# STUDIES ON THE MECHANISM OF BACTERIAL RESISTANCE TO COMPLEMENT-MEDIATED KILLING

## I. Terminal Complement Components Are Deposited and Released from *Salmonella minnesota* S218 without Causing Bacterial Death

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It has been recognized since 1895 (1) that some gram-negative bacteria are sensitive to the lytic action of fresh serum, whereas others are highly serum resistant. In general, serum-resistant organisms are more pathogenic than serum-sensitive bacteria in animal models of infection, and serum-resistant organisms are more commonly isolated from the bloodstream of patients with gram-negative bacteremia (2). In attempts to define the basis of this important virulence factor, characteristics of the outer membrane of serum-sensitive and serum-resistant organisms have been analyzed and compared (3–5). The presence of a complete lipopolysaccharide (LPS)[1] (i.e., the smooth phenotype) is the characteristic most clearly associated with serum resistance. Rough bacteria lacking a complete LPS are almost invariably serum sensitive.

The antibody and complement requirements for serum killing of bacteria also have been examined. It has been shown (6–8) that killing of gram-negative bacteria by serum requires the participation of terminal components of the complement system (C5–9). However, the mechanism of resistance of gram-negative bacteria to serum killing in the presence of adequate antibody is still unknown.

Resistance to serum killing could involve the inability to form a membrane attack complex on the organism. An alternative hypothesis, however, is that a membrane attack complex that forms on the bacterial surface may be functionally impotent either because of failure to insert into the bacterial outer membrane or because the inserted complex does not cause damage to vital outer or inner membrane structures.

Previous studies have examined a number of aspects of this issue. Studies have suggested that serum-sensitive and serum-resistant strains of *Escherichia coli* (9, 10) or *Salmonella typhimurium* (11) have equivalent amounts of C3 deposited. It is not resolved whether C5 is deposited on serum-resistant bacteria. Reynolds et al. (11) could not demonstrate deposition of functional C5 on serum-resistant *S. typhimurium* in $Mg^{++}$ saline after incubation in C6-deficient rabbit serum. On the other hand, Ogata and Levine (10) demonstrated equivalent C5 consumption by strains of *E. coli* that varied in complement sensitivity; however, evidence for levels of cell-bound C5 was not

[1] *Abbreviations used in this paper:* CFU, colony-forming unit; HBSS, Hanks' balanced salt solution; LPS, lipopolysaccharide; PNHS, pooled normal human serum; RT, room temperature.

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provided. No studies have quantitatively evaluated fixation of terminal complement proteins to the bacterial surface of serum-resistant gram-negative bacteria.

We examined the interaction of terminal complement components with serum-resistant bacteria in a systematic fashion. Using highly purified, functionally active, radiolabeled complement components, we examined deposition of C3, a component not associated with the terminal attack complex, as well as deposition of C5, C7, and C9 (the first, third, and fifth members of the five-member terminal attack complex). The results of this study demonstrate that there is no block in complement utilization by these serum-resistant organisms. On the contrary, the serum-resistant bacteria possess a unique mechanism allowing them to assemble and shed the intact terminal complex without sustaining lethal membrane damage.

## Materials and Methods

*Buffers.* The following buffers were used: veronal-buffered saline containing 0.1% gelatin, 0.15 mM $CaCl_2$, and 1.0 mM $MgCl_2$ ($VBSG^{++}$); Hanks' balanced salt solution (HBSS); and HBSS with 0.15 mM $CaCl_2$ and 1.0 mM $MgCl_2$ ($HBSS^{++}$).

*Bacteria.* *Salmonella minnesota* Re595 and *S. minnesota* S218 were kindly provided by Dr. Jacik Hawiger, Vanderbilt University, Nashville, TE. *S. minnesota* S218 is a smooth, wild-type organism containing a complete LPS. This organism is reported to be serum resistant (12). *S. minnesota* Re595 is a mutant of the above parent strain. This deep rough mutant contains only lipid A and 2-keto 3-deoxy octonate in the LPS and lacks the remainder of the core polysaccharide and O-specific polysaccharide moieties present in the parent strain (13). It is reported to be highly sensitive to the lytic action of serum (12).

Bacteria were inoculated from frozen stocks onto GC agar base plates containing horse blood and 1% Isovitalex. Plates were incubated overnight at 37°C. Organisms were then inoculated into trypticase soy broth and incubated for 6–6½ h at 37°C in a rotating rack. Bacteria were washed two times in HBSS at room temperature (RT) and suspended to $OD_{600} = 1.240$ in $HBSS^{++}$. This optical density corresponded to $1.46 \times 10^9$ colony-forming units (CFU)/ml for S218 and $1.12 \times 10^9$ CFU/ml for Re595.

*Serum.* Pooled normal human serum (PNHS) was obtained from 10 volunteers. Antibody titers in PNHS to S218 and Re595, measured by bacterial agglutination of $1 \times 10^9$ CFU/ml were 1:32 and 1:8, respectively. Sera were obtained from patients with a complete deficiency of C2 or C8, and plasma from a patient with complete deficiency of C5 was the kind gift of Dr. Henry Gewurz, Rush Medical School, Chicago, IL.

*Serum Bactericidal Test.* Equal volumes of various dilutions of PNHS in $HBSS^{++}$ at 4°C and a bacterial suspension adjusted to the desired optical density were mixed in 12 × 75-mm plastic tubes and immediately incubated at 37°C in a water bath with intermittent shaking. At varying times thereafter, 30- to 50-μl samples were removed for quantitative bacterial cultures. Aliquots were serially diluted in HBSS, and 40 μl was plated on GC agar base horse blood plates. Colonies were counted after overnight incubation, and 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 heat-inactivated serum minus $\log_{10}$ CFU/ml in unheated serum.

*Ab Preparation and Presensitization.* Antibody to Re595 and S218 was raised in rabbits as previously described (14). Immunoglobulin-containing fractions containing IgM and IgG were prepared by 5% polyethylene glycol precipitation and octanoic acid precipitation. Antibody titers of immune rabbit serum or immunoglobulin fractions were measured by bacterial agglutination at 4°C using $1 \times 10^9$ CFU/ml of viable organisms or at RT using an equivalent number of heat-killed organisms. Titers of immune serum were 1:128 for S218 and 1:32 for Re595. Titers of immunoglobulin fractions were 1:512 for S218 and 1:256 for Re595. Bacteria were presensitized in some experiments by incubation of $6 \times 10^8$ CFU of Re595 or $7 \times 10^8$ CFU of S218 with either 4 ml of heated immune rabbit sera diluted 1:32 for Re595 and 1:128 for S218 or 4 ml of a 1:512 dilution of the immunoglobulin fraction. Bacteria were incubated with antibody for 30 min at RT and washed twice in HBSS.

*Purification and Iodination of Complement Components.* Purification of functionally active C3, C5,

C7, C8, and C9 was performed with minor modifications, as previously described by Hammer et al. (15), from a 2-liter pool of fresh normal human EDTA-plasma. The C9 was brought to homogeneity by chromatography on Biogel A-0.5m, followed by passage over an immunoadsorbent column with antibodies to C4, IgG, IgA, and albumin. Purity of the isolated proteins was assessed, in part, by sodium dodecyl sulfate polyacrylamide gel electrophoresis and by immunoelectrophoresis in 1.0% agarose.

Radiolabeling of C3, C5, and C7 with $^{125}I$ was performed using Inman's modification of the Bolton-Hunter technique (16.) Radioiodination of C9 with $^{125}I$ or $^{131}I$ was done by the solid-phase glucose oxidase-lactoperoxidase method, (Enzymobeads, Bio-Rad Laboratories, Richmond, CA). There was no loss of hemolytic activity of C3, C7, or C9 with labeling, whereas C5 preparations sustained losses of hemolytic activity of ~15%. Specific radioactivity of labeled components was C3, $5.25 \times 10^5$ cpm/μg; C5, $4.99 \times 10^5$ cpm/μg; C7, $3.82 \times 10^5$ cpm/μg; and C9, $1.74 \times 10^6$ cpm/μg.

*Quantitation of Radiolabeled Component Binding to Bacteria.* Radioiodinated C3, C5, C7, or C9 were added to diluted serum before mixing with bacteria to attain between $5 \times 10^5$ and $2 \times 10^6$ cpm/ml. Mixtures of bacteria and serum prepared as described under Serum Bactericidal Test were incubated at 37°C. At designated times, 200 μl of reaction mixture was removed, added to 1.0 ml of ice-cold HBSS in a 1.5-ml high-speed centrifuge tube, and immediately centrifuged for 5 min at 12,500 *g* at RT (Eppendorf, Brinkman Instruments, Westbury, NY). The supernatant was removed by vacuum suction, and the bottom 5 mm of the tube containing the bacterial pellet was removed for counting. This method was shown in preliminary experiments to give the highest percentage of specific binding of radiolabeled components, calculated as:

$$\text{percent specific binding} = \frac{\text{cpm pellet (serum)} - \text{cpm pellet (heat-inactivated serum)}}{\text{cpm input}} \times 100.$$

The cpm in the pellet of heat-inactivated serum mixture was 0.3–0.5% of total input counts. Molecules of radiolabeled component bound per CFU were calculated from the specific cpm bound per pellet, the known original CFU per pellet, and the specific radioactivity of the labeled component. Total molecules of component bound (labeled plus unlabeled) were then derived from the ratio of hemolytic units of unlabeled component to hemolytic units of labeled component in the reaction mixture. The ratio of bound C9 to bound C7 was calculated from values for total molecules of each component per CFU. All measurements of binding of radiolabeled components were done in duplicate on at least two occasions.

This procedure allows one to quantitatively collect lysed as well as unlysed bacteria, as determined by an experiment in which the serum-sensitive Re595 organisms were incubated in C8D human serum. The uptake of C3, C5, and C7 on these viable bacteria after high-speed centrifugation was equivalent to results in normal human serum in which >99% of bacteria were killed.

*Consumption of Hemolytic Activity.* Consumption of hemolytic C3, C5, C7, C8, and C9 activity in reaction mixtures containing either serum-sensitive or serum-resistant organisms was measured. Hemolytic titrations on reaction mixture supernatants were performed within 1 h after collection, using the appropriate complement-cellular intermediate at $1.5 \times 10^7$ cells (EAC 1, 4, or EAC 1–7) in a total reaction volume of 0.5 ml after minor modifications of standard techniques (17). Controls included serum in $HBSS^{++}$ without bacteria, incubated and handled concomitantly with the test samples.

*Release of [$^{14}C$]Phospholipid from Re595 and S218.* For measurement of [$^{14}C$]phospholipid release, Re595 and S218 were initially grown overnight in trypticase soy broth (TSB). Then, 4.5 ml of Tris glucose media (18) containing 0.03% glucose and 2 μCi/ml [$^{14}C$]glucose (329 mCi/mmol; New England Nuclear, Boston, MA) was added to the pellet from 0.5 ml of the overnight TSB broth culture. The organisms were incubated for 4 h at 37°C, washed three times in HBSS, and suspended to OD = 1.240 in $HBSS^{++}$. Equal volumes of the bacterial suspension and varying concentrations of PNHS diluted in $HBSS^{++}$ were mixed. Samples of 500 μl were withdrawn at differing times, the bacterial pellet was sedimented at 12,500 *g* for 5 min, and the supernatant was removed. Lipids were extracted from some samples by the method of Bligh and Dyer (19). Aliquots of 50 μl were added to 10 ml of Aquasol (New England

Nuclear) and counted in a beta scintillation counter (LS8100; Beckman Instruments, Inc., Fullerton, CA).

*Electron Microscopy.* Bacteria in Tris buffer were negatively stained with 2% ammonium molybdate and examined by transmission electron microscopy at 75 Kv in a Hitachi instrument, model HU-11C.

## Results

*Serum Susceptibility of S. minnesota Re595 and S218 in PNHS, Adsorbed PNHS, MgEGTA Serum, and C2D Serum.* Initial experiments examined the serum sensitivity of *S. minnesota* strains. *S. minnesota* S218 was resistant to killing by normal human serum after incubation for 1 h at 37°C at serum dilutions between 0.31 and 40%; colony counts decreased by only 20% when S218 was incubated in 80% PNHS. In addition, there was no killing in 10% PNHS in three different buffer systems ($HBSS^{++}$, $VBSG^{++}$, and 0.05 M or 0.1 M Tris). *S. minnesota* Re595 bacterial lysis in PNHS followed a sigmoidal curve when plotted as log kill vs. percent serum (Fig. 1). In 10% PNHS, 99.9% of bacteria were killed (log killing ± SD in 10% PNHS was 2.84 ± 0.26 in 23 separate experiments). Presensitization of S218 with immune rabbit serum or with purified immune IgG and IgM for 30 min at RT, followed by washing and incubation with 10% PNHS for 60 min did not result in serum sensitivity. Killing of Re595 was decreased 20–30% in 10% PNHS, which was pre-adsorbed with $2.5 \times 10^{10}$ CFU/ml serum for 30 min at 0°C, suggesting that antibody augments killing of the serum-sensitive strain. Log killing of Re595 was 0.29 in 10% PNHS containing MgEGTA, 0.19 in C2D serum, and was almost 100-fold less than killing in 10% PNHS (2.45). These experiments confirm that S218 is resistant to killing by normal human serum in the presence of antibody (12) and show that the killing of Re595 in human serum is mediated predominantly by the classical complement pathway under these conditions.

*Consumption of C3 in PNHS.* The kinetics of C3 consumption was studied at three concentrations of serum: 2.5, 10, and 40%. Shown in Fig. 2 are the kinetics of consumption in 10% serum. The kinetics were similar at each of the three serum concentrations examined. Consumption of C3 proceeded rapidly and essentially reached an endpoint at 15–20 min with ~80% consumption of C3 in 10% PNHS.

*Uptake of $^{125}$IC3 on Re595 and S218 in PNHS.* The kinetics of radiolabeled C3 uptake on the bacterial surface was also examined in 2.5, 10, and 40% serum over a

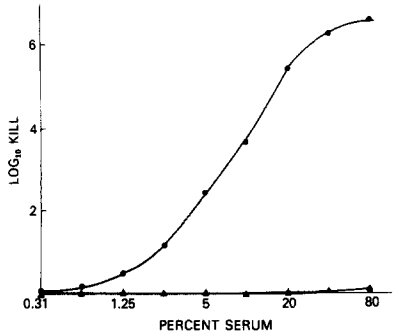


FIG. 1. Killing of *S. minnesota* Re595 and *S. minnesota* S218 by PNHS. Bacteria at a concentration of $5.6 \times 10^8$ cells/ml (Re595) or $7.3 \times 10^8$ cells/ml (S218) were incubated for 60 min at 37°C in dilutions of PNHS ranging from 0.31 to 80%. Re595 (●——●); S218 (▲——▲).

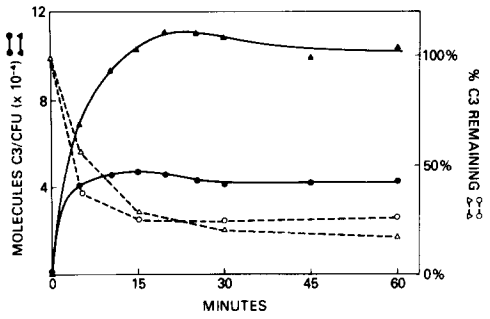


FIG. 2. Consumption and uptake of C3 by *S. minnesota* Re595 and *S. minnesota* S218 in 10% PNHS. Bacteria at a concentration of $5.6 \times 10^8$ cells/ml (Re595) or $7.3 \times 10^8$ cells/ml (S218) were incubated in 10% PNHS in $HBSS^{++}$ containing $^{125}I$ C3. The control tube for C3 consumption contained 10% PNHS in $HBSS^{++}$ without bacteria. Control tubes for $^{125}I$ C3 binding contained 10% heat-inactivated PNHS in $HBSS^{++}$ and either Re595 or S218 at the above concentration. Samples were removed at the designated times for measurement of C3 consumption and specific $^{125}I$ C3 binding, and total molecules of C3 bound per CFU were calculated. All values for C3 consumption were expressed relative to the control tube at each time. All experiments were repeated at least twice, and values between experiments did not vary by >10%. For C3 consumption, Re595 (O- - -O), S218 (Δ- - -Δ); for C3 binding, Re595 (●——●), S218 (▲——▲).

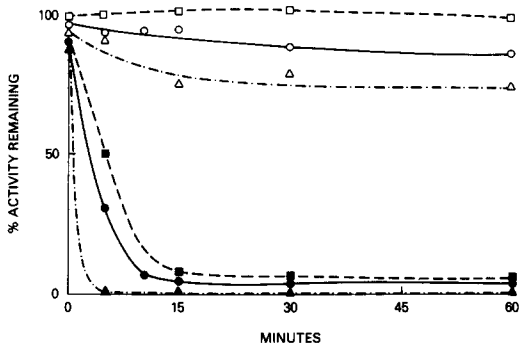


FIG. 3. Consumption of C5, C7, and C9 by *S. minnesota* Re595 and *S. minnesota* S218 in 10% PNHS. Experimental conditions were as outlined in the legend to Fig. 2, except that 10% PNHS did not contain $^{125}I$ C3. All values for percent consumption were expressed relative to the control tube at each time. Re595: C5 (□- - -□), C7 (O——O), C9 (Δ-·-·-Δ); S218: C5 (■- - -■), C7 (●——●), C9 (▲-·-·-▲).

60-min period of incubation at 37°C. Results for 10% serum are shown in Fig. 2, but kinetics were similar in 2.5% and 40% serum. Uptake of $^{125}I$ C3 by Re595 and S218 in PNHS was rapid and plateaued at a peak value for both organisms by 20 min. Twice as many molecules of C3 were bound per CFU of serum-resistant S218 as were bound per CFU of serum-sensitive Re595 in 10% PNHS.

*Consumption of C5, C7, and C9 by Re595 and S218.* Consumption of C5, C7, and C9 was examined in kinetic experiments in 10% PNHS (Fig. 3). No measurable depletion of C5, 10% C7 consumption, and 26% depletion of C9 occurred in the reactions containing the serum-sensitive organisms and 10% PNHS. In contrast, depletion of C5 and C7 in the reaction of S218 and 10% PNHS approached 95% by 15 min of incubation, and complete inactivation of hemolytic C9 was observed by 5 min. The kinetics of C9 consumption were also examined in 2.5% and 40% serum and were equivalent to those in 10% serum. No loss of C9 hemolytic activity occurred when

S218 was incubated in PNHS containing 0.01 M EDTA or in 10% C5D serum (Table I). Addition of purified C5 to C5D serum restored the ability of S218 to consume C9. Complete inactivation of C9 occurred when heat-killed, washed S218 organisms were incubated in 10% PNHS. These experiments demonstrated that inactivation of C9 by S218 was dependent on complement activation and was independent of bacterial viability. Complete consumption of terminal complement components by S218 demonstrates that serum resistance does not result from a block in complement activation.

*Uptake of $^{125}I$ C5, $^{125}I$ C7, and $^{125}I$ C9 on Re595 and S218.* The kinetics of deposition of terminal complement components on the serum-resistant and serum-sensitive organisms in 10% PNHS was studied next (Fig. 4). Binding of C5, C7, and C9 increased rapidly and progressively on serum-sensitive Re595. The maximum specific

TABLE I

*Consumption of C9 by S. minnesota 218 and S. minnesota Re595**

| | Re595 | S218 |
|---|---|---|
| | % | % |
| 10% PNHS | 29 | 100 |
| 10% PNHS in 0.01 M EDTA | 11 | 4 |
| 10% C5D | 0 | 0 |
| 10% C5D + 9640 U/ml C5 | 22 | 88 |
| 10% PNHS‡ | 20 | 98 |

* Percent of hemolytic activity consumed after 60-min incubation at 37°C; consumption calculated relative to control tubes without bacteria incubated concomittantly.

‡ Heat-killed bacteria (80°C for 10 min).

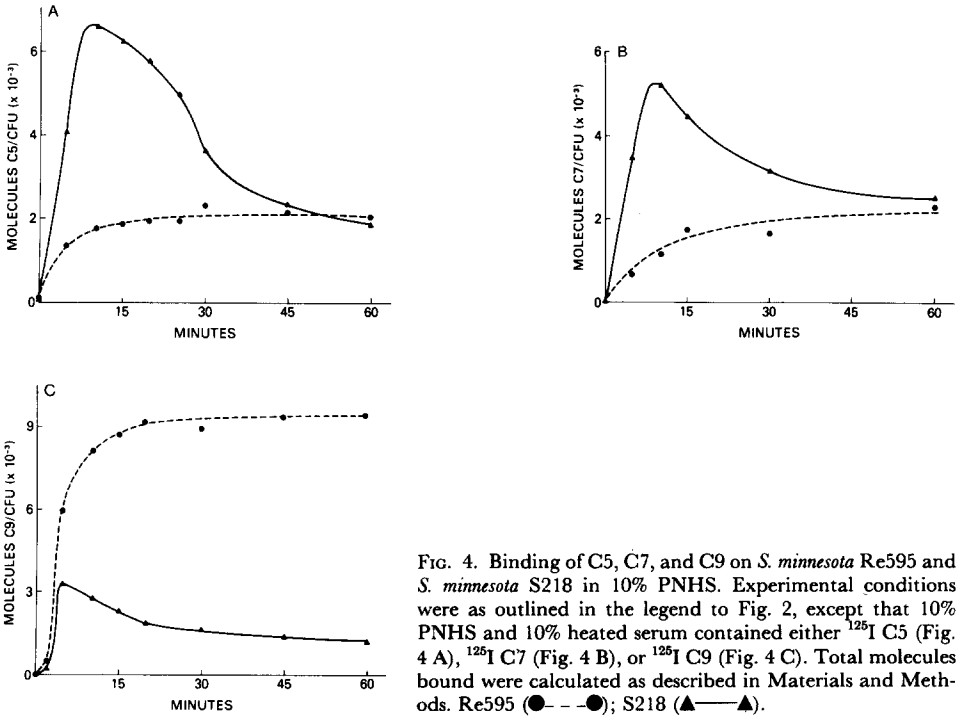


FIG. 4. Binding of C5, C7, and C9 on *S. minnesota* Re595 and *S. minnesota* S218 in 10% PNHS. Experimental conditions were as outlined in the legend to Fig. 2, except that 10% PNHS and 10% heated serum contained either $^{125}I$ C5 (Fig. 4 A), $^{125}I$ C7 (Fig. 4 B), or $^{125}I$ C9 (Fig. 4 C). Total molecules bound were calculated as described in Materials and Methods. Re595 (●- - -●); S218 (▲——▲).

uptake of labeled C5, C7, and C9 was 1.9%, 1.3%, and 5.1% on Re595. In contrast, maximum binding of C5, C7, and C9 on the serum-resistant S218 was 7.6%, 4.2%, and 2.4%. Surprisingly, binding of C5, C7, and C9 on the serum-resistant organism reached a peak at 5–10 min. Thereafter, progressive and striking loss of the bound components from S218 was observed with continued incubation. Release of $^{125}$I C9 from S218 was also demonstrated when heat-killed S218 was studied, demonstrating that loss of $^{125}$I C9 from the bacterial surface was not a function of bacterial growth or metabolism.

These experiments demonstrate that the serum-sensitive organism consumes relatively small amounts of C5, C7, and C9, and these components are efficiently and stably deposited on the bacterial surface. In contrast, all of the C5, C7, and C9 is consumed in the reaction with the serum-resistant organism. Binding of terminal components is less efficient than with the sensitive organism, and components are not stably bound to the bacterial surface.

*Uptake of $^{125}$I C7 and $^{131}$I C9 on Re595 and S218.* Experiments with $^{125}$I-labeled C5, C7, and C9 suggested that fewer molecules of C9 were bound per bound molecule of C5 or C7 on S218 than on Re595. This was examined in a double-label experiment using uptake of $^{131}$I C9 and $^{125}$I C7. An average of 4.3 C9:C7 was bound to serum-sensitive Re595 by 15 min, but the ratio of C9:C7 on S218 was 0.68. The ratios remain constant during the remainder of the incubation, indicating that C7 and C9 on S218 were being released at the same rate. Because C9 was totally consumed in the reaction of 10% PNHS and S218, the possibility that addition of excess C9 would increase the ratio of C9:C7 on S218 was investigated. Unlabeled C9 in increasing amounts was added to 10% PNHS to achieve final C9 titers ranging from 4,000 U/ml to 14,000 U/ml. Binding of $^{131}$I C9 and $^{125}$I C7 was measured, and the ratio of C9:C7 was calculated (Fig. 5). A linear decrease in $^{131}$I C9 molecules bound per CFU of Re595 was observed as the concentration of C9 increased (Fig. 5 A), but no change in the ratio of total C9 to C7 molecules bound occurred (Fig. 5 B). This demonstrates that

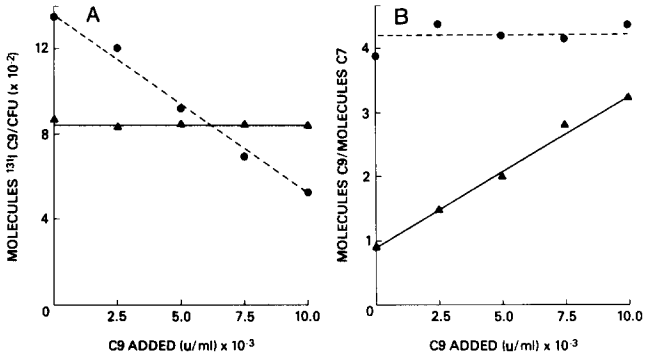


FIG. 5. (A) Molecules $^{131}$I C9 bound/CFU of *S. minnesota* Re595 or *S. minnesota* S218 in 10% PNHS with addition of purified, unlabeled C9 at t = 0. Bacteria at a concentration of 5.6 × $10^8$ cells/ml (Re595) or 7.3 × $10^8$ cells/ml (S218) were incubated in 10% PNHS (C9 titer = 4,000 U/ml) in HBSS$^{++}$ containing $^{125}$I C7 and $^{131}$I C9 and also containing an additional 0, 2.5 × $10^3$, 5 × $10^3$, 7.5 × $10^3$, or 1.0 × $10^4$ U/ml of purified, unlabeled C9 added at t = 0. Molecules of $^{125}$I C7 and $^{131}$I C9 bound per CFU were measured after incubation for 30 min at 37°C. Re595 (●– – –●); S218 (▲——▲). (B) Ratio of C9 molecules bound per C7 molecules bound on *S. minnesota* Re595 and *S. minnesota* S218 from part 5 A. Re595 (●– – –●); S218 (▲——▲). Total molecules of C9 and C7 were calculated from $^{125}$I C7 and $^{131}$I C9 binding.

TABLE II

*Release of $^{14}C$ from S. minnesota Re595 and S. minnesota S218 after Incubation in Varying Dilutions of PNHS*

| Percent PNHS | Percent total $^{14}C$ released* | | Percent [$^{14}C$]lipid released‡ | |
|---|---|---|---|---|
| | Re595 | S218 | Re595 | S218 |
| % | | | | |
| 5 | 21.4 ± 5.3 | 1.7 ± 1.3 | 10.0 ± 1.6 | 2.5 ± 0.5 |
| 10 | 32.0 ± 5.9 | 3.5 ± 0.4 | 21.1 ± 1.8 | 4.1 ± 0.8 |
| 20 | 54.7 ± 1.9 | 6.4 ± 1.4 | 33.9 ± 4.3 | 4.7 ± 0.7 |

Bacteria were prepared as described in Materials and Methods and incubated for 60 min in dilutions of PNHS. Total $^{14}C$ release and [$^{14}C$]lipid release, using the method of Bligh and Dyer (17), were measured. Results shown are mean ± SD for two experiments done in duplicate.

* $^{14}C$ released per total $^{14}C$ in reaction mixture.

‡ [$^{14}C$] lipid released per total [$^{14}C$]lipid in reaction mixture.

(*a*) the maximum achievable ratio of C9:C7 on Re595 is 4.3:1, and (*b*) the $^{131}I$ C9 molecules behave like unlabeled C9 molecules. The latter finding is a necessary condition for validity of calculation of total molecules of C9 bound. In contrast, with increasing inputs of C9, there was no change in molecules $^{131}I$ C9 bound to S218 (Fig. 5 A), and the C9:C7 ratio increased from 0.68 to 3.3 (Fig. 5 B). In a separate but otherwise identical experiment (not shown), up to 25,000 U/ml of unlabeled C9 was added to the reaction mixture with S218, and the C9:C7 ratio reached 4.4:1. There was no difference in the rate of release of $^{125}I$ C7 or $^{131}I$ C9 from S218 as the C9:C7 ratio increased. 90% consumption of C9 by S218 occurred at the highest concentration of added C9 in Fig. 5 A (10,000 U/ml). Therefore, the low C9:C7 ratio on S218 in 10% PNHS does not represent a defect in the capacity of C5b678 on the surface of S218 to bind multiple C9 molecules, but rather reflects depletion of C9 from the fluid phase. Even at the highest ratio of C9:C7 achieved, no killing of S218 occurred in any of the tubes.

*Release of $^{14}C$ from Re595 and S218.* Experiments measuring release of $^{14}C$ from Re595 and S218 by PNHS provided evidence that C5b-9 does not substantially damage the outer membrane of the serum-resistant organism. The percentage of $^{14}C$ released from Re595 was 10 times higher than from S218 at all serum dilutions tested (Table II). Only 3.5% of $^{14}C$ was released from S218 in 10% PNHS. However, smooth gram-negative bacteria contain a smaller amount of outer membrane phospholipid than rough organisms, and this factor might influence the above results. Therefore, the percent of total [$^{14}C$]lipid released was also measured as described in Materials and Methods. As shown in Table II, only 4.1% of total [$^{14}C$]lipid was released from S218 in 10% PNHS, compared with 21.1% release from Re595. These results suggest that C5b-9 on S218 neither disrupts the outer membrane nor releases outer membrane lipids.

*Electron Microscopy.* The serum-sensitive Re595 and serum-resistant S218 were examined by electron microscopy after incubation of the organisms in 10% PNHS for 30 min (Fig. 6). Extensive spheroplast formation and outer membrane damage with bleb formation was apparent for Re595. No evidence of outer membrane damage or spheroplast formation was noted for S218.

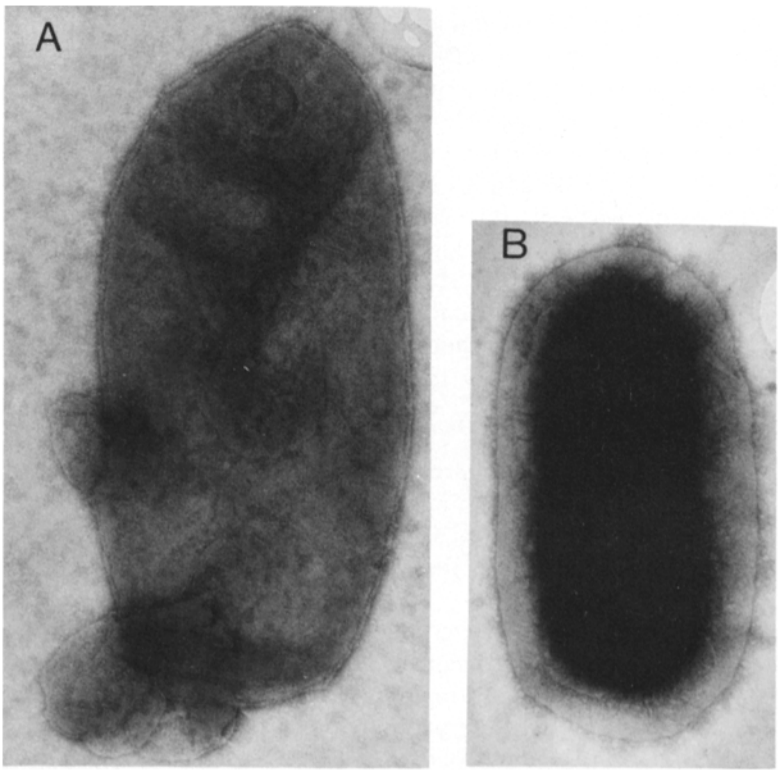


FIG. 6. Transmission electron microscopy of *S. minnesota* Re595 and *S. minnesota* S218 after incubation in 10% PNHS. (A) Re595; magnification, × 48,000. (B) S218; magnification, × 48,000.

## Discussion

Relative serum resistance is an important factor in determining the virulence of a number of gram-negative organisms. These experiments explore the molecular basis of such serum resistance using two closely related strains of *S. minnesota.* Our initial experiments confirmed earlier observations (12) that *S. minnesota* S218 is highly resistant to the lytic action of serum, whereas Re595 is highly sensitive. Our studies demonstrated that both organisms consume C3 from serum and that $^{125}$I C3 uptake parallels consumption. Twice as many molecules of C3 are bound to S218 as are bound to Re595. Once bound, the C3b binding appears stable during the period of observation.

Interestingly, there is total consumption of C5, C7, and C9 by the serum-resistant organism, but there is minimum consumption of these components by the serum-sensitive strain. This is reflected in kinetic experiments by a high initial rate of consumption of C5, C7, and C9 on the serum-resistant strain. These experiments proved that consumption of late components was associated with activation of the complement cascade and that bacterial proteases were not released that destroyed critical complement components in the fluid phase. It was also essential to study uptake of components on the bacterial surface because consumption of components in the fluid phase could have been mediated by released fragments of the bacterial outer membrane. Total depletion of terminal components in the fluid phase would

have prevented bacterial killing and lysis. However, our results showed that C5, C7, and C9 were rapidly bound to S218 with maximum uptake by 5–10 min, but thereafter a progressive loss of all components occurred from the bacterial surface. In contrast, binding of C5, C7, and C9 to Re595 was stable and was associated with minimum consumption of these components.

Experiments using $^{125}$I C7 and $^{131}$I C9 in 10% PNHS demonstrated that the ratio of C9:C7 on Re595 was 4.3:1 but was <1:1 for S218. However, experiments with addition of unlabeled exogenous C9 to 10% PNHS demonstrated that further incorporation of C9 can occur on the surface of S218. In this case, the C9:C7 ratio approached the ratio of 4.3:1 observed on Re595. Because consumption of C9 was essentially complete at all concentrations of added C9, this suggests that availability of hemolytically active C9 is the limiting factor in the C9:C7 ratio on S218. Nonetheless, even when the C9:C7 ratio on S218 was equivalent to the ratio on Re595, no killing of S218 occurred. Current evidence suggests that one molecule of C5, C6, C7, and C8 combines with three to six molecules of C9 for both the fluid-phase human SC5b-9 and dimeric human membrane attack complex (20, 21). Thus, our results are in general agreement with published data, although, because of the differences in methodology and in the membranes studied, the results may not be directly comparable.

Release of phospholipids from the cell membrane of serum-sensitive gram-negative bacteria by the action of complement has been demonstrated repeatedly (22, 23). Such release is consistent with the amphiphilic nature of the C5b-9 complex, which is capable of binding and releasing phospholipids from lipid vesicles, erythrocytes, and serum-sensitive gram-negative bacteria. Beckerdite-Quagliota et al. demonstrated that phospholipids were not released from a serum-resistant strain of *Serratia marcesens*, but substantial phospholipid release occurred from a serum-sensitive strain of *S. marcesens* (24). This finding is similar to the results of our studies and suggests that the amphiphilic C5b-9 on S218 does not interact with membrane phospholipids.

The results presented here suggest that serum resistance of *S. minnesota* S218 does not represent a defect in membrane attack complex formation on the surface of the organism. Rather, the formed complex does not remain associated with the bacterial surface, suggesting that the complex does not insert firmly into the outer membrane.

## Summary

The mechanism of resistance of gram-negative bacteria to killing by complement was investigated. Complement consumption and uptake of purified, radiolabeled complement components on bacteria was studied using a serum-sensitive and a serum-resistant strain of *Salmonella minnesota*. Twice as many molecules of $^{125}$I C3 were bound per colony-forming unit (CFU) of the smooth, serum-resistant *S. minnesota* S218 as were bound per CFU of the rough, serum-sensitive *S. minnesota* Re595 in 10% pooled normal human serum (PNHS), although 75–80% of C3 was consumed by both organisms. Hemolytic titrations documented total consumption of C9 by 5 min and >95% consumption of C5 and C7 by 15 min in the reaction with S218 with 10% PNHS. In contrast, negligible C5 depletion, 10% C7 consumption, and only a 26% decrease in C9 titer occurred with the serum-sensitive Re595. Binding of $^{125}$I C5, $^{125}$I C7, and $^{125}$I C9 to S218 and Re595 was measured in 10% PNHS. A total of 6,600 molecules C5/CFU, 5,200 molecules C7/CFU, and 3,100 molecules C9/CFU bound

to S218 after 5–10 min of incubation at 37°C, but 50–70% of the C5, C7, and C9 bound to S218 was released from the organism during incubation at 37°C for 60 min. Binding of 2,000 molecules C5/CFU, 1,900 molecules C7/CFU, and 9,000 molecules C9/CFU to Re595 was achieved by 20 min and was stable. The ratio of bound C9 molecules to bound C7 molecules, measured using $^{131}$I C9 and $^{125}$I C7, was constant for both organisms after 15 min and was 4.3:1 on Re595 and 0.65:1 on S218 in 10% PNHS. With addition of increasing amounts of purified, unlabeled C9 to 10% PNHS, there was no change in the C9:C7 ratio on Re595. However, with S218 there was a linear increase of the C9:C7 ratio, which approached the ratio on Re595. There was no $^{14}$C release from S218 incubated in PNHS, nor was there evidence by electron microscopy of outer membrane damage to S218. Therefore, *S. minnesota* S218 is resistant to killing by PNHS, despite the fact that the organism consumes terminal complement components efficiently and that terminal components are deposited on the surface in significant amounts. The C5b-9 complex is released from the surface of S218 without causing lethal outer membrane damage.

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