

## Alternate Complement Pathway Activation by Group A Streptococci: Role of M-Protein

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Avirulent strains of group A streptococci readily activate the complement system in normal human serum via the alternate complement pathway (ACP). Virulent M-positive group A streptococci are much less potent as activators of the ACP. The ability of M-positive streptococci to activate the ACP is enhanced by trypsinization or mild peptic digestion. The latter treatment removes the serologically active and antiphagocytic type-specific moieties of M protein, but retains the surface fuzzy layer. The phagocytosis of avirulent streptococci is markedly enhanced by preopsonization in serum chelated with Mg-ethylene glycol tetraacetic acid (classic complement pathway blocked) but not in serum devoid of heat-labile factors. These studies suggest that the function of M protein as a virulence factor may be mediated, at least in part, by its ability to retard interaction of ACP components with structures present on the streptococcal cell surface.

Group A streptococci (GrAS) are among the commonest bacterial pathogens of humans. They are of special interest because of their propensity to initiate delayed, nonsuppurative sequelae which are presumably mediated by immunological processes. The major virulence factor of GrAS is M protein, an antigen that is located on the bacterial cell surface and retards phagocytosis by mechanisms as yet unknown. The hyaluronate capsule of GrAS is a secondary virulence factor conferring additional resistance to phagocytosis (6). Avirulent GrAS lacking both M protein and capsule are phagocytosed even in antibody-deficient sera, such as those of newborn colostrum-deprived piglets (13), but such phagocytosis is markedly enhanced by the presence of thermolabile serum factors.

Recent studies have focused upon the interaction of GrAS somatic constituents and components of the complement system. Isolated streptococcal peptidoglycan, cell walls, cell membranes, and lipoteichoic acid have been reported to activate the alternate complement pathway (ACP) (3, 9, 15). Little is known, however, concerning the interactions of whole, intact GrAS with the human complement system. In the present study I have demonstrated differences between virulent and avirulent GrAS in their ability to activate the ACP. Such differences appear to be due to the fact that M protein present in virulent strains retards interaction of ACP components with the streptococcal cell surface.

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### MATERIALS AND METHODS

**Bacteria and cultural conditions.** GrAS of various serotypes were maintained on blood agar plates at 4°C or in Todd-Hewitt broth at -90°C. They were tested regularly by M and T typing, capsule preparation, and growth in fresh human blood. M-positive strains were mouse-passed periodically to maintain optimal virulence. M protein content of hot-acid extracts of GrAS was monitored semiquantitatively by determining the highest dilution of the extract capable of producing precipitin reactions with specific antisera in capillary tubes. Before use in complement absorption or phagocytosis experiments, organisms were grown overnight in Todd-Hewitt broth and washed three times in normal saline or in phosphate-buffered saline (pH 7.2).

**Complement activation.** Serum was donated by five healthy volunteers, pooled, divided into 1.0-ml samples, and frozen immediately at -90°C. The serum pool was tested in the indirect bactericidal assay (14) against strains of each of the M types employed in the study to assure that it did not contain detectable amounts of type-specific antibody. Just before use, pooled serum samples were thawed and chelated with 10 mM ethylene glycol tetraacetic acid and MgCl<sub>2</sub> (MgEGTA). Sera chelated in this manner failed to lyse amboceptor-coated sheep erythrocytes, thus verifying blockage of the classic complement pathway (5).

Pooled normal human serum (PNHS) samples were absorbed with streptococci, in a final concentration of approximately  $3 \times 10^9$  colony-forming units per ml, for

1 h at 37°C in a shaking water bath. Control tubes, all of which were also incubated at 37°C for 1 h, included: PNHS unabsorbed; MgEGTA-chelated PNHS absorbed with zymosan (2 mg/ml); MgEGTA-chelated PNHS unabsorbed. At the conclusion of the incubation period, all sera were assayed for hemolytic complement (HC) activity, expressed in 50% hemolytic complement units, by the method of Mayer (11). Results were expressed as percent of total HC removed.

Incubation of MgEGTA-chelated serum at 37°C in itself activated the ACP and removed a portion of the HC activity from the serum pool. This effect, which we verified by immunoelectrophoresis using anti-C3 activator serum (Behring Diagnostics, Somerville, N.J.), was previously described by Des Prez et al. (2). In each test, therefore, the MgEGTA-chelated control was used as the base-line value.

In some experiments GrAS were treated with hyaluronidase or proteolytic enzymes before use in complement activation tests. In these experiments organisms were first killed by 3-min ultraviolet irradiation to prevent regeneration of capsules or M-protein in vitro. Such irradiation did not in itself diminish the encapsulation, M typability, or resistance to phagocytosis of the streptococci. Hyaluronidase treatment (human umbilical cord hyaluronidase; Sigma Chemical Co., St. Louis, Mo.) was carried out for 15 min at 37°C using 100 turbidity units in a 1.0-ml suspension of  $3 \times 10^9$  colony-forming units of GrAS at pH 6.1. Trypsinization was performed at a concentration of 1 mg/ml at pH 8; trypsin lima bean inhibitor was added after 15 min of incubation at 37°C. Pepsin digestion was performed according to the method of Beachey et al. (1), using 50 µg of pepsin per ml at pH 5.8.

**Phagocytosis.** Before use in phagocytosis experiments,  $5 \times 10^6$  colony-forming units of GrAS were suspended in tubes containing Hanks balanced salt solution and 20% of fresh-frozen or heat-inactivated (56°C for 30 min) pooled human serum. This preopsonization step was carried out at 37°C for 30 min in a shaking water bath, either in the presence or absence of 10 mM MgEGTA. In tubes containing MgEGTA, Hanks balanced salt solution without calcium or magnesium was used as the buffer. After preopsonization, the organisms were washed in Hanks balanced salt solution and added to the leukocyte suspensions.

Lightly heparinized (10 U/ml) human blood was obtained from healthy volunteers and allowed to sediment at room temperature for 1 h. The leukocyte-rich supernatant was removed, washed in Hanks balanced salt solution, and adjusted to a final concentration of approximately  $10^7$  polymorphonuclear leukocytes per ml in Hanks balanced salt solution with 0.1% gelatin and 10% bovine serum albumin. The final phagocytosis mixture consisted of 0.5 ml of leukocyte suspension plus 0.5 ml of bacterial suspension with a ratio of streptococci to polymorphonuclear leukocytes of approximately 1:1. Phagocytosis was carried out at 37°C in plastic tubes rotated end over end at 8 rpm. At specified intervals, samples were removed, smeared onto glass slides, dried in air, fixed with methanol, and stained with Giemsa stain. Slides were counted under oil immersion at  $\times 100$  magnification. One hundred polymorphonuclear leukocytes were counted, and the proportion with associated streptococci was recorded as "percent phagocytosis."

## RESULTS

Virulent strains of GrAS (M positive, resistant to phagocytosis in fresh human blood) removed one-quarter to one-third of HC activity from MgEGTA-chelated PNHS (Table 1). Zymosan (2 mg/ml of serum), which is a potent activator of the ACP, resulted in consumption of all HC, as did two avirulent group A strains, C203S and D58X (both T type 3, M negative).

High concentrations of avirulent streptococci are required to completely deplete normal human serum. For example, strains C203S, D58X, and T1Av removed all HC activity when added to the serum pool in concentrations of  $3 \times 10^9$  colony-forming units per ml. At a 1:20 dilution ( $1.5 \times 10^8$  colony-forming units per ml), the three strains removed only 23, 25, and 29% of HC, respectively. These quantitative relationships are similar to those reported by Fine et al. (5) for ACP activation by *Streptococcus pneumoniae*.

Streptococcal cell walls and mucopeptide were found to activate the ACP, as reported by others (9, 15). Samples of both (1 mg per ml), kindly supplied by E. H. Beachey, completely removed HC from MgEGTA-chelated serum; lipoteichoic acid in the same concentration absorbed only 22% of HC activity.

I next studied the effect of enzyme treatment of ultraviolet-killed M-positive streptococci on complement activation. The test strain, type 3 Fleming (M3, R3, T3), initially removed 25% of HC from the serum pool (Fig. 1). After trypsinization, the Fleming strain removed all complement activity, thus behaving exactly like the avirulent type 3 strain, D58X (M negative, R3, T3). Treatment of the Fleming strain with hyaluronidase had no effect, despite the fact that log-phase cultures of this organism were heavily encapsulated. Similar results were demonstrated with two M-positive type 12 strains. Strains Au and Wa removed 26 and 20% of HC, respectively. Treatment with trypsin, but not hyaluronidase, resulted in complete removal of hemolytic activity.

TABLE 1. Removal of HC from Mg-EGTA-chelated PNHS by M-positive and M-negative GrAS

Reagent	% Decrease in HC <sup>a</sup>
Zymosan	100
M-positive streptococci	
Type 3	24
Type 11	36
Type 12	37
M-negative streptococci	
C203S	100
D58X	100

<sup>a</sup> As compared with MgEGTA-chelated control serum sample.

Virulent GrAS of M type 24 were subjected to peptic digestion at suboptimal pH. This treatment resulted in loss of M typability and of resistance to phagocytosis, but the surface fuzzy layer was preserved (Fig. 2). Before pepsin treatment, absorption of pooled human serum with ultraviolet-killed M24 streptococci resulted in removal of approximately one-third of HC (Fig. 3). Organisms whose surface had been altered by pepsin were capable of completely removing hemolytic activity.

The role of the ACP in opsonization of aviru-

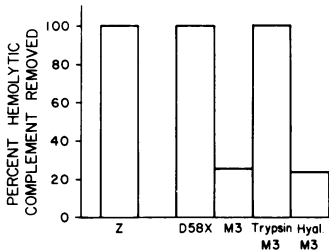


FIG. 1. Effect of trypsin treatment of M-positive type 3 GrAS upon their ability to activate the ACP in MgEGTA-chelated human serum. Z, Zymosan; Hyal., hyaluronidase.

lent streptococci was investigated. Strains T6 glossy, C203S, and T1Av were readily phagocytosed after preopsonization in fresh-frozen PNHS but not after preopsonization in heat-inactivated (56°C for 30 min) serum. When these avirulent organisms were opsonized in fresh-frozen serum chelated with 10 mM MgEGTA, phagocytosis was readily apparent, but the rate of phagocytosis was somewhat slower than in unchelated serum. A representative experiment is depicted in Fig. 4.

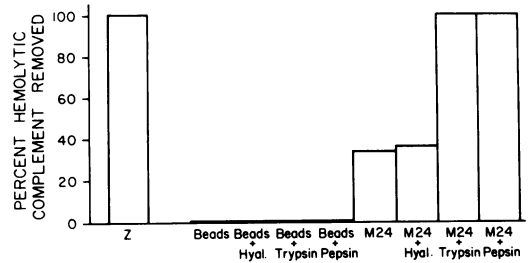


FIG. 3. Effect of trypsin and mild pepsin digestion of M24 streptococci upon their ability to activate the ACP in normal human serum chelated with MgEGTA. Controls include glass beads either untreated or treated with hyaluronidase, trypsin, or pepsin.

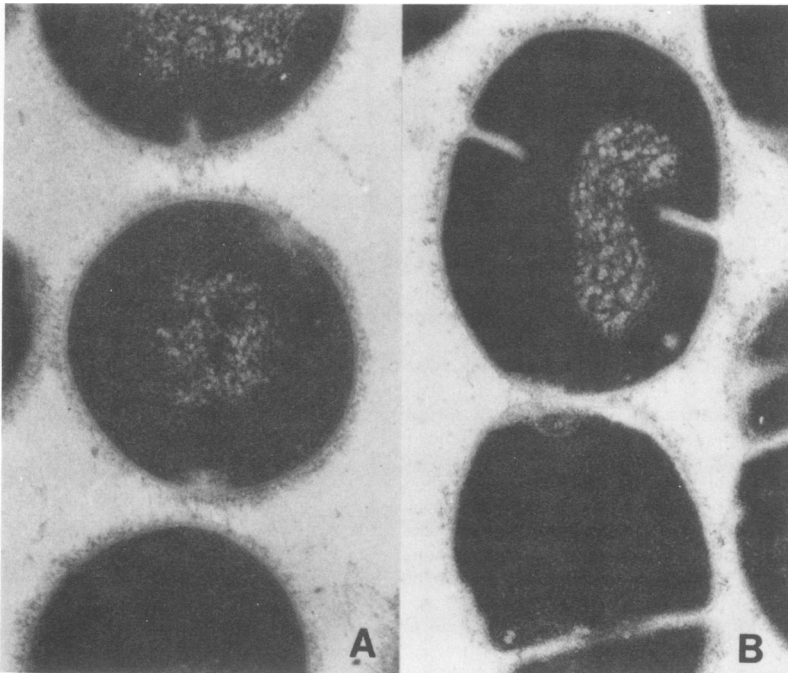


FIG. 2. Electron micrograph of ultraviolet-killed M24 GrAS before (A) and after (B) mild pepsin digestion. Pepsin treatment did not produce gross changes in surface morphology and left intact the surface fuzzy layer in which M protein ordinarily resides. Unlike the untreated organisms, pepsin-treated streptococci were neither M typable nor resistant to phagocytosis in fresh human blood, thus indicating loss of type-specific M antigen.

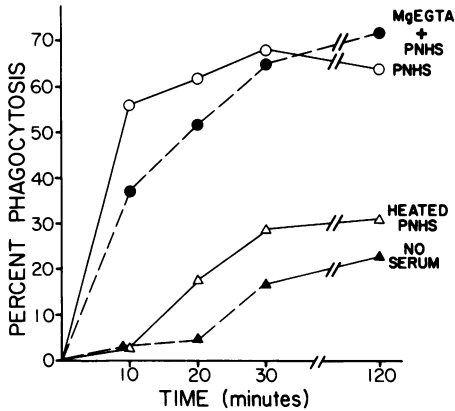


FIG. 4. Phagocytosis of avirulent (type 6 glossy) GrAS. Substances used for preopsonization are indicated in the figure.

## DISCUSSION

During the past few years there has been increasing interest in the interaction of bacterial and fungal organisms with the complement system (4, 7, 8, 10, 12). Considerable variability has been observed both from species to species (7, 10) and within strains of the same species (4, 10) with regard to the ability of various microorganisms to activate the ACP as well as their specific requirements for opsonization.

Despite the fact that GrAS are known to undergo marked variations in phase of virulence (as measured by resistance to phagocytosis, M typability, and encapsulation) under differing *in vivo* and *in vitro* conditions, the possible effect of such variations on interactions with the human complement pathway has not heretofore been studied systematically. The serum absorption experiments presented here yield only semiquantitative results, because of both the nature of the assay system and the complicating factor of partial spontaneous ACP activation by Mg-EGTA. Nevertheless, they indicate that avirulent GrAS are more potent activators of the alternate pathway than are M-positive strains.

The nature of the cell wall substances which activate the ACP in whole, intact organisms is unknown. Considerable work has been done, however, with isolated somatic constituents of GrAS. Greenblatt et al. (9), utilizing a kinetic assay of complement activation, determined that GrAS peptidoglycan was the most active material, on a weight basis, in activating the ACP, followed by cell walls, protoplast membranes, and whole cells. Peptidoglycan was more active than lipopolysaccharide from gram-negative bacilli or zymosan in ACP activation. The group-specific carbohydrate showed no activity. Tauber et al. (15), using a somewhat different

assay system, found both GrAS cell walls and cell membranes to activate the ACP, but the latter did so more avidly. The ACP-activating factor in membranes was identified as a protein of molecular weight greater than 40,000 to 60,000 which activated complement by a properdin-independent mechanism. Lipoteichoic acid is also represented upon the cell surface, where it plays a critical role in binding of GrAS to mucosal surfaces (1). Fiedel and Jackson (3) have reported that lipoteichoic acid activates the alternate pathway, but this effect was dose dependent, occurred over a narrow range of lipoteichoic acid concentrations, and could not be detected at higher concentrations.

That the type-specific moiety of M protein is the substance that retards ACP activation is suggested by the known role of this antigen in virulence and by its location on the surface hairs. Moreover, treatments known to destroy M protein, e.g., proteolytic digestion by trypsin and pepsin, greatly enhanced the ability of virulent GrAS to activate the ACP. It is of course conceivable that the observed effects were due to derangement of some other surface structure(s) by the enzymatic treatment. However, the mild peptic digestion procedure employed is the gentlest known for removing surface M protein. As indicated above, this procedure resulted in the loss of serological and functional M-protein activity without causing gross morphological distortion of the organisms or denuding them of surface hairs. The other major virulence factor on the streptococcal surface is the hyaluronate capsule. A role for this structure in the complement interactions described here was not apparent, because hyaluronidase digestion of the capsule had no effect. Additional studies utilizing early-log-phase cultures might, however, cast further light upon this issue.

Interest in the potential biological significance of these findings is enhanced by the finding that M-negative GrAS are readily opsonized via the ACP. These findings are consistent with older studies indicating that heat-labile factors but not antibodies are required for phagocytosis of such avirulent organisms (13). In contrast, M-positive strains resist phagocytosis in the presence of fresh human blood unless type-specific anti-M antibodies are present (14). Taken together, the data on complement activation and opsonization indicate that the function of M protein as a virulence factor may be mediated, at least in part, by its ability to mask complement receptors present on the streptococcal cell surface and thus to prevent opsonization via the ACP.

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#### ADDENDUM

Since submission of this paper, Peterson et al. (*J. Infect. Dis.*, **139**:575-585, 1979) have reported studies on opsonization of M-rich and M-negative strains derived from a single parent strain (CS44) of type 12 GrAS. Their results are in accord with the concepts presented above, in that the M-rich strain was poorly opsonized by all sera tested but the M-negative variant was efficiently opsonized via the ACP.

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