

Activation of the Alternative Pathway of Human Complement by the Extracellular Slime Glycolipoprotein of *Pseudomonas aeruginosa*

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The capability of the extracellular slime glycolipoprotein (GLP) of *Pseudomonas aeruginosa* to activate human complement was investigated. When slime GLP was added to type AB human serum, C3 and factor B were converted to their respective major cleavage fragments, C3b and Bb. This activation also occurred when slime GLP was incubated with serum-ethylene glycol bis(trichloroacetate)-Mg⁺⁺, a result which indicates that the alternative complement pathway is involved. Additional support for the hypothesis of alternative pathway activation was provided by the fact that when serum-ethylene glycol bis(trichloroacetate)-Mg⁺⁺ was preheated to inactivate factor B, slime GLP did not induce conversion of C3. The activation of the alternative pathway of human complement by slime GLP may represent an early nonimmune defense against *P. aeruginosa* infection.

Activation of the alternative pathway of human complement by a variety of bacteria [1, 2], fungi [3], parasites [4], and virus-infected cells [5] may well represent a nonspecific first line of defense against invading organisms. This defense may be operating through the generation of opsonins and of factors that are chemotactic for phagocytes [6]. In addition, bacterial products have been shown to be mitogenic for B lymphocytes [7, 8] and to activate macrophages [6].

Pseudomonas aeruginosa is a significant cause of hospital-acquired infections. It has been reported as the cause of infection in patients debilitated by severe burns [9, 10], leukemia [11], and treatment with immunosuppressive drugs [12], and it is frequently isolated from patients with cystic fibrosis [13].

Different factors of *P. aeruginosa* have been proposed as the likely sources of the organism's virulence. Among these factors, the extracellular slime layer is considered to be a specific characteristic of *P. aeruginosa*. Extracellular slime glycolipoprotein (GLP) produces effects similar to those that accompany a lethal infection with via-

ble bacilli [14] and is produced in mice after ip injection of viable cells [15]. Active immunization with GLP or passive immunization with rabbit antiserum to GLP protects mice against a lethal challenge with viable cells [16, 17].

In view of the importance of *P. aeruginosa* as an infectious agent in humans and the functions of the complement system in host responses to invading organisms, a study of the biologic activities of the extracellular slime GLP would be incomplete without an examination of the interaction of slime GLP with the complement system.

Materials and Methods

Microorganisms. The organism used, *P. aeruginosa* strain C2, was isolated from a human clinical specimen and has been described [18].

Materials. GVB is Veronal-buffered saline, pH 7.3, containing 0.1% gelatin. GVB-EDTA is GVB with 10 mM EDTA. GVB-EGTA-Mg⁺⁺ is GVB with 10 mM EGTA and 5 mM Mg⁺⁺.

Freshly obtained type AB serum was used as a source of normal human complement. The blood was clotted at 4 C for 60 min, and the serum was separated by centrifugation at 1,000 g for 15 min at 4 C.

To block both classical and alternative pathway activity, serum was chelated with 20 mM EDTA (Sigma Chemical Co., St. Louis). To block only

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classical pathway activity, serum was chelated with 10 mM EGTA (Sigma) and supplemented with 1 mM MgCl₂ (serum-EGTA-Mg⁺⁺).

Extraction of slime GLP. The GLP fraction was obtained from the extracellular slime layer of *P. aeruginosa* strain C2 [14, 19]. Slime was extracted in 0.15 M NaCl from bacterial cultures grown for 18 hr on sheets of cellophane overlying trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.). The extract was precipitated with ethanol, clarified by centrifugation at 16,300 g for 30 min, and dialyzed overnight against distilled water. The dialyzate was centrifuged at 105,000 g for 3 hr, and the supernatant containing the GLP fraction was lyophilized. The GLP was then filtered through gels and subjected to anion-exchange chromatography; it eluted at 0.3–0.4 M KCl. Purity and homogeneity were demonstrated by chromatography, sedimentation pattern, and immunodiffusion [14]. Lyophilized GLP was stored in vacuo at 4 C.

Assays for conversion of C3 and activation of factor B. The presence of C3, converted to its more anodally migrating form, was determined by immunoelectrophoresis. Test serum was treated with GLP or zymosan (Sigma) at 37 C for 30 min and then analyzed by electrophoresis in agarose gel containing barbital buffer (10 mM EDTA), pH 8.6. A monospecific antiserum to human C3 (Behring Diagnostics, Somerville, N.J.) was used to detect the conversion of C3.

Activation of factor B was determined by immunoelectrophoresis using the agarose-barbital buffer described above and a monospecific antiserum to factor B (Atlantic Antibodies, Westbrook, Me.).

Hemolytic assay. Various amounts of GLP or zymosan were added to 25 μ l of serum-EGTA-Mg⁺⁺, and the mixture was incubated at 37 C for 20 min. Rabbit erythrocytes in GVB-EGTA-Mg⁺⁺ were added, and incubation with shaking was continued for an additional 6 min. After the addition of 1 ml of 0.01 M GVB-EDTA at 0 C and centrifugation at 400 g for 10 min, the percentage of cells that were lysed was determined by the release of hemoglobin, measured by the value for *A* at 412 nm. The percentage of cells that were lysed was calculated in relation to that giving 60% hemolysis.

The four controls were a cell control containing all of the reactants except serum, a control giving

100% lysis (H₂O), a control containing serum-EGTA-Mg⁺⁺ that had not been treated with GLP, and a control containing serum that had been treated with EDTA.

Results

Complement activation by slime GLP of *P. aeruginosa*. When GLP was added to type AB human serum, conversion of C3 occurred as detected by immunoelectrophoresis (figure 1, top). Incubation of GLP with human serum also produced altered forms of factor B, so that fragments with slower electrophoretic mobility were generated (figure 1, bottom).

Conversion of C3 and factor B to their respective major cleavage fragments, C3b and Bb, occurred when GLP was incubated with human serum that had been pretreated with EGTA to block CF via the classical pathway (figure 1). Incubation of zymosan with serum-EGTA-Mg⁺⁺ produced altered forms of C3 and factor B that were similar in position to those seen in GLP-activated serum.

Additional support for the hypothesis of alternative pathway activation was derived from experiments showing that although slime GLP induced conversion of C3 in serum-EGTA-Mg⁺⁺, it was unable to do so when the same serum was preheated to 50 C for 30 min to inactivate factor B (figure 1, top). In addition, no activation of C3 could be detected in serum that was chelated with 20 mM EDTA.

To investigate further whether there is any enzyme that cleaves C3 or factor B, pure C3 and factor B [20] were preincubated with 1 mg of GLP and analyzed by immunoelectrophoresis. No detectable cleavage was found after preincubation for 2 hr.

Inhibition of alternative pathway-dependent hemolysis of rabbit erythrocytes. Various concentrations of slime GLP were preincubated with serum-EGTA-Mg⁺⁺ at 37 C for 20 min and subsequently tested with the use of rabbit erythrocytes in GVB-EGTA-Mg⁺⁺ for any remaining activity of the alternative complement pathway. GLP at a concentration of 3.2 μ g/ml blocked 92% of the alternative pathway activity after incubation for 20 min (figure 2, left). When the inhibition of lysis of rabbit erythrocytes induced by GLP was com-

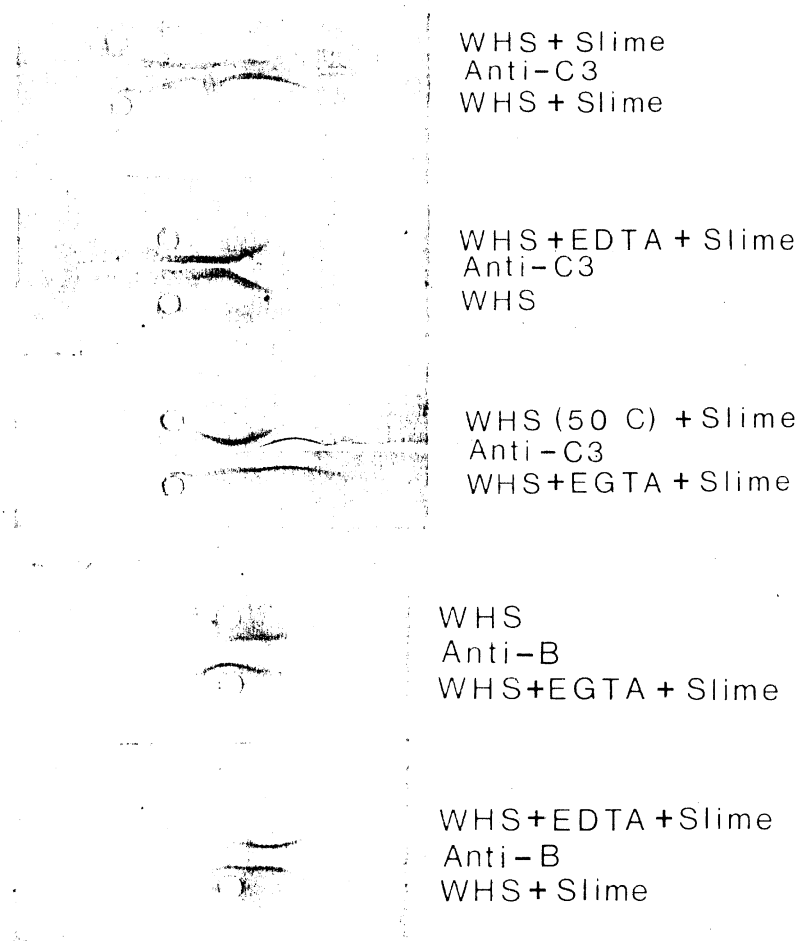


Figure 1. Immunoelectrophoretic profiles of human serum incubated with slime glycolipoprotein from *Pseudomonas aeruginosa* and (top) rabbit antiserum to human C3 (Anti-C3) or (bottom) rabbit antiserum to human factor B (Anti-B). WHS = whole human serum.

pared with that seen with zymosan, we found that 3.2 μg of GLP/ml and 3.1 μg of zymosan/ml blocked the same percentage of complement activity (data not shown).

The time kinetics of blocking the alternative pathway activity by GLP are shown in figure 2, right. Nearly 100% of the activity was blocked after incubation for 20 min of serum-EGTA- Mg^{++} with 3.2 μg of GLP/ml.

Discussion

The results of the present study demonstrate that the extracellular slime GLP of *P. aeruginosa*, when added to human serum, is able to convert C3 to its activated fragments. When GLP was incubated with unchelated serum or serum-EGTA- Mg^{++} , C3 and factor B were converted to their respective major cleavage fragments, C3b and Bb, a

result which indicates complement activation via the alternative pathway. Although GLP induced conversion of C3 in serum-EGTA- Mg^{++} , it was unable to do so when the same serum was preheated to 50 C to destroy factor B. The aforementioned experiments, coupled with the fact that no conversion of C3 could be detected in serum chelated with EDTA, provide strong evidence that slime GLP from *P. aeruginosa* activates human complement via the alternative pathway, although these results do not rule out completely some activation of the classical pathway.

There has recently been increasing interest in the interaction of microorganisms with the complement system. Various microbes, including gram-positive [21, 22] and gram-negative [23, 24] bacteria, are known to activate complement. Also, various microbial components such as zymosan from the yeast *Saccharomyces cerevisiae* [25], the

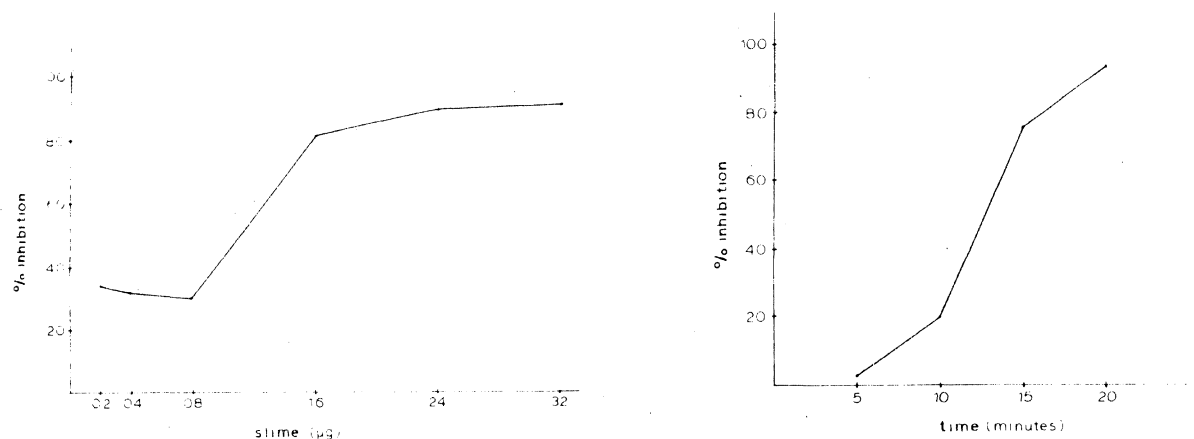


Figure 2. Effect on hemolysis of rabbit erythrocytes of variation in (left) concentration of slime glycolipoprotein from *Pseudomonas aeruginosa* and (right) length of incubation period in human serum-EGTA-Mg⁺⁺.

lipopolysaccharide of *Escherichia coli* [26], the glucans from streptococci [27], and the cell-wall teichoic acid of pneumococci [28] have been shown to activate the complement system. It is conceivable that complement, whether acting by the classical or the alternative pathway, provides a general mechanism of destruction of invading organisms. Furthermore, complement activation via the alternative pathway might represent an early mechanism of defense against infection that operates in the absence of, and thus before, the manifestation of the immune response.

The slime layer of *P. aeruginosa* seems to contribute heavily to the pathogenesis of infection with the organism [14, 17]. It has been reported that antibody to slime GLP effectively neutralizes the inhibitory effect of GLP on phagocytosis of viable cells of *P. aeruginosa* [14], and antisera to the various GLPs protect mice against infection by homologous strains [17]. Recently, it has been demonstrated that slime GLP exerts a mitogenic effect on human peripheral blood and cord blood lymphocytes [29]; this nonspecific mitogenic effect may play a role in the host response to infections with *P. aeruginosa*.

The finding presented in this report—that slime GLP activates the alternative pathway of human complement—suggests another nonspecific mechanism for the protection of the host against the invading cells of *P. aeruginosa*. Mitogenicity and complement activation by slime GLP may represent two early events that can provide natural, nonimmune defense against *P. aeruginosa* infection.

The activation of human complement by slime

GLP suggests that an examination of the role of complement in opsonization and in intracellular and extracellular killing of *P. aeruginosa* in both the immune and nonimmune host would be of further interest.

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