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Serum Sensitivity of a *Pseudomonas aeruginosa* Mucoid Strain

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The susceptibility of *Pseudomonas aeruginosa* 144M (a mucoid strain isolated from the sputum of a cystic fibrosis patient) to the bactericidal activity of pooled fresh normal human serum (FHS) was examined. FHS at concentrations of ≥2.5% was capable of killing >95% of strain 144M. Strain 144M was killed by FHS in a dose-dependent manner. Although either immunoglobulin M (IgM) or IgG was bactericidal in the presence of complement, IgM was about 10 times as effective as IgG. However, optimal killing activity required both IgM and IgG and complement, activated by the classical pathway. A role for lysozyme in the killing of 144M was demonstrated only when low concentrations of FHS were used. In contrast to 144M, *P. aeruginosa* strains 144NM and 144M(SR) were totally resistant to FHS at all of the concentrations tested (up to 50%). Neither the FHS susceptibility of 144M nor the FHS resistance of 144NM or 144M(SR) was altered by choice of growth medium, growth phase, or temperature of growth. Results of absorption studies with whole organisms, isolated outer membrane preparations, or lipopolysaccharide (LPS) from each strain suggest that the antigen(s) which binds the bactericidal immunoglobulins is accessible on the surface of 144M but not on the surface of 144NM or 144M(SR), is insensitive to trypsin treatment, and is believed to be LPS. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the three LPS preparations demonstrated that 144M LPS contained primarily lipid-A–core polysaccharide components, whereas the LPSs from 144NM and 144M(SR) were heterogeneous, with various degrees of O-side-chain substitution. These results suggest that at least one target for bactericidal antibody on the surface of 144M is contained in the rough LPS of this strain.

In comparison with infections caused by gram-positive bacteria, the incidence of gram-negative bacterial infections has been on the rise. Of particular concern is the increasing problem of infections caused by *Pseudomonas aeruginosa* among patients with burn wounds, cystic fibrosis (CF), acute leukemia, organ transplants, and intravenous drug addiction (3). Hospitalized patients are particularly susceptible to *P. aeruginosa* infections. In the hospital surveillance program of the Centers for Disease Control, *P. aeruginosa* accounted for 10% of urinary tract infections, 9% of surgical wound infections, 17% of lower respiratory tract infections, and 11% of all bacteremias (1).

To improve patient survival, a better understanding of the role of natural host defense mechanisms against *P. aeruginosa* is essential. The ubiquitous nature of *P. aeruginosa* explains why fresh human serum (FHS) contains antibodies directed against this organism (12). The bactericidal activity of antibody and complement in FHS is regarded as a significant component of host defenses against many gram-negative bacteria (36). However, the susceptibility of *P. aeruginosa* strains to the bactericidal activity of FHS appears to be quite heterogeneous. Whereas most strains isolated from patients with bacteremia or endocarditis are serum resistant (30, 33, 47), mucoid and nonmucoid strains of *P. aeruginosa* isolated from the sputum of CF patients are much more susceptible to the bactericidal activity of FHS than are strains isolated from non-CF patients (17, 26, 33, 38).

This study examines the components in FHS responsible for the killing of an FHS-susceptible strain of *P. aeruginosa* and the mechanism by which this process takes place. In addition, the bacterial surface determinants responsible for the susceptibility of this strain to antibody-plus-complement-mediated killing is examined. The results suggest that the susceptibility of *P. aeruginosa* 144M to FHS is due to the binding of bactericidal immunoglobulin M (IgM) and IgG to lipopolysaccharides (LPS) exposed on the surface of 144M, followed by complement activation via the classical pathway.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. aeruginosa* 144M, a mucoid strain obtained from the sputum of a patient with CF, its spontaneous nonmucoid derivative 144NM, and a serum-resistant mucoid mutant, 144M(SR), isolated by passage of 144M in the presence of increasing concentrations of FHS (32a), were maintained on brain heart infusion (BHI) agar (BBL Microbiology Systems, Cockeysville, Md.) at 37°C in 5% CO_2 and transferred every 18 to 24 h. All three strains had colonial phenotypes which were stable on BHI agar.

OM isolation. Each strain was grown overnight at 37°C with shaking in 500-ml BHI broth lots in 1-liter flasks. A total of 4.5 liters of bacteria was harvested by centrifugation at 10,000 × *g* for 20 min at 4°C and washed twice with 0.01 M phosphate-buffered saline (PBS [pH 7.4]), and the pellet was resuspended in distilled water and lyophilized. The details for outer membrane (OM) isolation have been described previously (33). The protein content was determined by the Folin phenol method of Lowry et al. (21).

LPS isolation and SDS-polyacrylamide gel electrophoresis. Each strain was grown overnight at 37°C with shaking in 500-ml BHI broth lots in 1-liter flasks, and a total of 12 liters was harvested by centrifugation at 10,000 × *g* for 20 min at 4°C and washed twice with PBS; the pellet was then resuspended in distilled water and lyophilized. LPS was isolated from each strain by the recently described protocol of Darveau

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and Hancock (7). The final LPS suspension was extensively dialyzed against distilled water and then lyophilized. Protein content in these LPS preparations was determined to be 2.5% (microgram of protein per microgram of LPS) for 144M LPS, 1.5% for 144NM LPS, and 2.8% for 144M(SR) LPS. These isolated LPS preparations were examined by electrophoresis on a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel containing 4 M urea (41) and stained by the silver stain procedure described by Tsai and Frasch (41) with a few modifications. Details of these techniques are presented in a separate report (32a).

Serum. Blood was obtained by venipuncture of four normal healthy adult male volunteers (with no known previous history of *P. aeruginosa* infection) and was allowed to clot at room temperature for 30 min and overnight at 4°C. After centrifugation at 1,000 × *g* for 15 min at 4°C, FHS was removed, pooled, sterile filtered (pore size, 0.45 μm), and either used immediately or stored at −70°C in small portions until use.

IgM- and IgG-rich pools (pools A and B, respectively) were obtained by fractionation of FHS on a Bio-Gel A column (1.5 m, 100 to 200 mesh; Bio-Rad Laboratories, Richmond, Calif.) and characterized as to immunoglobulin content as described previously (4, 32). Pool A contained 39 mg of IgM per dl and no detectable IgG or IgA, whereas pool B contained 25 mg of IgM, 720 mg of IgG, and 105 mg of IgA per dl.

Serum bactericidal microassay. For these experiments, 18- to 24-h BHI agar-grown cultures of 144M, 144M(SR), or 144NM were suspended in PBS and centrifuged at 10,000 × *g* for 10 min at 4°C. The pellet was suspended in PBS, the bacterial concentration was determined spectrophotometrically at 550 nm, and the suspension was diluted in PBS to a final concentration of ca. 10^4 CFU/ml. The bactericidal microassay was carried out in 96-well microtiter plates (Becton, Dickinson Co., Oxnard, Calif.) exactly as described previously (33). Briefly, 100 μl of bacterial suspension and 100 μl of pooled FHS (at final concentrations ranging from 0.1 to 50%) were incubated at 37°C for 60 min with gentle rotation. Afterwards, the percent bacterial survival was determined by plating 100- and 10-μl samples from each well onto BHI agar plates; the number of CFU per milliliter was determined after overnight incubation at 37°C with 5% CO_2 and compared to that in control wells which contained PBS in place of FHS.

In the experiments designed to examine the kinetics of serum bactericidal activity, the same bactericidal microassay was employed, except that 10 wells were inoculated with each bacteria-serum mixture. At various times, 100- and 10-μl samples from one well of each combination were plated onto BHI agar and incubated overnight at 37°C with 5% CO_2, and the number of CFU per milliliter was determined. The percent bacterial survival was then determined as described previously (33).

Treatment of serum. For some experiments, FHS was decomplemented by heating at 56°C for 30 min. To selectively decomplement the alternative pathway, factor B was inactivated by treatment of FHS at 50°C for 20 min (10). FHS treated with 0.01 M Mg-EGTA [0.01 M $MgCl_2$, 0.01 M ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] was used to assess the activity of the alternative pathway while selectively inactivating the classical pathway (11). For those experiments with FHS and Mg-EGTA, Hanks balanced salt solution without Ca^{2+} or Mg^{2+} ions (Microbiological Associates, Bethesda, Md.) replaced PBS as the nonserum control.

Absorption of serum. FHS was sequentially absorbed five times with ca. 10^{10} CFU/ml of FHS. The first four suspensions were tumbled slowly end-over-end at 4°C for 1 h, and the last suspension was tumbled at 4°C overnight. Bacteria were pelleted by centrifugation at 10,000 × *g* for 10 min at 4°C, and the supernatant was sterile filtered (pore size, 0.45 μm). The absorbed serum was either used immediately or stored at −70°C in small portions until use.

Absorption of FHS with isolated LPS was performed in a manner similar to that described by Young (46). An LPS solution (500 μg/ml in distilled water) was mixed with an equal volume of FHS and tumbled at 4°C for 60 min. The mixture was then centrifuged at 15,000 × *g* for 30 min at 4°C. The supernatant was collected, sterile filtered (pore size, 0.45 μm), and kept at −70°C until use.

FHS was absorbed with isolated OM preparations by mixing an OM preparation (3.0 mg/ml in distilled water) with an equal volume of FHS. After the mixture was tumbled at 4°C for 60 min, it was centrifuged at 15,000 × *g* for 30 min at 4°C. The supernatant was collected, sterile filtered (pore size, 0.45 μm), and kept at −70°C until use.

Trypsin treatment. Each strain was grown overnight on BHI agar, suspended in PBS, and centrifuged at 10,000 × *g* for 10 min at 4°C. Each pellet was suspended in PBS; the bacterial concentration was determined spectrophotometrically, and the suspension was diluted in PBS to a concentration of 10^9 bacteria per ml. Trypsin (Sigma Chemical Co., St. Louis, Mo.) was added to one half of this suspension to a final concentration of 2.5 mg/ml; the other half was left untreated. Both samples were incubated for 1 h at 37°C with tumbling. Soybean trypsin inhibitor (Sigma) was then added to the trypsin-treated sample to a final concentration of 2 mg/ml. Both tubes were reincubated at 37°C with tumbling for 15 min, after which they were centrifuged at 10,000 × *g* for 15 min at 4°C, washed twice with PBS, and suspended in PBS. The trypsin-treated and untreated bacterial suspensions were then used to absorb FHS as described above except that the fifth overnight absorption step was not done. The two bacterial suspensions were also used as test strains in the FHS bactericidal microassay.

Lysozyme assay. The levels of active lysozyme in FHS were determined by the spectrophotometric method of Nerurkar (27). FHS or chicken egg white lysozyme (Sigma) was added to a suspension of *Micrococcus lysodeikticus* (Sigma), and the optical density at 450 nm was recorded for 1 to 3 min. A standard curve was established with lysozyme concentrations ranging from 1 to 20 μg/ml. The lysozyme activity in the FHS samples was then determined by comparison to the standard curve.

Absorption of serum with bentonite. FHS was depleted of lysozyme by absorption with bentonite by the method of Wardlaw (43). Bentonite (Sigma) was washed in distilled water as previously described (43) and added to FHS at a concentration of 5 mg/ml of FHS. The mixture was tumbled for 15 min at 4°C and centrifuged at 10,000 × *g* for 30 min at 4°C, and the supernatant was filter sterilized (pore size, 0.45 μm) and stored at −70°C until use.

RESULTS

Susceptibility to FHS. The susceptibility of strains 144M, 144NM, and 144M(SR) to FHS was determined by incubating each strain for 60 min at 37°C in the presence or absence of FHS at concentrations ranging from 0.31 to 20%. FHS at concentrations of ≥2.5% was capable of killing >95% of strain 144M (Fig. 1). In contrast, both 144NM and 144M(SR) were resistant to the bactericidal activity of FHS and ap-

peared to grow at the higher serum concentrations. Although not shown here, 144NM and 144M(SR) were also found to be resistant to FHS in concentrations of up to 50% (the highest concentration tested), even when the incubation period with FHS was increased to 2, 3, or 22 h at 37°C.

The susceptibility of 144M to the bactericidal activity of FHS was the same whether the bacteria were grown in BHI broth, on a BHI agar plate, or in MVBM, a chemically defined broth medium which promotes slime production (20). Growth of 144M in BHI broth at 25, 30, or 37°C did not alter its susceptibility to FHS, nor did growth at these temperatures affect the resistance of 144M(SR) to FHS. Finally, the susceptibility of 144M to FHS was the same whether the bacteria were examined in log phase (4-h culture) or stationary phase of growth (24-h culture). Thus, for the remainder of the studies, 18- to 24-h BHI agar-grown cultures (at 37°C) were used in the serum bactericidal microassay.

Kinetics of bactericidal activity. The kinetics of the bactericidal activity of FHS for strain 144M is shown in Fig. 2. A 5-min incubation in FHS at concentrations of ≥10% was sufficient to kill all 144M, whereas equivalent bactericidal activity required 10 min for 5% FHS, 30 min for 4% FHS, and 50 min for 2% FHS. With 1 and 0.5% FHS, ca. 34 and 93% of the bacteria survived the 60-min incubation period, respectively.

Serum components responsible for bactericidal activity. A newly developed whole bacterial cell enzyme-linked immunosorbent assay (5) indicated that the IgG and IgM antibody titer to 144M in pooled FHS is ca. 1:100; as described in an earlier paper (4), either IgG or IgM can be bactericidal in the presence of complement. With 10% 144M-absorbed FHS (MabsS) as a source of complement (4), the relative efficacies of IgM (pool A) and IgG (pool B) as bactericidal immunoglobulins for strain 144M were determined. Although both IgM and IgG were bactericidal, IgM was about 10 times more effective as a bactericidal immunoglobulin than was IgG, i.e., to kill 90% of 144M, 12 μg of IgM or 115 μg of IgG was required plus 10% MabsS (Fig. 3). However, neither immunoglobulin alone was as effective as heat-inactivated serum (HIS), a fact which was particularly apparent at the lower immunoglobulin concentrations. Since HIS contains both IgG and IgM, the greater activity observed with HIS was probably the result of the combined action of both immunoglobulin classes.

Role of complement in killing by FHS. An examination of the relative roles of the classical and alternative pathways of complement activation for killing of 144M by FHS is shown in Table 1. Heating FHS at 56°C for 30 min completely abolished its bactericidal activity, whereas incubation at

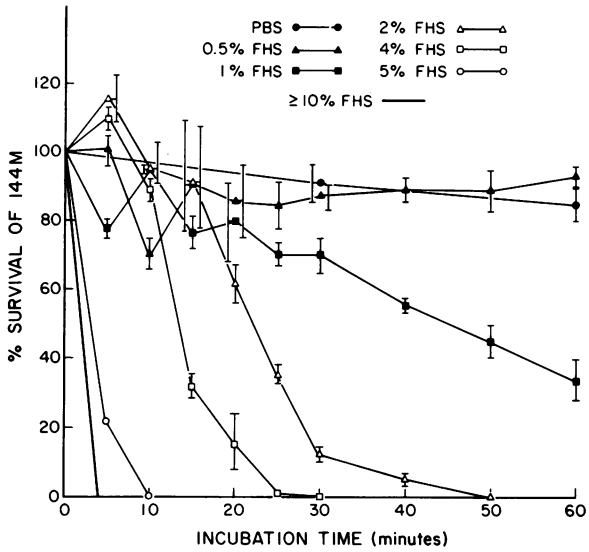

FIG. 2. Kinetics of serum bactericidal activity for 144M. The percent survival in FHS in various concentrations was determined at several times during a total of 60 min of incubation at 37°C. Values represent the mean ± SEM based on three experiments.

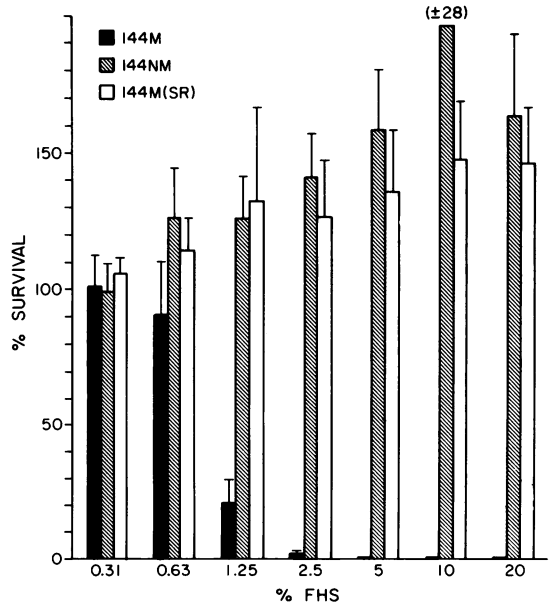

FIG. 1. Bactericidal activity of FHS for strains 144M, 144NM, and 144M(SR). The percent survival of each strain in FHS in various concentrations was determined, after 60 min of incubation at 37°C, with an FHS bactericidal microassay. Values represent the mean plus or minus standard error of the mean (SEM) based on five experiments.

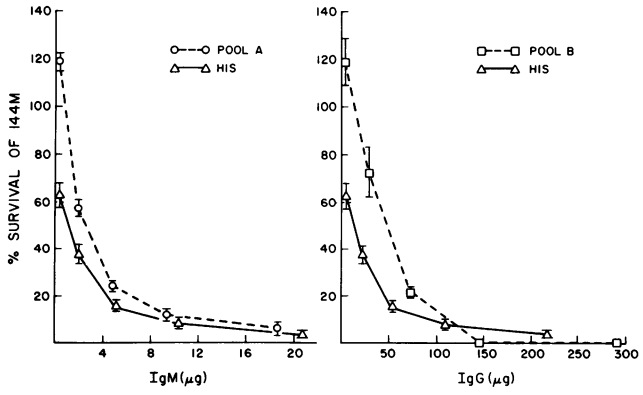

FIG. 3. Role of immunoglobulin in the bactericidal activity of FHS for 144M. Bacteria were incubated with pool A (IgM), pool B (IgG), or HIS (56°C, 30 min) at the various concentrations listed. In addition, MabsS was added to a final concentration of 10% as a source of complement. These mixtures were incubated at 37°C for 60 min, and the percent survival was determined. Values represent the mean ± SEM based on three experiments.

TABLE 1. Role of complement in the killing of strain 144M by FHS

Serum	% Survival[a]	No. of expt
10% FHS	<1	4
10% FHS (56°C, 30 min)	137.6 ± 13.5	7
10% FHS (50°C, 20 min)	<1	4
10% FHS + Mg-EGTA	132.7 ± 12.4	4

[a] Survival is determined by comparison of the number of CFU in the test well with that in the PBS control, which is considered to be 100% survival.

50°C for 20 min (to inhibit the alternative pathway) had no effect. The addition of 10 mM Mg-EGTA to 10% FHS, a procedure which renders the classical pathway nonfunctional, completely blocked the bactericidal activity of FHS, implicating the importance of the classical complement pathway for killing of 144M by FHS.

Role of lysozyme in killing by FHS. The role of lysozyme in the killing of 144M by FHS was examined (Table 2). Absorption of FHS with bentonite, which removed 93% of its lysozyme activity, had no observable effect on the killing activity of 10% FHS. However, the removal of lysozyme significantly decreased the bactericidal activity of 1% FHS ($P < 0.001$, by Student's two-tailed t test).

Bactericidal activity of FHS for 144M after absorption with OM preparations from 144M, 144M(SR), or 144NM. FHS was absorbed with OM preparations isolated from 144M, 144M(SR), or 144NM as described above. The presence of residual complement activity in the absorbed FHS was verified by the 50% hemolytic complement assay of Mayer (23). Residual bactericidal activity in the absorbed FHS was examined by comparing the percent survival of 144M incubated in the presence of either FHS or absorbed FHS for 60 min at 37°C. FHS at concentrations of ≥2.5% was sufficient to kill >90% of 144M (Fig. 4). However, after absorption with 144M OM preparations, the absorbed FHS, at concentrations of 2.5, 5, or 10% was no longer bactericidal for 144M. This suggests that the determinant(s) on the surface of 144M to which bactericidal antibody binds is present on these OM preparations. Absorption of FHS with 144NM OMs also removed some bactericidal activity, although much less than did 144M OMs, from FHS, increasing the concentration of serum needed to kill 90% of 144M from 2.5%, for FHS, to 10%, for absorbed FHS. In contrast, absorption of FHS with 144M(SR) OMs did not appear to have any appreciable effect on the bactericidal activity of FHS for 144M.

Effect of trypsin treatment on bacteria. Whole 144M organisms pretreated with trypsin were as capable of absorbing the bactericidal activity from FHS as non-trypsin-treated organisms (Table 3). In contrast, neither 144NM nor trypsin-treated 144NM absorbed any bactericidal activity from FHS. Furthermore, strain 144M, when treated with trypsin and used as the target organism in an FHS bactericidal assay, was still highly susceptible to FHS. (Although not shown here, trypsin treatment did not alter the resistance of 144NM to FHS). Therefore, the target for the bactericidal activity of FHS is present on 144M and is not sensitive to the protein-cleaving effects of trypsin.

Examination of the LPS from 144M, 144M(SR), and 144NM. Previous studies have demonstrated that 144M serotyped weakly with serotype 3 and 14 antisera (Bacto-*Pseudomonas aeruginosa* antiserum set, Difco Laboratories, Detroit, Mich.), whereas 144NM agglutinated strongly with serotype 6 antisera (33). Schiller et al. found that 144M(SR) also serotyped strongly as type 6 (32a). Since the serotype is based on the O-antigenic side chain of the LPS (6), the alteration in serotype configuration in 144NM and 144M(SR) compared with that in the parental strain 144M, suggested a variation in the LPS composition which might be related to susceptibility to FHS.

To explore possible differences in LPS composition, we isolated LPS of *P. aeruginosa* strains 144M, 144M(SR), and 144NM and electrophoresed them on a 15% SDS-polyacrylamide slab gel; the patterns were developed with a periodic acid-silver stain. The mobility of the LPS bands have been shown to correspond to the degree of roughness of the LPS, with increases in mobility correlating to the shortening of the O-side chains or oligosaccharide core or both (7, 14, 16, 29). LPS of the FHS susceptible parental strain 144M is mainly rough, short LPS, as the dark-staining material near the gel bottom is believed to be mainly lipid-A-core polysaccharide (Fig. 5). In contrast, LPS preparations from serum-resistant strains 144NM and 144M(SR) display size heterogeneity, as evidenced by the various bands in the middle to upper regions of the gel, which are thought to represent LPS with various numbers of side-chain lengths.

Susceptibility of 144M to FHS absorbed with LPS from 144M, 144M(SR), or 144NM. FHS was absorbed with LPS

TABLE 2. Role of lysozyme in the bactericidal activity of FHS for strain 144M[a]

Serum	% Survival
10% FHS	<1
10% Bentonite-absorbed FHS[b]	<1
1% FHS	56.9 ± 4.6
1% Bentonite-absorbed FHS[b]	90.7 ± 3.3

[a] Four experiments were performed for each type of serum.
[b] Bentonite absorption resulted in a 93% loss of lysozyme activity in FHS.

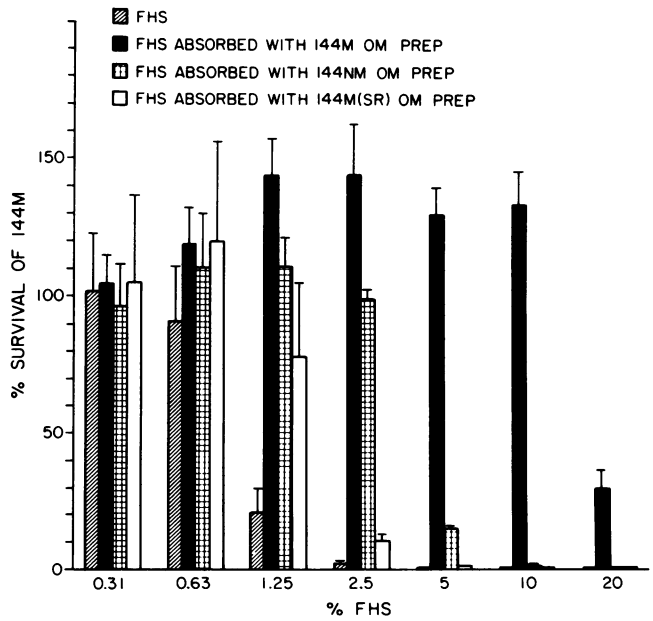

FIG. 4. Bactericidal activity of FHS for 144M before and after absorption with either 144M OMs, 144NM OMs, or 144M(SR) OMs. The percent survival of 144M in FHS or absorbed-FHS in various concentrations was determined after 60 min of incubation at 37°C. Values represent the mean ± SEM based on three experiments.

TABLE 3. Effect of trypsin treatment on the ability of 144M or 144NM to absorb bactericidal activity from FHS or to alter the FHS susceptibility of 144M

Serum[a]	Test bacteria	% Survival	No. of expt
FHS	144M	0	10
FHS	144M[b]	0	10
144M-absorbed FHS	144M	132.6 ± 7.1	16
144M-absorbed[b] FHS	144M	130.2 ± 7.3	21
144NM-absorbed FHS	144M	0	4
144NM-absorbed[b] FHS	144M	0	4

[a] All sera were used at a 10% final concentration.
[b] Treated with trypsin, as described in the text.

isolated from 144M, 144M(SR), or 144NM, as described above, and the presence of residual complement activity in the absorbed FHS was verified by the 50% hemolytic complement assay. Residual bactericidal activity in the absorbed FHS was determined by comparing the percent survival of 144M incubated in the presence of FHS with that of 144M in the presence of absorbed FHS for 60 min at 37°C. At a concentration of ≥2.5%, FHS, FHS absorbed with 144NM LPS, or FHS absorbed with 144M(SR) LPS was sufficient to kill >90% of 144M (Fig. 6). In contrast, 2.5% FHS absorbed with 144M LPS killed only about 40% of 144M, which suggests that LPS from 144M can bind bactericidal immunoglobulin(s) present in FHS.

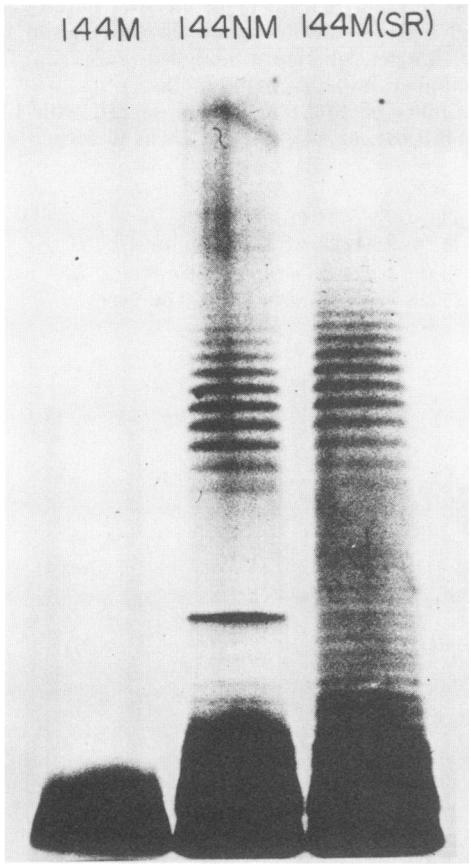

FIG. 5. SDS-polyacrylamide gel electrophoresis of lipopolysaccharide preparations prepared from 144NM, 144M, and 144M(SR). Each lane was loaded with ca. 15 μg of bacterial LPS preparation.

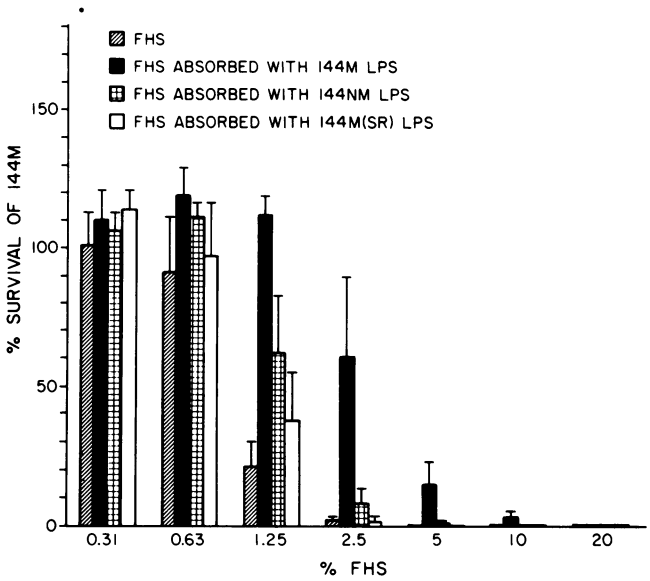

FIG. 6. Bactericidal activity of FHS for 144M before and after absorption of FHS with 144M LPS, 144NM LPS, or 144M(SR) LPS. The percent survival of 144M in FHS or absorbed-FHS in various concentrations was determined after 60 min of incubation at 37°C. Values represent the mean ± SEM based on three experiments.

DISCUSSION

The bactericidal activity of serum is regarded as a significant component of natural host defense mechanisms against gram-negative bacteria, since bacilli isolated from localized infections are often susceptible to serum, whereas those isolated from cases of bacteremia are resistant to serum (31, 42). The results of many studies, reviewed by Taylor (36), suggest that deposition of the assembled terminal complement components of the membrane attack complex (MAC) onto the surface of susceptible gram-negative bacteria is responsible for serum-mediated killing and is a necessary prerequisite for lysozyme-mediated bacteriolysis. Although bacteriolysis occurs to a significant extent only in the presence of lysozyme (18), an enzyme which hydrolyzes the glycosidic linkages in the peptidoglycan layer, its presence is not required for serum bactericidal activity (19, 34).

P. aeruginosa strains isolated from CF patients have been shown to be more susceptible to FHS than were strains from non-CF patients (17, 26, 33, 38). In this study, the bactericidal activity of FHS was examined for three *P. aeruginosa* strains: 144M, originally isolated from the sputum of a CF patient; 144NM, a spontaneous nonmucoid derivative of 144M; and 144M(SR), a mucoid strain isolated by passage of 144M in the presence of increasing concentrations of FHS (32a). Whereas FHS in concentrations of ≥2.5% was capable of killing >95% of strain 144M, strains 144NM and 144M(SR) were resistant to 50% FHS. These strains were not simply exhibiting delayed susceptibility (39, 40), since they still appeared serum resistant even after the incubation with FHS was increased from 1 h to 22 h at 37°C.

Unlike some studies in which the growth medium (9, 35), phase of growth (8, 9), or temperature of growth (22) were found to affect the response of a strain to serum, these parameters altered neither the FHS susceptibility of 144M nor the FHS resistance of 144NM or 144M(SR).

Strain 144M was killed by FHS in a dose-dependent manner and was killed faster by FHS at high concentrations

than by FHS at low concentrations. Wright and Levine (44, 45) have suggested that effective lesions occur only when the MACs are deposited on the bacterial surface at the point of contact between the cytoplasmic and outer membranes. Therefore, the chance of getting an effective bactericidal lesion would increase with higher concentrations of FHS, and the kinetic response would thus be dose dependent.

Complement was shown to be a necessary requirement for killing by FHS, since heat treatment of FHS at 56°C for 30 min (HIS) abolished all bactericidal activity; however, complement by itself was not sufficient for killing, since MabsS was not bactericidal, yet it retained most of its complement activity. Bactericidal activity was restored to MabsS by the addition of either IgG or IgM, although IgM was found to be about 10 times as effective a bactericidal immunoglobulin as IgG. Michael and Rosen (25) have demonstrated that the predominant natural antibodies to gram-negative bacteria are found in the IgM fraction of human plasma. Bjornson and Michael (2) found that as little as 6.5 ng of purified IgM from rabbits immunized with *P. aeruginosa* was effective in bactericidal assays with the homologous strain, whereas little or no bactericidal activity was observed with as much as 39 μg of IgG. In this study, ca. 12 μg of IgM or 115 μg of IgG from nonimmune human serum was required to kill 90% of 144M in the presence of complement. Since pool B (used as an IgG source) had an IgG/IgM ratio of ca. 29:1, there was not enough IgM in this pool to account for the bactericidal activity observed, suggesting that nonimmune IgG was bactericidal for 144M. The bactericidal killing of 144M, however, was optimal when HIS (which contains both IgG and IgM) was added to MabsS.

Activation of complement via the classical complement pathway was found to be essential for killing by FHS, since the addition of Mg-EGTA to serum abolished all bactericidal activity. Inactivation of the alternative pathway via 50°C incubation for 20 min had no effect on killing by FHS in this microassay, although the results of earlier experiments with larger bacterial concentrations (4) suggested that the alternative pathway had a minor, delayed bactericidal reaction. The importance of the classical pathway in killing of *P. aeruginosa* by FHS has also been reported by Thomassen and Demko (38), whereas Meshulam et al. (24) and Offredo-Hemmer et al. (28) have suggested a supportive role for the alternative pathway.

Although removal of lysozyme from FHS by bentonite absorption did not affect the bactericidal activity of 10% FHS, it did significantly reduce the bactericidal activity of 1% FHS. As described by Taylor in his review (36), the role of lysozyme in the bactericidal reaction is somewhat controversial. Although some investigators have determined that lysozyme is not required for serum bactericidal activity (19, 34), Glynn and Milne (13) and Taylor and Kroll (37) found that neutralization of lysozyme activity with antilysozyme antiserum or removal by bentonite adsorption resulted in reduced rates of killing of certain *Escherichia coli* strains by serum. Since the bactericidal activity of serum is due to complement-mediated damage to both the OM and cytoplasmic membranes, the ability of lysozyme to split glycosidic linkages in the peptidoglycan layer should, theoretically, enhance the complement-mediated destruction of the cytoplasmic membrane, especially at low serum concentrations. At higher serum concentrations there is a greater likelihood of effective lesions occurring by the deposition of the MAC on the bacterial surface at the point of contact between the cytoplasmic and outer membranes (44, 45). Alternatively, as suggested by Glynn and Milne (13) and Taylor and Kroll (37), bentonite absorption, in addition to removing lysozyme, might have also removed an additional serum component, distinct from antibody and complement, required for maximal bactericidal activity. Further studies are required to resolve the role of lysozyme in the killing of 144M by FHS.

To determine the bacterial component(s) to which the bactericidal immunoglobulins bind, we performed a series of absorption studies. Absorption of FHS with either 144NM or 144M(SR) whole organisms did not decrease the bactericidal activity of serum (data not shown). In contrast, absorption of FHS with 144M whole organisms totally removed the bactericidal activity of 10% FHS. These studies suggest that the antigen(s) involved with binding the bactericidal antibodies is present on the cell surface of 144M, but is (i) at low epitope density, (ii) not present, (iii) altered, or (iv) inaccessible to antibody in strains 144NM and 144M(SR).

To identify the bactericidal immunoglobulin binding site(s) on the surface of 144M, we isolated the OM of this strain. The gram-negative outer envelope structure has been depicted as LPS embedded in a continuum of proteins and phospholipids. Serum absorbed with 144M OM preparations lost most of the bactericidal activity for 144M, implying that the target(s) for bactericidal immunoglobulin binding is present on the outer membrane and could be either an OM protein or LPS. In contrast, absorption of FHS with OM preparations from 144M(SR) did not decrease its bactericidal activity. Although it was inapparent at FHS concentrations of 10% or greater, absorption of FHS with 144NM OMs did reduce the bactericidal activity of serum, especially at a serum concentration of 2.5%. Therefore, it is possible that 144NM has a bactericidal immunoglobulin binding site in common with 144M but at a much lower epitope density and probably inaccessible on the surface of intact 144NM.

Although an OM protein could be one bactericidal immunoglobulin binding site on 144M, it is considered unlikely to be the main determinant, since trypsin treatment of 144M did not alter its susceptibility to FHS nor inhibit its ability to absorb the bactericidal activity from FHS. On the basis of the difference in serotype response between the parental strain 144M (weakly serotype 3 and 14) and the two serum-resistant strains 144NM and 144M(SR) (strong serotype 6), we took a closer look at LPS from these strains.

FHS absorbed with 144M LPS showed decreased bactericidal activity for 144M as compared to unabsorbed FHS, whereas absorption with LPS from either 144NM or 144M(SR) did not reduce the bactericidal activity of FHS. These results suggest that at least one target for bactericidal antibody on the surface of 144M is LPS.

SDS-polyacrylamide gel electrophoresis of LPS isolated from 144M, 144NM, and 144M(SR) demonstrated that the LPS of the FHS-susceptible parental strain 144M is mainly rough, short LPS, whereas the LPS from the serum-resistant strains 144NM and 144M(SR) display size heterogeneity, including some with a moderate to high number of substituted O-antigenic side-chain moieties, representative of smooth, long LPS types. This observation explains the alteration in serotype configuration in 144M(SR) and 144NM compared to the parental strain 144M, and is consistent with the observation that gram-negative bacteria with smooth LPS are usually more resistant to serum than are strains with rough LPS (36). Examination of two additional FHS-susceptible strains, ByM and WcM, have also demonstrated the presence of rough LPS, whereas their serum-resistant counterparts, ByM(SR) and WcM(SR) (adapted to serum resistance by passage through increasing concentrations of FHS), contained smooth LPS (32a). In addition, Hancock et al. (15)

have recently reported similar LPS gel profiles for FHS-susceptible isolates from CF patients.

Taylor (36) has postulated that serum resistance in gram-negative bacteria is probably due to the interference of bacterial structures with the formation, attachment, and subsequent activity of the MAC. In smooth *Pseudomonas* strains, such as 144NM and 144M(SR), antibody may be bound to the O-antigenic side chain of long LPS, too far from the bacterial cell wall to permit the MAC to interact with the cell surface to effect lethal damage. Alternatively, the long O-antigen side chains may inhibit the action of the MAC by blocking receptors to which activated components must bind in the reaction sequence which causes cell death, or the side chains may mask the bactericidal immunoglobulin binding sites. Studies are in progress to take a closer look at the serum resistance of strains 144NM and 144M(SR).

In summary, the susceptibility of 144M to FHS is thought to be due to the binding of bactericidal IgM and IgG immunoglobulins to rough LPS exposed on the surface of 144M, followed by complement activation via the classical pathway.

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