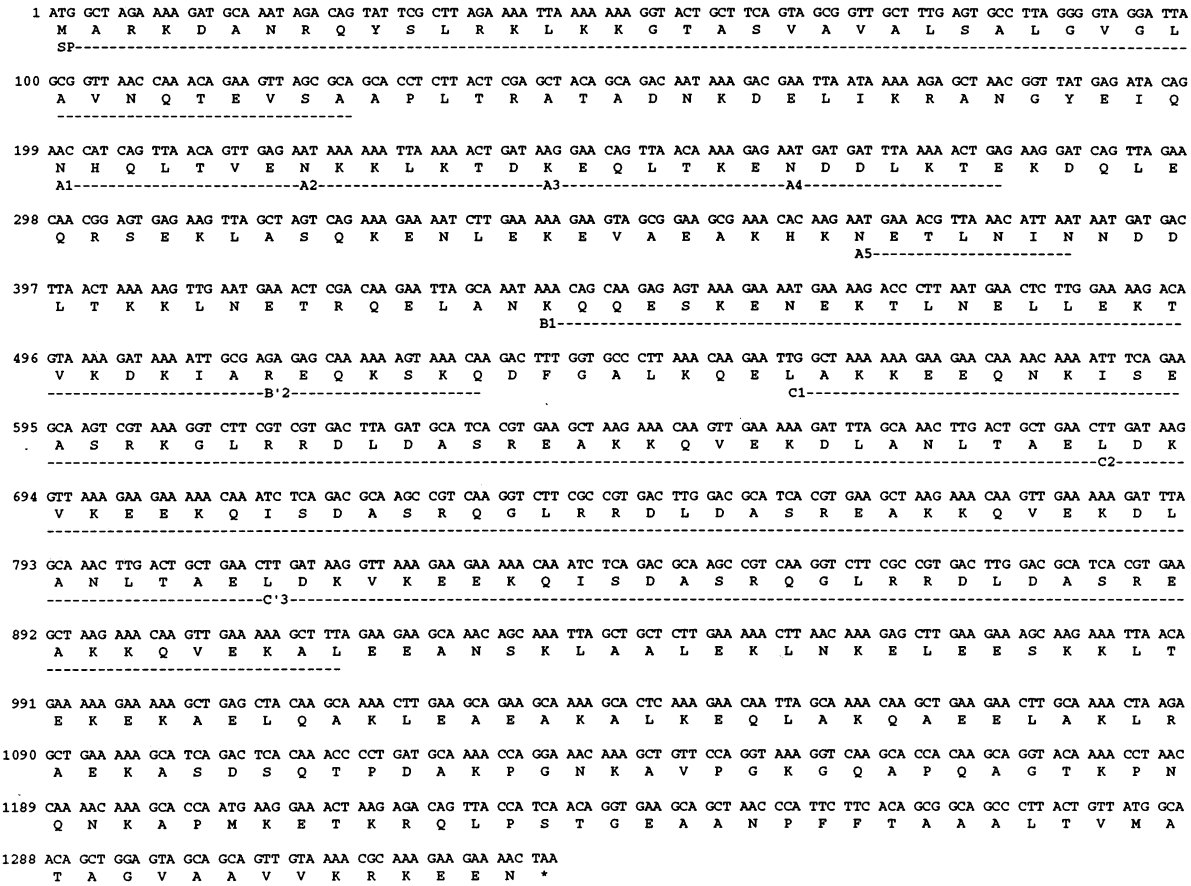


FIG. 4. Primary structure of type 18 M protein deduced from the *emm18* gene sequence. The signal peptide is assumed to be 42 amino acids in length. A, B, and C repeats are indicated.

prevent phagocytic ingestion and killing. Type 24 streptococci are fully resistant to phagocytosis only in blood containing fibrinogen, whereas type 18 streptococci bind significantly less fibrinogen and are fully resistant to phagocytosis in its absence. For both serotypes, the hyaluronate capsule is required for full resistance to phagocytosis, as measured by growth in whole blood. We recognize that our results do not allow a general conclusion regarding differences in resistance to opsonization between all type 24 and type 18 streptococci because only a single representative strain of each serotype was used in these studies. It has previously been shown by Fischetti et al. (9) that M proteins from different strains within the same serotype may show variations in size which result from deletions or additions of internal repeating structures. The different functional characteristics of M24 and M18 proteins, particularly related to fibrinogen binding, could be serotype specific, but it is also possible that they only reflect differences among strains of the same serotype. Further studies of additional strains within each serotype are necessary to draw general conclusions about virulence mechanisms used by different serotypes.

Although we have identified differences in the ways in which strains of type 18 and type 24 streptococci prevent opsonization, our studies also raise some unanswered questions that illustrate the complexities of the bacterial cell surface. First, the amounts and patterns of C3 deposited on the surface of encapsulated and unencapsulated type 18 streptococci were not significantly different. However, the unencapsulated mutant was unable to resist phagocytosis, suggesting that the hyaluronate capsule may serve as a physical barrier that prevents C3b bound to the bacterial surface from interacting with the phagocyte receptor. This hypothesis is consistent with previous observations of encapsulated *Staphylococcus aureus* cells that were not phagocytosed (24) despite deposition of C3 on the bacterial surface, indicating that the two processes are not always concordant. This observation raises another question related to the function of the hyaluronate capsule on type 24

TABLE 4. C3 deposition on group A streptococcal strains incubated in human serum or plasma

| Strain | Condition | C3 bound[a] | % PMNs with associated streptococci[b] |
|---|---|---|---|
| Type 24 | Serum | — | 58 |
| | Plasma | 14 ± 18 | 2 |
| 24-72 | Serum | 110 ± 17 | 66 |
| | Plasma | 66 ± 18 | 44 |
| Type 18 | Serum | — | 4 |
| | Plasma | 65 ± 21 | 2 |
| TX72 | Serum | 60 ± 29 | 64 |
| | Plasma | 42 ± 43 | 62 |

[a] Binding is expressed as percent cpm bound (mean ± standard deviation) to the parent strain (type 18 or type 24) in serum in the same experiment. Each value represents the mean of at least four determinations in a minimum of two experiments. —, 100% bound.

[b] Data are from Table 2 and are shown again for comparison to C3 binding.

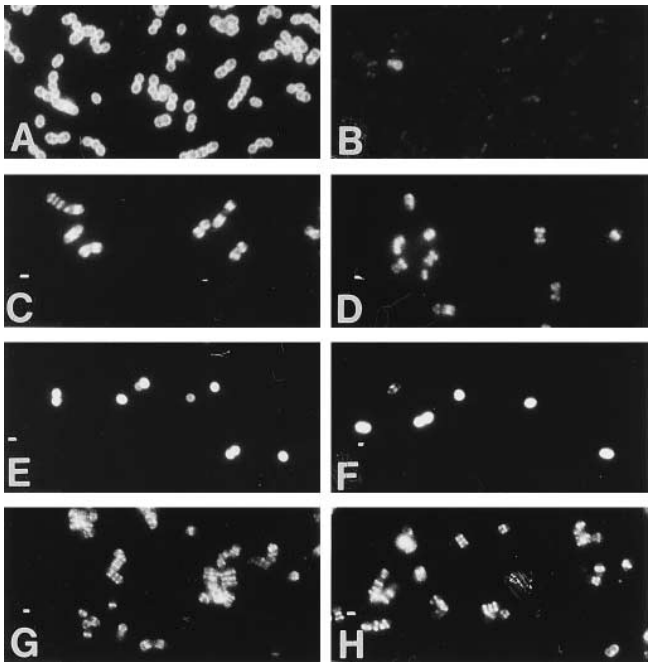


FIG. 5. Immunofluorescence analysis of C3 binding to type 18 and type 24 streptococci. Type 24 (A, B, E, and F) and type 18 (C, D, G, and H) streptococci were incubated in either fresh serum (A, C, E, and G) or plasma (B, D, F, and H) and then incubated in fluorescein-labeled goat anti-human C3. Encapsulated type 24 (A and B), unencapsulated type 24 (strain 24-72) (E and F), encapsulated type 18 (C and D), and unencapsulated type 18 (strain TX72) (G and H) are shown.

streptococci. The type 24 Vaughn strain appears to express capsule in quantities similar to those of the encapsulated type 18 strain, as determined by India ink preparations and quantitative chemical analyses performed previously (31–33). However, the capsule of type 24 streptococci does not appear to function as a physical barrier to phagocyte binding as it does with type 18 streptococci. Encapsulated type 24 streptococci that are opsonized in serum are more susceptible to phagocytosis and killing than are (similarly encapsulated) type 18 streptococci. The combination of capsule expression, the absolute quantity of C3b on the surface of the organism, and, as shown previously (16), the pattern of C3 deposition may all be important determinants of resistance to phagocytosis and may differ among different strains of group A streptococci.

Another difference between these two organisms is that unencapsulated type 24 streptococci incubated in plasma bound C3b in a circumferential pattern, similar to the pattern observed when the parent strain was incubated in serum. The unencapsulated strain expresses M protein in quantities similar to those of the parent (31), yet even when the bacteria were incubated in fibrinogen (plasma), the absence of capsule influenced the pattern of C3 binding. In contrast, with type 18 streptococci, the patterns of C3 binding were qualitatively the same on the parent and mutant in either serum or plasma. The reason for the two patterns of C3 deposition on type 18 streptococci, some at cross walls only and some in a circumferential pattern, is not clear. The experiments were performed by use of log-phase organisms originally grown from single colonies. Thus, the phenotypic differences may be related to the different ages of individual cocci in the culture.

Earlier studies have identified differences in opsonic requirements between highly encapsulated and unencapsulated group A streptococci (13, 29). Hirsch and Church (13) identified a plasma factor present in human blood but not rabbit blood that was necessary for effective phagocytic killing of highly encapsulated streptococci. Stollerman et al. (29) extended this observation and showed that some individuals lacked the accessory plasma factor that was needed for the opsonic effect with highly encapsulated strains. We also noted that blood from some donors was not sufficient to promote optimal killing of type 18 streptococci but the same blood was effective in killing type 24 streptococci. To avoid complicating the interpretation of the results of this study, we did not use blood donors that had deficient opsonic activity against type 18 streptococci. It should be noted that the accessory plasma factors reported in these earlier studies were required for M antibody-mediated bactericidal activity (13, 29). Nevertheless, the results point to important differences in requirements for opsonization and phagocytosis between different serotypes of group A streptococci.

Recent studies using type 3 streptococci support the concept that there may be a range of mechanisms used by group A streptococci to prevent opsonization (4). Type 3 organisms activated only minimal levels of complement in serum via the alternate pathway. The addition of fibrinogen to opsonization assays reduced the levels of opsonization of type 3 streptococci (4) but not to the same levels observed for type 24 streptococci in the present studies. Type 3 streptococci also bind fibrinogen in amounts that are intermediate between those of type 18 and type 24 streptococci (our unpublished data).

Previous studies have indicated that there are several potential mechanisms by which group A streptococci prevent effective opsonization, phagocytosis, or both (8, 14, 15). M protein (14) and fibrinogen (15) have been shown to bind factor H, which may limit the amount of C3b deposited on the bacterial cell surface (15). The combined effects of fibrinogen binding to M protein and factor H binding may explain the almost total absence of C3b on the surface of type 24 streptococci (34). Fibrinogen also has been shown to bind to hyaluronic acid (21), which in turn could amplify the role of the streptococcal capsule in preventing opsonization and phagocytosis. In support of this hypothesis is the finding that the unencapsulated mutants of type 24 and type 18 streptococci showed slightly reduced binding of $^{3}$H-fibrinogen per CFU compared with that of their parent strains (unpublished observation). Recent studies also have shown that M-like proteins of some serotypes of group A streptococci bind C4b-binding protein, which inhibits the classical pathway of complement activation and may function to limit opsonization of particular serotypes (30).

In the present study, we have shown that not all group A streptococci use the same mechanisms to prevent phagocytic killing. Differences in M protein structures and their ability to bind fibrinogen together with apparent functional differences in hyaluronate capsule may lead to different mechanisms of control of opsonization. Detailed analyses of the binding and functional activity of plasma control proteins, such as factor H and C4b-binding protein, to different serotypes and different strains within a serotype may lead to a more complete understanding of the complex spectrum of mechanisms involved in the ability of the organisms to resist phagocytosis in the nonimmune host.

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## REFERENCES


1. **Beachey, E. H., and I. Ofek.** 1976. Epithelial cell binding of group A streptococci by lipoteichoic acid on fimbriae denuded of M protein. J. Exp. Med. **143:**759–771.
2. **Beachey, E. H., G. H. Stollerman, E. Y. Chiang, T. M. Chiang, J. M. Seyer, and A. H. Kang.** 1977. Purification and properties of M protein extracted from group A streptococci with pepsin: covalent structure of the amino terminal region of the type 24 M antigen. J. Exp. Med. **145:**1469–1481.
3. **Blaser, M. J., E. Wang, M. K. R. Tummuru, R. Washburn, S. Fujimoto, and A. Labigne.** 1994. High-frequency S-layer protein variation in *Campylobacter fetus* revealed by sapA mutagenesis. Mol. Microbiol. **14:**453–462.
4. **Campo, R. E., D. R. Schultz, and A. L. Bisno.** 1995. M proteins of group G streptococci: mechanisms of resistance to phagocytosis. J. Infect. Dis. **171:** 601–606.
5. **Dale, J. B., E. Y. Chiang, and J. W. Lederer.** 1993. Recombinant tetravalent group A streptococcal M protein vaccine. J. Immunol. **151:**2188–2194.
6. **Dale, J. B., J. M. Seyer, and E. H. Beachey.** 1983. Type-specific immunogenicity of a chemically synthesized peptide fragment of type 5 streptococcal M protein. J. Exp. Med. **158:**1727–1732.
7. **Fischetti, V. A.** 1989. Streptococcal M protein: molecular design and biological behavior. Clin. Microbiol. Rev. **2:**285–314.
8. **Fischetti, V. A., R. D. Horstman, and V. Pancholi.** 1995. Location of the complement factor H binding site on streptococcal M protein. Infect. Immun. **63:**149–153.
9. **Fischetti, V. A., K. F. Jones, and J. R. Scott.** 1985. Size variation of the M protein in group A streptococci. J. Exp. Med. **161:**1384–1401.
10. **Foley, M. J., and W. B. Wood.** 1959. Studies on the pathogenicity of group A streptococci. II. The antiphagocytic effects of the M protein and the capsular gel. J. Exp. Med. **110:**617–628.
11. **Haanes-Fritz, E., W. Kraus, V. Burdett, J. B. Dale, and E. H. Beachey.** 1988. Comparison of the leader sequences of four group A streptococcal M protein genes. Nucleic Acids Res. **16:**4667–4677.
12. **Hammer, C. H., G. H. Wirtz, L. Renfer, H. D. Gresham, and B. F. Tack.** 1981. Large scale isolation of functionally active components of the human complement system. J. Biol. Chem. **256:**3995–4006.
13. **Hirsch, J. G., and A. B. Church.** 1960. Studies of phagocytosis of group A streptococci by polymorphonuclear leucocytes in vitro. J. Exp. Med. **111:** 309–322.
14. **Horstman, R. D., H. J. Sievertsen, J. Knobloch, and V. A. Fischetti.** 1988. Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H. Proc. Natl. Acad. Sci. USA **85:**1657–1661.
15. **Horstman, R. D., H. J. Sievertsen, M. Leippe, and V. A. Fischetti.** 1992. Role of fibrinogen in complement inhibition by streptococcal M protein. Infect. Immun. **60:**5036–5041.
16. **Jacks-Weis, J., Y. Kim, and P. P. Cleary.** 1982. Restricted deposition of C3 on M+ group A streptococci: correlation with resistance to phagocytosis. J. Immunol. **128:**1897–1902.
17. **Johnson, D. R., D. L. Stevens, and E. L. Kaplan.** 1992. Epidemiologic analysis of group A streptococcal serotypes associated with severe systemic infections, rheumatic fever, or uncomplicated pharyngitis. J. Infect. Dis. **166:** 374–382.
18. **Kass, E. H., and C. V. Seastone.** 1944. The role of mucoid polysaccharide (hyaluronic acid) in the virulence of group A hemolytic streptococci. J. Exp. Med. **79:**319–330.
19. **Lancefield, R. C.** 1948. Differentiation of group A streptococci with a common R antigen into three serological types with special reference to the bactericidal test. J. Exp. Med. **106:**525–544.
20. **Lancefield, R. C.** 1962. Current knowledge of the type-specific M antigens of group A streptococci. J. Immunol. **89:**307–313.
21. **LeBoeuf, R. D., R. H. Raja, G. M. Fuller, and P. H. Weigel.** 1986. Human fibrinogen specifically binds hyaluronic acid. J. Biol. Chem. **261:**12586–12592.
22. **Marques, M. B., D. L. Kasper, M. K. Pangburn, and M. R. Wessels.** 1992. Prevention of C3 deposition is a virulence mechanism of type III group B streptococcus capsular polysaccharide. Infect. Immun. **60:**3986–3993.
23. **Mouw, A. R., E. H. Beachey, and V. Burdett.** 1988. Molecular evolution of streptococcal M protein: cloning and nucleotide sequence of the type 24 M protein gene and relation to other genes of *Streptococcus pyogenes*. J. Bacteriol. **170:**676–684.
24. **Peterson, P. K., Y. Kim, B. J. Wilkinson, D. Schmeling, A. F. Michael, and P. G. Quie.** 1978. Dichotomy between opsonization and serum complement activation by encapsulated staphylococci. Infect. Immun. **20:**770–775.
25. **Phillips, G. N., P. F. Flicker, C. Cohen, B. N. Manjula, and V. A. Fischetti.** 1981. Streptococcal M protein: alpha-helical coiled-coil structure and arrangement on the cell surface. Proc. Natl. Acad. Sci. USA **78:**4689–4693.
26. **Podbielski, A., B. Melzer, and R. Lutticken.** 1991. Application of the polymerase chain reaction to study the M protein (-like) gene family in beta-hemolytic streptococci. Med. Microbiol. Immunol. **180:**213–227.
27. **Rothbard, S.** 1948. Protective effect of hyaluronidase and type-specific anti-M serum on experimental group A infections in mice. J. Exp. Med. **88:**325–333.
28. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**4767–4771.
29. **Stollerman, G. H., M. Rytel, and J. Ortiz.** 1963. Accessory plasma factors involved in the bactericidal test for type-specific antibody to group A streptococci. II. Human plasma cofactor(s) enhancing opsonization of encapsulated organisms. J. Exp. Med. **117:**1–17.
30. **Thern, A., L. Stenberg, B. Dahlback, and G. Lindahl.** 1995. Ig-binding surface proteins of *Streptococcus pyogenes* also bind human C4b-binding protein (C4BP), a regulatory component of the complement system. J. Immunol. **154:**375–386.
31. **Wessels, M. R., and M. S. Bronze.** 1994. Critical role of the group A streptococcal capsule in pharyngeal colonization and infection in mice. Proc. Natl. Acad. Sci. USA **91:**12238–12242.
32. **Wessels, M. R., J. B. Goldberg, A. E. Moses, and T. J. DiCesare.** 1994. Effects on virulence of mutations in a locus essential for hyaluronic acid expression in group A streptococci. Infect. Immun. **62:**433–441.
33. **Wessels, M. R., A. E. Moses, J. B. Goldberg, and T. J. DiCesare.** 1991. Hyaluronic acid capsule is a virulence factor for mucoid group A streptococci. Proc. Natl. Acad. Sci. USA **88:**8317–8321.
34. **Whitnack, E., and E. H. Beachey.** 1982. Antiopsonic activity of fibrinogen bound to M protein on the surface of group A streptococci. J. Clin. Invest. **69:**1042–1048.
35. **Whitnack, E., and E. H. Beachey.** 1985. Biochemical and biological properties of the binding of human fibrinogen to M protein of group A streptococci. J. Bacteriol. **164:**350–358.
36. **Whitnack, E., A. L. Bisno, and E. H. Beachey.** 1981. Hyaluronate capsule prevents attachment of group A streptococci to mouse peritoneal macrophages. Infect. Immun. **31:**985–991.


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