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Interaction of Complement with Serum-Sensitive and Serum-Resistant Strains of *Pseudomonas aeruginosa*

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The interaction of complement with the following two strains of *Pseudomonas aeruginosa* was examined: 144M, a mucoid, serum-sensitive strain bearing short lipopolysaccharide O chains, and 144M-SR, a mucoid, serum-resistant strain bearing long lipopolysaccharide O chains isolated by repeated passage of 144M in increasing concentrations of pooled normal human serum (PNHS). While significant killing of 144M occurred in 5 to 40% PNHS, no killing of 144M-SR was observed. Both strains activated complement, especially 144M-SR which consumed 88.7, 96.4, and 100% of the available complement 3 (C3), C5, and C9, respectively, in 10% PNHS during a 60-min incubation at 37°C. Although it activated more C3 than did 144M (54.9% consumption), 144M-SR bound only half as much C3 as 144M. Similarly, although 144M-SR activated more C9 than did 144M (50.0% consumption in 60 min), there was considerably less C9 attached to 144M-SR (2,990 molecules of C9 per bacterium) than to 144M (13,700 molecules per bacterium) after 60 min of incubation. Furthermore, only 162 molecules of the C9 bound to 144M-SR remained bound after treatment with 0.1% trypsin, while 5,692 molecules of the C9 bound to 144M remained bound under similar conditions. These results show that the serum resistance of 144M-SR does not represent a failure to activate complement efficiently, but instead reflects failure of the assembled terminal complement complex C5b-9 to insert stably into the outer membrane of this strain.

Optimal killing of gram-negative bacteria in serum requires the participation of terminal components of the complement system, presumably through the formation of a complement 5b-9 (C5b-9) complex containing complement components C5b6789 (7, 11, 29). Resistance to complement-mediated killing is an attribute of many gram-negative bacteria possessing a smooth phenotype, that is, containing lipopolysaccharide (LPS) molecules with long polysaccharide side chains (O antigen). This conclusion is based on studies in which rough isogenic mutants were compared with smooth parental strains of *Escherichia coli* and *Salmonella* spp. (22, 23, 25).

Most strains of *Pseudomonas aeruginosa* isolated from blood, wounds, urine, or burns are serum resistant (24, 28, 34), although the nature of this resistance is not known. In contrast, most strains of *P. aeruginosa* isolated from the sputum of patients with cystic fibrosis are serum sensitive and deficient in LPS O side chains (9, 10, 19, 21, 28, 33). To examine the nature of serum susceptibility or resistance in *P. aeruginosa*, a series of serum-resistant derivative strains were isolated from serum-sensitive parental cystic fibrosis strains (27). In contrast to the parental strains, these derivative strains contained smooth-type LPS containing O side chains. In this study the interaction of complement with one of these derivative strains, 144M-SR, and its serum-sensitive parental counterpart, 144M, was examined to characterize the mechanism of serum resistance of the derivative strain.

MATERIALS AND METHODS

The following buffers were used (14): Hanks balanced salt solution (HBSS; GIBCO Laboratories, Chagrin Falls, Ohio) containing 0.15 mM $CaCl_2$ and 1.0 mM $MgCl_2$ ($HBSS^{++}$); isotonic Veronal-buffered saline containing 0.1% gelatin, 0.15 mM $CaCl_2$, and 1.0 mM $MgCl_2$ ($VBSG^{++}$); and $VBSG^{++}$ diluted with 5% glucose to 0.060 μ ($DVBSG^{++}$).

Bacterial strains and culture conditions. *P. aeruginosa* 144M, a serum-sensitive mucoid strain obtained from the sputum of a patient with cystic fibrosis, and its serum-resistant mucoid derivative, 144M-SR, isolated by passage of 144M in the presence of increasing concentrations of human serum (27), were maintained on brain heart infusion (BHI) agar (England Labs, Beltsville, Md.) at 37°C and transferred daily. Both strains had stable mucoid phenotypes.

Each strain was grown to mid-log phase (5 to 6 h) in BHI broth at 37°C with agitation, harvested by centrifugation at 20,000 × *g* for 10 min at 4°C, washed twice with $HBSS^{++}$, and resuspended in $HBSS^{++}$ to an optical density of 1.0 at 600 nm. This optical density corresponded to approximately 8×10^8 CFU/ml for 144M and 1×10^9 CFU/ml for 144M-SR.

Pooled normal human serum. Blood was obtained by venipuncture of 5 normal healthy adult male volunteers and was allowed to clot at room temperature for 30 min. After centrifugation at 1,000 × *g* for 15 min at 4°C, the serum was pooled, filter sterilized through a 0.22-μm-pore-size filter, and stored at −80°C in small fractions until use. For some experiments, complement in pooled normal human serum (PNHS) was inactivated by heating at 56°C for 30 min.

Serum bactericidal assay. Equal volumes of various dilutions of PNHS in $HBSS^{++}$ at 4°C and organisms at an optical density of 1.0 at 600 nm in $HBSS^{++}$ were mixed in plastic tubes (12 by 75 mm) and immediately incubated at 37°C in a water bath with intermittent shaking. At various times thereafter, 30-μl samples were removed and serially diluted in HBSS, and 50 μl was plated on BHI agar plates. Colonies were counted after overnight incubation at 37°C, and the results were expressed as $\log_{10}$ CFU/ml. The extent of killing was expressed as $\log_{10}$ kill, calculated as $\log_{10}$ CFU/ml in

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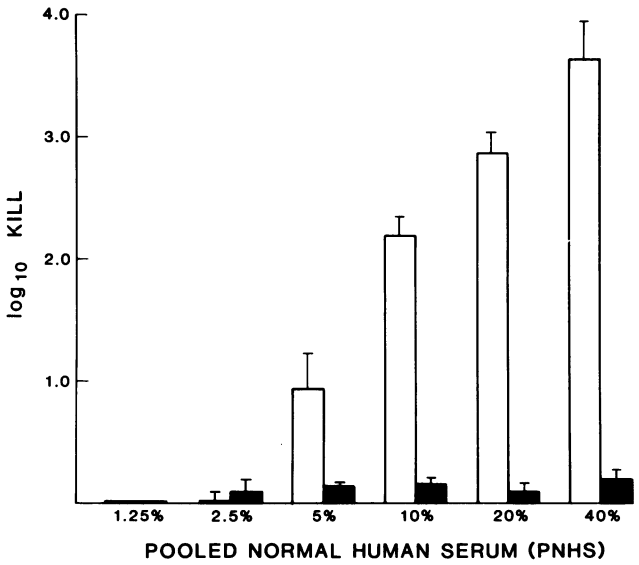

FIG. 1. Bactericidal activity of PNHS for strains 144M and 144M-SR. Bacteria at a concentration of ~4 × 10^8 cells per ml (144M) or ~5 × 10^8 cells per ml (144M-SR) were incubated in various concentrations of PNHS or heat-inactivated PNHS for 60 min at 37°C. The bactericidal activity of PNHS was determined by calculating the $\log_{10}$ kill at each serum concentration, where $\log_{10}$ kill = $\log_{10}$ CFU in heat-inactivated PNHS − $\log_{10}$ CFU in PNHS. Values represent the mean ± standard error of the mean based on three experiments (except for values for 10% PNHS which were derived from 11 experiments). Symbols: □, 144M; ■, 144M-SR.

heat-inactivated serum minus $\log_{10}$ CFU/ml in unheated serum.

Purification and radiolabeling of complement components. C3 and C9 were purified from fresh human plasma by using modifications of the procedure published by Hammer et al. (8). C3 and C9 were radiolabeled with $Na^{125}I$ by using Iodobeads (Pierce Chemical Co., Rockford, Ill.) to specific activities of 7.2 × 10^5 or 8.4 × 10^5 cpm/μg for C3 and 1.3 × 10^6 cpm/μg for C9.

Consumption of C3, C5, and C9. Consumption of hemolytic C3, C5, and C9 in reaction mixtures containing bacteria and serum was measured. Reaction mixture supernatants were collected by centrifugation of reaction mixtures at 12,500 × *g* for 5 min at 4°C to pellet the bacteria. Hemolytic titrations on reaction mixture supernatants were performed within 1 h after collection by using the appropriate complement-cellular intermediates at 1.5 × 10^7 cells (EAC 1,4 or EAC 1-7) in a total reaction volume of 0.5 ml after minor modifications of standard techniques (5). Controls included serum in $HBSS^{++}$ without bacteria, which was incubated and handled concomitantly with the test samples.

Binding of C3 and C9. Quantitation of C3 and C9 binding to the bacterial surface was measured as described previously (14). Briefly, mixtures of serum and bacteria were prepared as described above for the serum bactericidal assay, except that ~1 to 3 μg of either ^{125}I-labeled C3 or ^{125}I-labeled C9 was added per ml of reaction mixture. Samples were incubated at 37°C. At designated intervals samples of 200 μl were removed and added to microcentrifuge tubes containing 1 ml of cold $HBSS^{++}$, and the tubes were centrifuged for 8 min at 12,500 × *g* at 4°C. The supernatants were aspirated, and the pellets were counted in a gamma counter. Controls for nonspecific binding of ^{125}I-labeled C3 and ^{125}I-labeled C9 were bacteria that were incubated in serum that had been heated previously for 30 min at 56°C to block complement activation. The total number of molecules of C3 and C9 bound to the bacterial surface were calculated as described previously (14).

Elution of C9 from 144M or 144M-SR. Susceptibility of bound ^{125}I-labeled C9 to removal from the bacterial surface by buffers, salt, or trypsin was measured. A 1.0-ml fraction was removed from the reaction mixture after incubation in 10% PNHS in $HBSS^{++}$ containing ^{125}I-labeled C9 for 30 min (144M-SR) or 60 min (144M) at 37°C. The bacterial pellet was sedimented by centrifugation at 12,500 × *g* for 8 min at 4°C and washed twice in $DVBSG^{++}$, $VBSG^{++}$, or 1 M NaCl in $VBSG^{++}$. Pellets were suspended to the original volume after the second wash in the same solution that was used to wash the pellets. In addition, one pellet washed twice in $DVBSG^{++}$ was suspended to the original volume in 0.1% trypsin, tosyl-L-phenyl chloroketone treated. (Worthington Diagnostics, Freehold, N.J.) in $VBSG^{++}$. These samples were incubated at 37°C for 30 min, and counts of ^{125}I-labeled C9 remaining in the bacterial pellet were calculated as described above.

RESULTS

Serum bactericidal assays. The susceptibility of strains 144M and 144M-SR to PNHS was determined by incubating each strain for 60 min at 37°C in the presence of PNHS or heat-inactivated PNHS at concentrations ranging from 1.25 to 40% serum. Killing of 144M occurred in a dose-dependent fashion, with significant killing (>0.5 $\log_{10}$ kill) resulting from incubation in PNHS concentrations of 5 to 40% (Fig. 1). In contrast, no significant killing of 144M-SR was observed in any concentration of PNHS examined. For the remainder of the experiments described, a concentration of 10% PNHS was employed, with a mean ± standard error of the mean $\log_{10}$ kill of 2.20 ± 0.16 for 144M and 0.16 ± 0.05 for 144M-SR (based on 11 experiments) after incubation for 60 min at 37°C. To address the mechanism of serum resistance in 144M-SR, the ability of this strain to activate and bind individual complement components was compared to that of its serum-sensitive parental strain 144M.

Binding and consumption of C3 by 144M and 144M-SR. The kinetics of ^{125}I-radiolabeled C3 binding to the bacterial surface of these two strains was examined in 10% PNHS over a 120-min period at 37°C (Fig. 2). The binding of C3 to both strains began immediately, started to plateau at 60 min for 144M and 30 min for 144M-SR, and remained stable for up to 120 min. There were more molecules of C3 bound to the serum-sensitive strain 144M than to the serum-resistant strain 144M-SR at each time point we examined.

We next examined whether the greater binding of C3 to 144M represented more efficient activation of complement by this strain. In these experiments, the loss of C3 hemolytic activity in 10% PNHS incubated with either 144M or 144M-SR was monitored as a function of incubation time at 37°C. 144M-SR consumed C3 more rapidly and completely than did 144M (Fig. 2). After 60 min of incubation at 37°C, 144M-SR consumed an average of 88.7% of the available C3 compared with only 54.9% consumed by 144M. These experiments suggest a dissociation between C3 consumption and C3 deposition when 144M and 144M-SR are compared, and experiments are currently under way to examine this issue in greater detail.

Consumption of C5 and C9 by 144M and 144M-SR. The next series of experiments examined whether strains 144M and 144M-SR could activate C5 and C9, both terminal

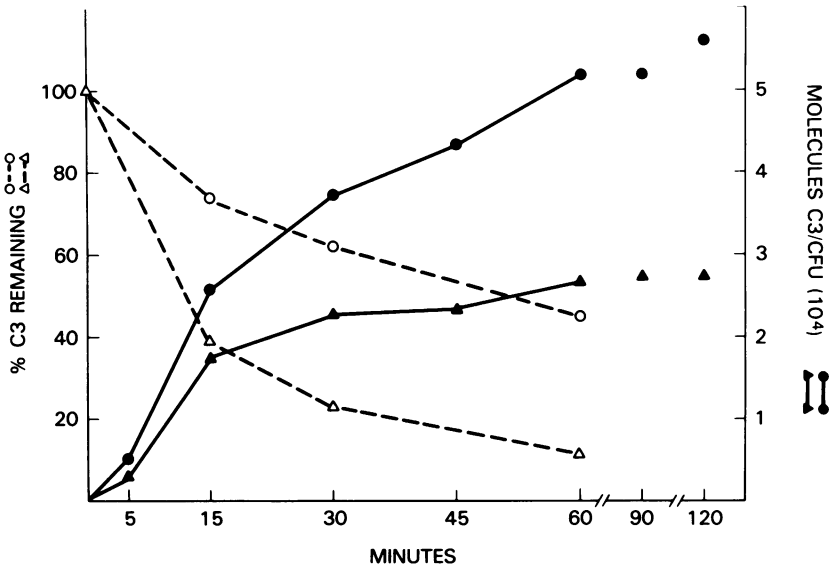

FIG. 2. Consumption and uptake of C3 by *P. aeruginosa* 144M and 144M-SR. Bacteria, at a concentration of ~4 × 10^8 cells per ml (144M) or ~5 × 10^8 cells per ml (144M-SR), were incubated in 10% PNHS in $HBSS^{++}$ containing ^{125}I-labeled C3. The control tube for C3 consumption contained 10% PNHS in $HBSS^{++}$ without bacteria. Control tubes for ^{125}I-labeled C3 binding contained 10% heat-inactivated PNHS in $HBSS^{++}$ and either 144M or 144M-SR at the concentration indicated above. Samples were removed at the designated times for measurement of C3 consumption and specific ^{125}I-labeled C3 binding, and the total number of molecules of C3 bound per CFU were calculated. All values for C3 consumption were expressed relative to the control tube at each time point. Values for C3 consumption represent the average of two experiments; values for C3 binding represent the average of three experiments, each of which was done in duplicate. Symbols for C3 consumption: ○, 144M, △, 144M-SR. Symbols for C3 binding: ●, 144M; ▲, 144M-SR.

complement components, and essential members of the C5b-9 complex. As with C3, 144M-SR was found to consume both C5 and C9 more rapidly and completely than 144M, with 96.4 and 100% consumption of C5 and C9, respectively, by 144M-SR after 60 min of incubation at 37°C (Fig. 3). In contrast, 144M consumed only 27.4% of C5 and 50.0% of C9 under the same conditions. The greater consumption of C5 and C9 by 144M-SR compared with 144M suggests that the mechanism of serum resistance for 144M-SR does not reflect a failure to activate the terminal complement cascade.

Binding of C9 to 144M and 144M-SR. To test the binding of C9 to 144M and 144M-SR, we compared the kinetics of ^{125}I-labeled C9 uptake by the two test strains. There was an initial deposition of C9 on the surface of 144M-SR which peaked at 15 min, followed by a gradual decline over the next 105 min (Fig. 4). In contrast, a lag in C9 binding occurred on 144M, but deposition increased rapidly after 15 min and continued to increase over the 120-min incubation period. At 60 min there was 4.6 times more C9 on 144M (13,700 molecules of C9 per CFU) than on 144M-SR (2,990 molecules of C9 per CFU).

The kinetics of C9 deposition correlate with the kinetics of C9 consumption by these strains (Fig. 3). With 144M-SR greater than 99% of the available C9 was consumed by 30 min of incubation, a point at which no further C9 deposition was noted on this strain. In contrast, 144M consumed only 11.2% of the C9 by 15 min (a point at which there were only 1,330 molecules of C9 deposited on 144M) and used only 50.0% of the available C9 by 60 min, at which time C9 deposition on 144M was still in progress.

Elution of C9 from 144M-SR. The nature of the attachment of C9 to 144M and 144M-SR was investigated next. The bacteria were incubated in 10% PNHS containing ^{125}I-labeled C9 for 60 min for 144M and 30 min for 144M-SR (which was the time observed for maximum C9 deposition on this strain). After these mixtures were centrifuged, the bacterial pellet with C9 attached was washed twice and suspended to the original concentration in $DVBSG^{++}$, $VBSG^{++}$, 1 M NaCl in $VBSG^{++}$, or 0.1% trypsin in $VBSG^{++}$ for 30 min at 37°C. The amount of C9 left on the bacterial surface was then determined as described above and compared with that in the control tube, which represents

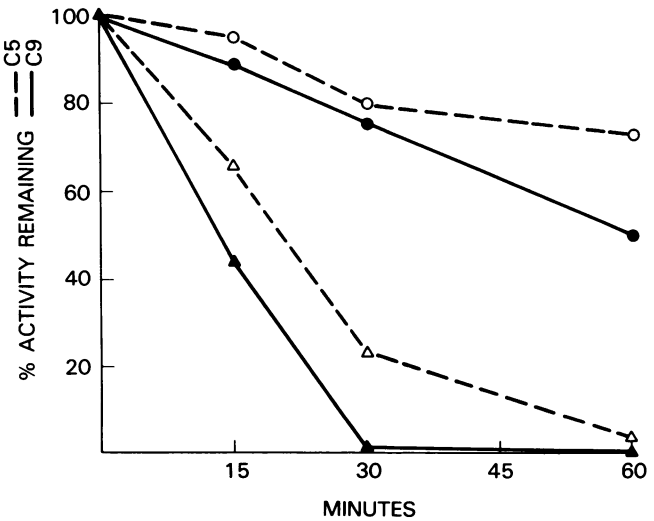

FIG. 3. Consumption of C5 and C9 by *P. aeruginosa* 144M and 144M-SR. Experimental conditions were as outlined in the legend to Fig. 2. All values for percent consumption were expressed relative to the control tube at each time, and represent the average of two experiments. Symbols for 144M: ○, C5; ●, C9. Symbols for 144M-SR: △, C5; ▲, C9.

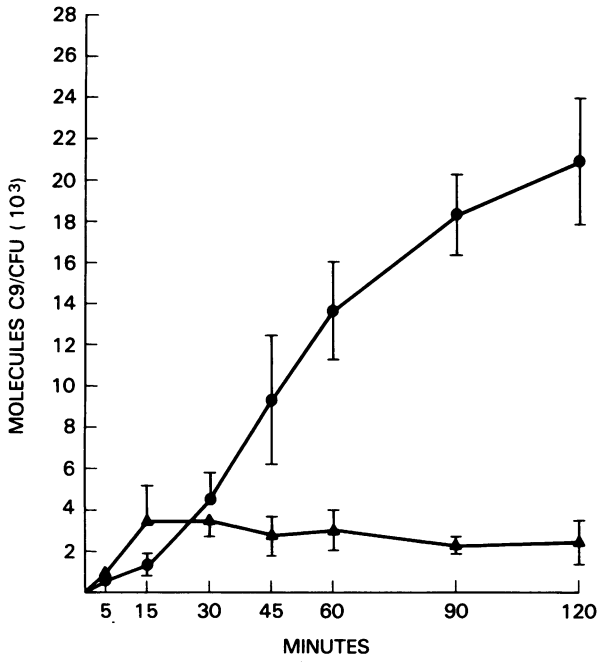

FIG. 4. Uptake of C9 by *P. aeruginosa* 144M and 144M-SR in 10% PNHS. Experimental conditions were as outlined in the legend to Fig. 2, except serum contained ^{125}I-labeled C9. Values represent the mean ± standard error of the mean based on four or five separate experiments, each of which was done in duplicate (except for the 5-min time point, which represents the average of two experiments, done in duplicate). Symbols: ●, 144M; ▲, 144M-SR.

the number of C9 molecules per CFU before washing and resuspension (Fig. 5).

First, washing and resuspension of 144M bearing ^{125}I-labeled C9 caused a loss of C9 of 21.2% (DVBSG^{++}), 26.5% (VBSG^{++}), 30.2% (NaCl), or 39.7% (trypsin). However, even under conditions leading to the most extensive release of ^{125}I-labeled C9, there were still approximately 5,700 molecules of C9 associated with 144M. In contrast, there was a greater loss of C9 from 144M-SR following these treatments, with decreases of C9 of 19.8% (DVBSG^{++}), 42.9% (VBSG^{++}), 68.5% (NaCl), and 93.4% (trypsin) observed. Considering the relatively few molecules of C9 bound per 144M-SR to begin with (control had 2,460 molecules of C9 per CFU), these treatments greatly reduced the C9 on 144M-SR, leaving only 162 molecules of C9 per CFU after trypsin treatment. These data suggest that most of C5b-9 is not inserted into the outer membrane of 144M-SR, a prerequisite for the bactericidal activity of the terminal components of the membrane attack complex (7, 11, 29).

DISCUSSION

The central question with regard to complement resistance is whether this resistance represents inefficient complement activation or instead represents subversion of the membrane attack process at some later step. There is growing evidence that the latter mechanism is operative under most circumstances. For example, C5b-9 complexes are formed and deposited on the surface of serum-resistant *Salmonella minnesota* but are not effectively inserted into the bacterial membrane and are therefore released (14, 15). Similar findings were also observed with serum-resistant strains of *E. coli* (6, 16, 31).

In a previous study, the susceptibility of 144M to PNHS had been demonstrated to be due to the binding of bactericidal immunoglobulin G (IgG) or IgM to rough LPS exposed on the surface of 144M, followed by complement activation via the classical pathway (26). In this study the resistance of strain 144M-SR to killing by serum was not attributable to an inability to activate complement; on the contrary, 144M-SR activated complement more rapidly and efficiently than did its serum-sensitive counterpart 144M, based on examination of C3, C5, and C9 consumption experiments. However, this activation by 144M-SR did not directly translate into complement component deposition on the surface of this strain. In fact, although 144M-SR consumed more C3, C5, and C9 per organism than did 144M, it deposited only about half as much C3 and much less C9 on its surface than did 144M. Furthermore, most of the C9 deposited on the surface of 144M-SR was removable with buffer, salt, or trypsin. This suggests that the C9 on the surface of 144M-SR was not inserted into the hydrophobic domain of the outer membrane of this bacterium. In contrast, more than 60% of the C9 deposited on 144M (~5,700 molecules of C9 per CFU) was not removable by these treatments, suggesting that these C9 molecules were inserted into the membrane. Our results are reminiscent of those of previous studies (14–16) demonstrating that killing of *S. minnesota* and *E. coli* is associated with increased binding of C5b-9 to the outer membrane in a form resistant to salt and protease release. These findings suggest that inhibition of C9 insertion into the outer membrane is one common mechanism of serum resistance among gram-negative bacteria.

Results of previous studies have demonstrated that 144M-SR differs from 144M in the presence of a long LPS O side chain in 144M-SR (26, 27). Results of studies with other gram-negative bacteria have suggested that the presence of long O side chains is critical for serum resistance (22, 23, 25, 32). By using a strain of *Salmonella montevideo*, in which the length and distribution of O side chains of LPS could be

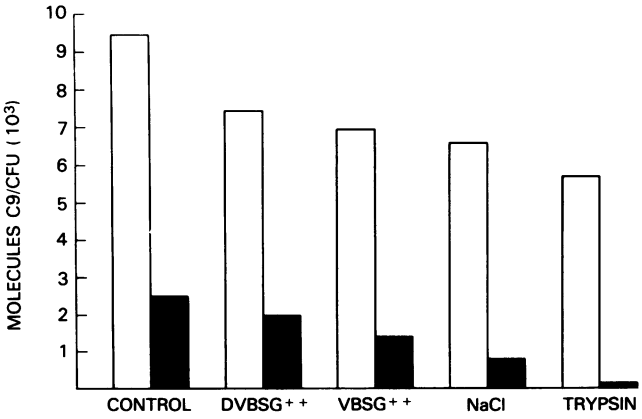

FIG. 5. Release of bound C9 from *P. aeruginosa* 144M and 144M-SR. Bacteria at a concentration of ~6 × 10^8 cells per ml (144M or 144M-SR) were incubated in 10% PNHS in HBSS^{++} containing ^{125}I-labeled C9 for either 60 min (144M) or 30 min (144M-SR) at 37°C. After the bacteria bearing ^{125}I-labeled C9 were centrifuged, the pellets were washed twice and then suspended to the original concentration in DVBSG^{++}, VBSG^{++}, 1 M NaCl, or 0.1% trypsin and incubated for an additional 30 min at 37°C. The molecules of C9 remaining bound were then determined and compared with the control tube, which represents the number of C9 molecules bound per cell before washing and resuspension. Values represent the average of three experiments. Symbols: □, 144M; ■, 144M-SR.

controlled, C3 was found to preferentially attach to the small subset of LPS molecules bearing the longest O side chains (13). These results suggest that C3 is sterically hindered from binding to short-chain LPSs. Using the same strain, Leive et al. (18) found that subtle alterations in either the O side chain length or coverage of lipid A core molecules with O side chains dramatically affected serum resistance, supporting the concept that steric hindrance to complement attack is the central factor leading to serum resistance for gram-negative bacteria. In support of this, rendering a serum-resistant isolate of *E. coli* serum sensitive by preincubation in 0.01 M EDTA, a procedure known to release LPS (17), resulted in enhanced and stable binding of C5b-9 to this organism (12). Preliminary studies have demonstrated that EDTA treatment of 144M-SR also dramatically increases C9 attachment to the bacterial surface (N. L. Schiller, unpublished data).

Interestingly, there appeared to be a clear dissociation between complement consumption (such as C3 or C9) and the amount of C3 or C9 deposited on the bacterial surface when 144M and 144M-SR were compared. While 144M-SR consumed more C3, C5, and C9 than did 144M, 144M-SR bound considerably less C3 and C9. This suggests that the presence of long LPS O side chains on 144M-SR interferes with the binding of C3 or C9 to the bacterial surface and the subsequent insertion of C9 into the outer membrane. Furthermore, the C9 associated with the surface of 144M-SR can be removed by incubating the bacteria in buffers or salt solutions or by treatment with trypsin.

With regard to C3, it appears that only a very small fraction of the C3 activated by 144M-SR can find a suitable binding site on the surface of these bacteria, whereas a much higher percentage of activated C3 attaches to the surface of 144M. It remains to be determined whether 144M-SR simply has fewer C3 acceptor sites than 144M, or whether many of the C3 acceptor sites on 144M-SR are covered by the long LPS O side chains. An alternative explanation is that C3 is enzymatically digested by bacterial proteases, rather than activated through the classical pathway, and thus is unable to bind to the bacterial surface. Schultz and Miller (30) have reported that *P. aeruginosa* elastase is capable of inactivating several complement components, including C3. However, neither 144M nor 144M-SR bacterial pellets nor culture supernatants directly consumed C3, as measured by depletion of hemolytic activity (data not shown). Furthermore, magnesium EGTA treatment of PNHS, which blocks the classical pathway, totally blocked the consumption of C3 by 144M-SR, suggesting that the consumption of C3 was a result of complement activation by the classical pathway (data not shown). Recently, Engels et al. (2, 3) also described a lack of correlation between C3 fixation and C3 consumption on strains of *P. aeruginosa*. They postulated that C3 might be released from the surface of bacteria by the solubilizing effect of complement on preformed IgG-C3b complexes (2). Moreover, Fries et al. (4) have demonstrated that C3b complexed with IgG is significantly more effective in alternative pathway consumption of C3 than is fluid-phase C3b. By using an *E. coli* strain carrying the plasmid-encoded outer membrane protein, *traT*, a protein conferring resistance to the antibacterial activity of serum (20), Aguero et al. (1) found that this protein also reduces the deposition of C3 on the bacterial surface and alters its distribution, thus antagonizing the complement-mediated opsonization of this strain. Further studies are in progress to explain the basis for this discrepancy between C3 consumption and binding observed on these two *P. aeruginosa* strains.

In summary the serum resistance of *P. aeruginosa* 144M-SR does not represent a failure to activate complement effectively, but instead reflects a failure of the assembled terminal complement complex C5b-9 to insert stably into the outer membrane of this strain. This mechanism of serum resistance is similar to that previously described for other strains bearing long LPS O side chains, such as certain *S. minnesota* and *E. coli* strains, suggesting that a common strategy is employed among these gram-negative bacteria for avoiding the effects of this important host defense mechanism.

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