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

II. C8 and C9 Release C5b67 from the Surface of *Salmonella minnesota* S218 because the Terminal Complex Does Not Insert into the Bacterial Outer Membrane

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Optimum killing of gram-negative bacteria in serum requires the participation of terminal components of the complement system, presumably through formation of a membrane attack complex (MAC)[1] containing complement components C5b6789 (1–3). Although it is clear that some gram-negative bacteria are highly resistant to serum killing in the presence of adequate antibody, the mechanism of this resistance is not known.

In the preceding paper (4), we reported results of uptake and consumption of C3 and terminal complement components by a smooth, serum-resistant strain of *Salmonella minnesota* (S218) and a rough, serum-sensitive mutant (Re595) of the above parent strain. We concluded that the mechanism of serum resistance in *S. minnesota* S218 does not involve a block in complement activation or in terminal complement component deposition on the bacterial surface but appears to be associated with a failure of the components to remain surface bound.

In this paper, we report studies on the mechanism of terminal component consumption and release from *S. minnesota* S218. Our results show that binding of C8 and C9 to stably bound C5b67 on the bacterial surface of S218 results in release of the MAC without bacterial killing. The release of C5b-8 and C5b-9 appears to be associated with failure of the complexes to bind hydrophobically in the outer membrane.

Materials and Methods

Buffers. Veronal-buffered saline, containing 0.1% gelatin, 0.15 mM $CaCl_2$, and 1.0 mM $MgCl_2$ ($VBSG^{++}$) was prepared; Hanks' balanced salt solution (HBSS) was purchased (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY); and HBSS with 0.15 mM $CaCl_2$ and 1.0 mM $MgCl_2$ ($HBSS^{++}$) was prepared.

Bacteria. *S. minnesota* Re595 and *S. minnesota* S218 were kindly provided by Dr. Jacik Hawiger, Vanderbilt University, Nashville, TE. Characteristics of these organisms and procedures for their growth are as previously reported (4).

[1] *Abbreviations used in this paper:* CFU, colony-forming unit; DOC, deoxycholate; HBSS, Hanks' balanced salt solution; $HBSS^{++}$, HBSS with 0.15 mM $CaCl_2$ and 1.0 mM $MgCl_2$; LPS, lipopolysaccharide; MAC, membrane attack complex; PNHS, pooled normal human serum; $VBSG^{++}$, veronal-buffered saline containing 0.1% gelatin, 0.15 mM $CaCl_2$, and 1.0 mM $MgCl_2$.

Serum. Serum was pooled from 10 normal volunteers. For some studies, C8-deficient serum was prepared. Pooled normal human serum (PNHS) was depleted of C8 immunochemically for these studies because of the recent finding that genetically deficient sera may not be deficient in all chains of the C8 molecule (5). PNHS was immunochemically depleted of hemolytic C8 activity using a specific anti-C8 immunoabsorbant column. The IgG fraction of monospecific burro anti-human C8 serum was prepared by octanoic acid precipitation. The IgG pool was adjusted to OD_{280} = 15.0 and was coupled to Sepharose 4BCL by cyanogen bromide. Serum depleted of C8 with this immunoabsorbant had <0.001% of the starting C8 titer but a normal CH_{50} after C8 repletion.

Quantitative Cultures. Quantitative cultures were performed as described previously (4).

Consumption of C8 and C9 Hemolytic Activity. Measurements of hemolytic C8 and C9 titers were performed using 1.5×10^7 EAC 1-7 cells in a total reaction volume of 0.5 ml after minor modifications of standard techniques (4).

Purification and Iodination of Complement Components. Purification of C5, C7, C8, and C9 was performed with minor modifications, as previously described by Hammer et al. (6), from a 2-liter pool of fresh normal human plasma. The specific hemolytic activity per μg of the purified components and the corresponding titers in PNHS were: C5, 1,783 U/μg (275,000 U/ml); C7, 2,096 U/μg (223, 450 U/ml); C8, 2,530 U/μg (150,000 U/ml); and C9, 331 U/μg (40,000 U/ml).

Radiolabeling of C5 and C7 with ^{125}I was performed with Bolton-Hunter reagent (New England Nuclear, Boston, MA) by the Inman modification (4). Radioiodination of C9 with Na ^{125}I or with Na ^{131}I was done by the solid-phase glucose oxidase-lactoperoxidase method, (Enzymobeads; Bio-Rad Laboratories, Richmond, CA). Specific radioactivity of labeled components was: C5, 4.99×10^5 cpm/μg; C7, 3.82×10^5 cpm/μg; ^{125}I C9, 1.74×10^6 cpm/μg; ^{131}I C9, 7.56×10^5 cpm/μg. Radiolabeling was accomplished with minimum loss of functional activity. In the case of C9, the purified, unlabeled component competed on a 1:1 basis with the radiolabeled component for bacterial binding.

Quantitation of Radiolabeled Component Binding to Bacteria. Binding of radiolabeled components to bacteria was determined as previously described (4). Molecules of radiolabeled component bound per colony forming unit (CFU) were calculated from the specific cpm bound, the known original CFU, 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 C9 molecules to C7 molecules in fractions from sucrose density gradients was calculated from the ratio of total molecules of each component per fraction. All measurements were done in duplicate on at least two occasions.

Elution Experiments. Susceptibility of bound ^{125}I C5 or ^{125}I C9 to removal from the bacterial outer membrane by salt, detergent, or protease was measured. Aliquots of 1–3 ml were removed after 15 min of incubation at 37°C from the reaction mixture of 10% PNHS or 10% C8D with S218 or Re595. The bacterial pellet was sedimented by centrifugation at 4,800 *g* for 10 min at 4°C and washed twice in $VBSG^{++}$. Pellets were suspended to the original volume after the second wash in either (*a*) $VBSG^{++}$ diluted 1:5 in 5% dextrose ($DVBSG^{++}$), (*b*) $HBSS^{++}$, (*c*) $VBSG^{++}$, (*d*) 1 M NaCl with 0.15 mM $CaCl_2$ + 1 mM $MgCl_2$ (pH 7.4), (*e*) 0.01 M EDTA in PBS, (*f*) 1.0% triton X-100, (*g*) 1.0% Na deoxycholate (pH 8.0), (*h*) 0.5% SDS, or (*i*) 1 mg/ml trypsin TPCK in $VBSG^{++}$. These samples were incubated at 37°C for 30 min, and counts of ^{125}I C5 or ^{125}I C9 remaining in the bacterial pellet at various times were measured as described under quantitation of radiolabeled component binding to bacteria. Counts in the supernatant were also measured to verify total input counts.

Sucrose Density Gradient Ultracentrifugation. A 10–40% sucrose density gradient in $VBSG^{++}$ was prepared in 9/16″ × 3½″ polyallomer tubes. Samples of 0.5 ml were layered onto the gradient and sedimented in an SW 50.1 rotor (Beckman Instruments, Inc., Fullerton, CA) for 16 h at 33,000 rpm at 4°C. Seven-drop fractions were collected and counted in a well-type gamma counter (Beckman Instruments, Inc.).

Results

Consumption of C8 and C9 by Cell-bound C5b-7. Our previous experiments had shown that the serum-resistant *S. minnesota* S218 activated terminal complement components

when incubated in serum. It was essential to determine whether consumption of the latter components was a function of C5b-9 formation on the bacterial surface or was occurring predominantly via activation in the fluid phase. To address this question, S218 was incubated for 15 min at 37°C in 10% C8D serum in $VBSG^{++}$. The reaction mixture was then centrifuged for 10 min at 4,800 *g* at 4°C, the supernatant was removed onto ice, and the bacterial pellet was washed two times in $VBSG^{++}$ at 4°C. Purified C8 was added to the supernatant from the original reaction mixture, C8 and C9 were added to the bacterial pellet, and both tubes were incubated for an additional 30 min at 37°C. There was 56% depletion of C8 in the tube containing the bacteria but only 18% depletion of C8 in the supernatant tube (Table I). Total depletion of C9 was observed in the tube containing the bacterial pellet, whereas only a 26% fall in C9 titers occurred in the tube containing the supernatant. Therefore, the extensive consumption of C8 and C9 by S218 was apparently the result of interaction of these two components with C5b67 on the bacterial surface, yet as previously shown, such interaction resulted in inefficient binding of C9 to the organism. The following

TABLE I

Consumption of C8 and C9 by the Supernatant and Pellet from S. minnesota S218 Incubated in C8D Serum

	C8		C9	
	Supernatant	Pellet	Supernatant	Pellet
U/ml added	5,800	5,800	3,800*	10,300
U/ml remaining after 30-min incubation	4,500 (18%)‡	2,400 (56%)	2,600 (26%)	8 (100%)

S. minnesota S218 was incubated in 10% C8D serum, and supernatant and pellet samples were prepared as described in Results. Purified C8 and C9 were added as indicated, samples were incubated for 30 min at 37°C, and C8 and C9 titers were measured.

* C9 titer in 10% C8D serum after incubation with S218 (no additional C9 added).

‡ Numbers in parentheses represent percent depletion relative to control tubes containing C8 or C9 in $VBSG^{++}$

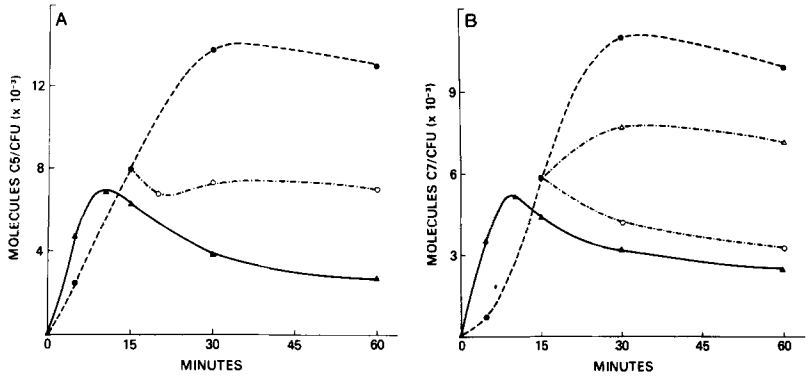


FIG. 1. Uptake of C5 and C7 by *S. minnesota* S218 in 10% C8D serum. *S. minnesota* S218 (7.3×10^8 cells/ml) was incubated in 10% C8D or 10% PNHS serum, containing either ^{125}I C5 (A) or ^{125}I C7 (B). After 15 min, varying quantities of purified C8 were added to aliquots from the mixtures containing C8D serum. Binding of $^{125}IC5$ and $^{125}IC7$ was measured at the indicated times. 10% C8D (●- - -●); 10% PNHS (▲——▲). For (A), 10% C8D + 11,500 U/ml C8 (○—·—○); for (B), 10% C8D + 4,700 U/ml C8/ml (△—·—·△), and 10% C8D + 23,000 U/ml C8/ml (○—·—·○).

experiments investigated the possibility that binding of C8 and/or C9 to cell-bound C5b67 was responsible for release of the terminal complex from the organism.

Uptake of ^{125}I C5 and ^{125}I C7 in C8D Serum. Measurement of ^{125}I C5 and ^{125}I C7 uptake in C8D serum (Fig. 1 A, B) demonstrated that nearly twice as many molecules of C5 and C7 were bound to S218 in 10% C8D as in 10% PNHS. Moreover, the kinetics of binding were totally different in C8D serum. Uptake of ^{125}I C5 and ^{125}I C7 by S218 in 10% C8D serum increased to a maximum that was not reached until 30 min of incubation at 37°C; binding remained relatively stable during an additional 30 min of incubation. In contrast, there was no difference in kinetics or extent of C5 and C7 uptake on Re 595 when uptake in 10% PNHS and 10% C8D serum was compared (data not shown).

Therefore, binding of C5 and C7 on S218 in C8D serum was more extensive and more stable than was binding of these components in PNHS. To prove that labile binding of C5 and C7 on S218 in PNHS was a consequence of subsequent interaction with C8 and C9, purified C8 in various concentrations was added to C8D serum after 15 min of incubation with S218. Addition of 11,500 U/ml of C8 prevented further uptake of C5 (Fig. 1 A). Addition of as little as 4,700 U/ml C8 reduced deposition of C7, and 23,000 U/ml of C8 caused release of C7, paralleling the release observed in PNHS (Fig. 1 B). These results suggested that binding of C8 to C5b67 on S218 is

Release of C5b67 from Washed S218 by Addition of C8, C9, or C8 plus C9 in the Absence of Serum. The next experiments investigated whether purified C8 alone was sufficient or the combination of purified C8 and C9 was required for rapid release of C5b67 from S218. For these experiments, Re 595 and S218 were incubated in 10% C8D serum containing either ^{125}I C5 or ^{125}I C7. The mixture was incubated for 20 min at 37°C, the bacteria were washed twice in $VBSG^{++}$ at 4°C and suspended in $VBSG^{++}$ alone, $VBSG^{++}$ with C8, $VBSG^{++}$ with C9, or $VBSG^{++}$ with C8 and C9. 13–18% of bound ^{125}I C5 (Fig. 2A) and ^{125}I C7 (Fig. 2B) was released from S218 during the subsequent incubation at 37°C in either $VBSG^{++}$ or $VBSG^{++}$ with C9. Much more

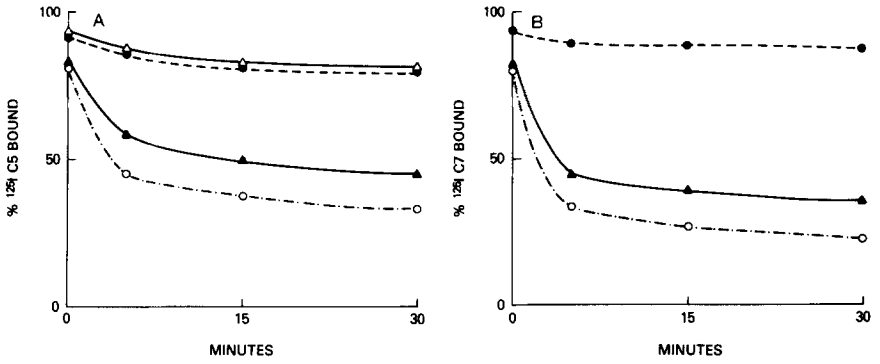


FIG. 2. Release of C5b67 from *S. minnesota* S218 by addition of C8 or C8 plus C9. *S. minnesota* S218 (7.3×10^8 cells/ml) was incubated for 30 min in 10% C8D serum, containing either ^{125}I C5 (A) or ^{125}I C7 (B). Bacteria were washed twice in $VBSG^{++}$ and suspended in $VBSG^{++}$ (●- - -●), $VBSG^{++}$ with 1.5×10^4 U/ml C8 ▲——▲, $VBSG^{++}$ with 1×10^4 U/ml C9 (△——△), or $VBSG^{++}$ with 1.5×10^4 U/ml C8 and 1×10^4 U/ml C9 (○—·—○). Mixtures were incubated at 37°C, and samples were removed for measurement of $^{125}IC5$ or $^{125}IC7$ bound. The difference between release of $^{125}IC5$ or $^{125}IC7$ by C8 and release by C8 plus C9 was statistically significant ($P < 0.05$).

extensive and rapid loss of bound ^{125}I C5 and ^{125}I C7 from S218 occurred during the first 15 min of incubation in tubes containing either C8 or C8 and C9, in parallel with the loss of fluid-phase C8 and C9 hemolytic activity (not shown). The percent release was consistently 10% greater with C8 and C9 than with C8 alone ($P < 0.05$). No difference in ^{125}I C7 release at 30 min was noted when three concentrations of C9 (2,500 U/ml, 5,000 U/ml, 10,000 U/ml) were compared in the presence of 15,000 U/ml of C8 (data not shown). In contrast to *S. minnesota* S218, <6% release of ^{125}I C5 from Re595 was detected with addition of C8 or C8 and C9 (data not shown).

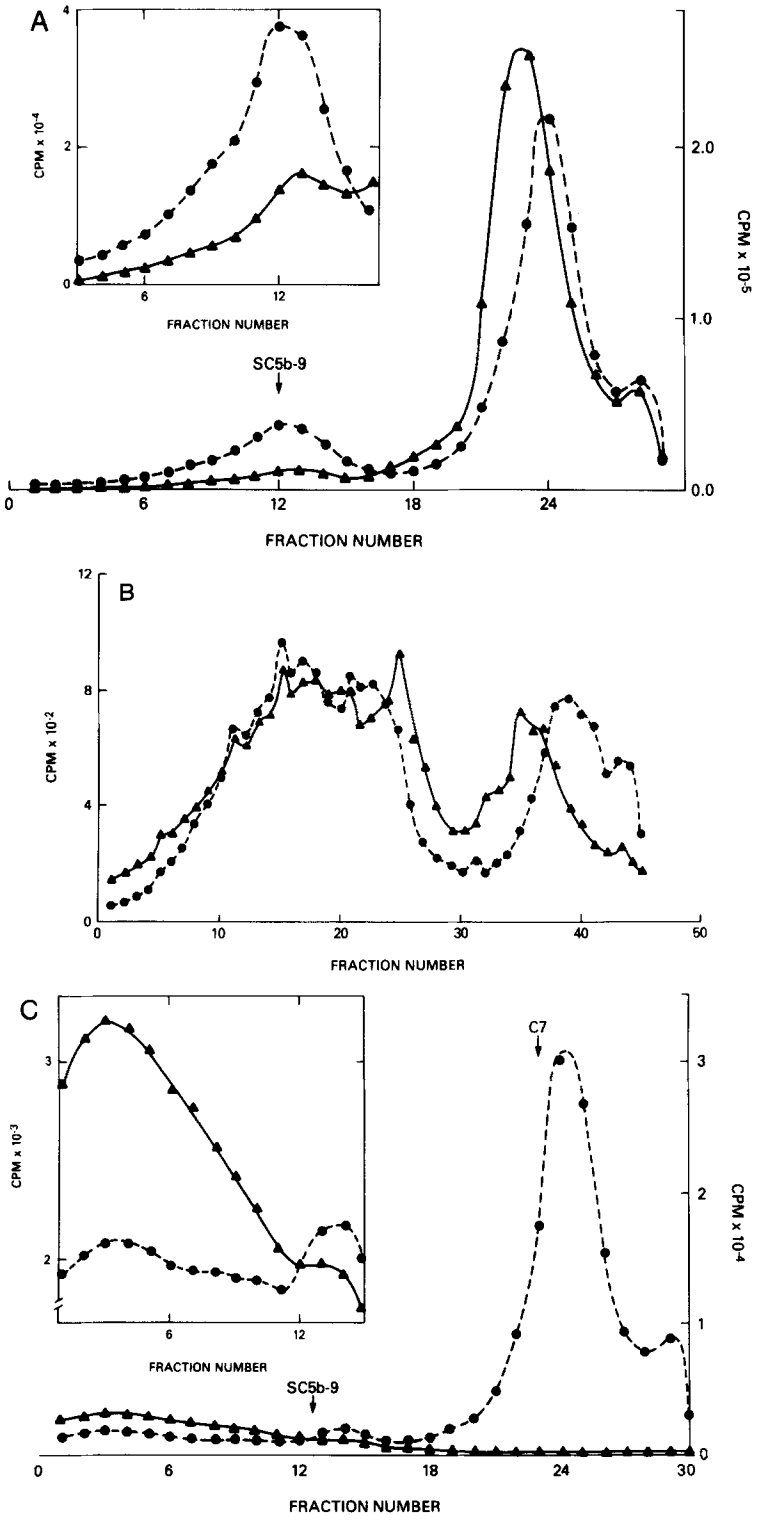
These experiments indicated that binding of C8 was primarily responsible for release of C5b67 from S218. This was further explored by determining the concentrations of C8 required to effect release of ^{125}I C7 bound to S218 by previous incubation in C8D serum. The percent of ^{125}I C7 released increased markedly at low concentrations (1,000–2,500 U/ml) of added C8, but release approached ~80% assymtotically at higher concentrations (5,000–14,000 U/ml) of C8.

Sucrose Density Gradient Ultracentrifugation. The size distribution of the terminal components bound to S218 and that of the released complex were examined by sucrose density gradient ultracentrifugation. The supernatant from the reaction of 10% PNHS containing ^{125}I C7 and ^{131}I C9 and either Re595 or S218 was centrifuged in a linear 10–40% sucrose gradient. A high molecular weight peak containing both C7 and C9 was observed for S218 (Fig. 3 A), sedimenting in the same region as the SC5b-9 peak from 10% PNHS incubated with 4 mg/ml of zymosan for 60 min. In the case of S218, the ratio of C9:C7 in each tube of the left one-half of the C5b-9 peak, chosen to preclude contribution from free C7 or free C9, was a constant 3.3:1.

The bacterial pellet from the incubation of S218 and 10% PNHS was washed twice in VBSG^{++} and then incubated in 1.0% Na deoxycholate (DOC), pH 8.0, for 30 min at 37°C. Sodium DOC does not disrupt the MAC (7) and was shown in other studies (see elution experiments) to extract 70% of bound C5 and C9 counts from the surface of S218. The bacterial pellet was removed, and the supernatant was centrifuged on a 10–40% linear sucrose gradient containing 1.0% DOC. The C7 and C9 sedimented in a large, broad peak in which the ratio of C9:C7 molecules never exceeded 1.2:1 (Fig. 3 B). Small peaks for the native molecules were also observed and are consistent with DOC elution of nonspecifically bound C7 and C9.

The complex released by addition of purified C8 and ^{131}IC9 to S218, previously incubated in C8D serum containing ^{125}I C7, was also examined. The ^{125}I C7 counts sedimented to the bottom of the gradient (Fig. 3 C), suggesting aggregation of the C7 containing complexes. The ratio of C9:C7 in the complexes was 0.5:1, consistent with a large fraction of the ^{125}I C7 counts being in C5b-7 or C5b-8 complexes.

Elution of C5b-7 and C5b-9 from S218 and Re 595 by Salt, EDTA, Detergent, and Trypsin. The ability of salt, EDTA, detergent, or trypsin to elute C5b-7 and C5b-9 from S218 and Re595 was examined. Elution of C5b-7 was tested after incubation of the organisms in 10% C8D serum containing ^{125}I C5 (Fig. 4 A). Elution of C5b-9 was evaluated after incubation of S218 and Re595 in 10% PNHS containing either ^{125}I C5 (Fig. 4 B) or ^{125}I C9 (Fig. 4 C). Incubation in low ionic strength buffer led to release of neither C5b67 nor C5b-9 from Re595 and S218. However, a substantial increase in elution of C5b-7 and C5b-9 from S218 was observed with incubation in buffers of increasing ionic strength and after incubation in 0.01 M EDTA. In contrast, <11% of C5b-7 and C5b-9 eluted from the serum-sensitive Re595 in 1 M NaCl or 0.01 M


A
CPM x 10⁻⁴
FRACTION NUMBER
SC5b-9
CPM x 10⁻⁵
FRACTION NUMBER
B
CPM x 10⁻²
FRACTION NUMBER
C
CPM x 10⁻³
FRACTION NUMBER
SC5b-9
C7
CPM x 10⁻⁴
FRACTION NUMBER

EDTA buffer. The nonionic detergent triton X-100 was less effective in eluting C5b-7 and C5b-9 from either organism than were the ionic detergents, sodium DOC and sodium dodecyl sulfate. Trypsin cleaved 54–57% of ^{125}I C5 (C5b-7 and C5b-9) counts and 25% of ^{125}I C9 counts from Re595, but cleaved >90% of ^{125}I C5 (C5b-7 and C5b-9) and ^{125}I C9 counts from S218. The addition of 2-mercaptoethanol after incubation with 0.1% trypsin resulted in no significant change in release of C5 and C9 from Re595 or S218. The results from this series of experiments suggest that binding of C5b67 and C5b-9 to S218 is mediated in large part by ionic interactions, whereas attachment of C5b67 and C5b-9 to Re595 is mediated through predominantly hydrophobic bonds. Both C5 and C9 were more accessible to trypsin cleavage on the surface of S218 than on the surface of Re595, a result consistent with partial membrane shielding of C5b67 and C5b-9 on Re595.

Discussion

Incubation of serum-resistant *S. minnesota* S218 in serum leads to activation of serum complement, deposition of terminal complement components on the organism, and release of the terminal components without bacterial killing (4). These studies explore the mechanism of terminal component consumption and release. Initial studies demonstrated that consumption of C8 and C9 occurred predominantly after interaction with C5b-7 bound to the bacterial surface. Subsequent experiments showed that C5b-7 bound extensively and stably in C8-deficient serum, but addition of C8 caused dose-dependent release of the terminal complex from the bacterial surface.

There are several possibilities that may explain this finding. Part of the bacterial surface might be shed, carrying the terminal component complex into the fluid phase. However, we previously demonstrated <5% release of endogenously incorporated ^{14}C and [^{14}C]lipid from S218 when the organism was incubated in 10% PNHS. This fact, in conjunction with our demonstration that C3 remains firmly bound to S218 and the lack of electron microscopic evidence for outer membrane damage on S218, suggests that gross outer membrane damage or membrane loss does not explain the terminal component release.

A second possibility is that proteolysis of the surface-bound complement proteins ensued with the addition of C8 and C9 to C5b67 on the bacterial surface. This possibility seems unlikely. The proteolytic enzyme would have to spare C3 bound to the bacteria. Moreover, the complex released from S218 in 10% PNHS sedimented as a typical SC5b-9 complex containing intact C9, as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (not shown). Furthermore, release of terminal components from S218 was observed with bacteria that were killed by heating for 10 min at 80°C, conditions that would inactivate many bacterial proteases.

A third and likely possibility to explain the release of C5b67 is that addition of C8

FIG. 3. Sucrose density gradient ultracentrifgation of terminal component complexes released or eluted from *S. minnesota* S218. Samples were prepared as described below and were centrifuged for 16 h at 33,000 rpm at 4°C. ▲——▲, ^{125}I C7; ●- - -●, ^{131}I C9. Panel A, *S. minnesota* S218 (7.3×10^8 cells/ml) was incubated for 20 min in 10% PNHS, containing ^{125}I C7 and ^{131}I C9. The supernatant was applied to a 10–40% sucrose gradient in VBSG^{++}. Panel B, the bacterial pellet from the reaction described in panel A was incubated for 30 min at 37°C in 1% NaDOC. The supernatant was applied to a 10–40% sucrose gradient in VBSG^{++} with 1% NaDOC. Panel C, S218 was incubated for 30 min in C8D serum containing ^{125}I C7 and ^{131}I C9. The bacterial pellet was washed in VBSG^{++}, and 14,000 U/ml C8 and 4,000 U/ml C9 were added. After incubation for 30 min, the supernatant was applied to a 10–40% sucrose gradient in VBSG^{++}.

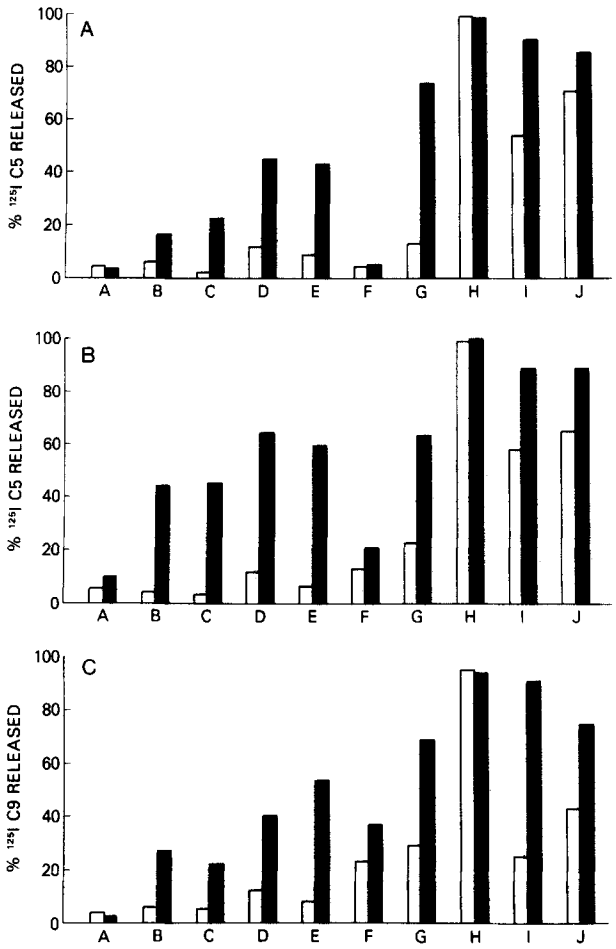


FIG. 4. Elution of C5b67 and C5b-9 from *S. minnesota* Re595 and *S. minnesota* S218 by salt, EDTA, detergent, or trypsin. A, C5 (C8D serum); B, C5 (PNHS); C, C9 (PNHS). Bacteria were prepared as described under elution experiments in Materials and Methods. Washed organisms with bound C5b67 (A) or C5b-9 (B and C) were then incubated for 30 min at 37°C in (*a*) $DVBSG^{++}$, (*b*) $HBSS^{++}$, (*c*) $VBSG^{++}$, (*d*) 1 M NaCl, pH 7.4, with 0.15 mM $CaCl_2$ and 1.0 mM $MgCl_2$, (*e*) 0.01 M EDTA in PBS, (*f*) 1.0% triton X-100, pH 7.4, (*g*) 1.0% NaDOC, pH 8.0, (*h*) 0.5% SDS, and (*i*) and (*j*) 0.1% trypsin in $VBSG^{++}$. At 30 min, the percent of ^{125}I C5 or ^{125}I C9 released was measured (tubes A–I). To tube J, 2-mercaptoethanol was added to a concentration of 1%, and released ^{125}I C5 and ^{125}I C9 counts were determined after an additional 10-min incubation. Results shown are means of data from two to three experiments done in duplicate. Re595, □; S218, ■.

or C8 and C9 increases the hydrophobicity and decreases the degree of ionic binding of the resultant complex. Release of C5b-8 and C5b-9 then ensues because, as discussed below, these complexes are not attached by strong hydrophobic interactions to the outer membrane of the serum-resistant organism. Podack and Müller-Eberhard (8) demonstrated that C5b-6 and C5b-7 bound, respectively, 24 and 27 mol DOC/mol complex, but C5b-8 bound 63 mol, and C5b-9 bound 86 mol of DOC/mol of complex. Analogous results were obtained when phospholipid binding to complexes in DOC-phospholipid vesicles was studied (9). The molar phospholipid/protein ratios were C5b-7, 388:1; C5b-8, 841:1; C5b-9, 918:1; and C5b-9 dimer (MAC), 1460:1.

Our results, showing 13% release of C5b-7, 65% release of C5b-8, and 76% release of C5b-9 from S218, during incubation for 30 min at 37°C, are consistent with the idea that increased hydrophobicity leads to decreased ability of terminal complexes to remain bound to the surface of S218.

These results are consistent with the concept that a C5b-9 complex forms on the surface of S218, but the formed complex does not insert into hydrophobic regions in the outer membrane of the serum-resistant bacterium. Experiments on complement lysis of erythrocytes suggest that MAC insertion into hydrophobic membrane domains is necessary for lysis. For example, the MAC on erythrocyte membranes cannot be eluted by salt (10). Our results demonstrating minimum elution of C5b-7 and C5b-9 from serum-sensitive Re595 by incubation in buffers of increasing ionic strength support the idea that ionic interactions are not prominent in binding of terminal components to Re595. In contrast, C5b67 and C5b-9 are eluted from S218 by salt, suggesting that these complexes are ionically bound to the bacterial surface. Further evidence for MAC inserton into the lipid bilayer of erythrocyte membranes was provided by Hammer et al. (10, 11). These workers showed that erythrocyte-bound C5b-7, C5b-8, or C5b-9 were partially resistant to trypsin-mediated proteolysis and release and suggested that insertion into the lipid bilayer protected terminal components from trypsin attack. More recent findings (7, 12, 13) have raised questions as to the susceptibility of fluid-phase and membrane-bound C5b-9 to proteolytic attack. Nevertheless, it is striking that the C5b-7 and C5b-9 complexes on the serum-resistant organism are more sensitive to trypsin-mediated release than are the complexes on the serum-sensitive bacteria. Either the molecular form of the terminal complex on S218 is different from that on Re595, or the complex on S218 is not inserted into the lipid bilayer. Our finding that <5% of [^{14}C]lipid is released from S218 during incubation in 10% PNHS implies that C5b-9 is not interacting with and displacing outer membrane phospholipids.

The fluid-phase complex released from the reaction of S218 and 10% PNHS sedimented as a typical SC5b-9 complex. In contrast, the complex released by addition of C8 and C9 to S218 previously incubated in C8D serum sedimented as an aggregate with a C9:C7 ratio of 0.5:1. This suggests that the major form of C5b-7 bound to S218 lacks S protein, because S protein bound to C5b-7, C5b-8, or C5b-9 prevents aggregation of these complexes in aqueous solution (8). If this supposition is correct, the results imply that C8 releases C5b-7 from the resistant organism in the absence of S protein.

The lipopolysaccharides (LPS) of smooth, gram-negative bacteria contain long polysaccharide side chains that extend externally to the lipid portion of the outer membrane. The polysaccharide portion of bacterial LPS may activate the human alternative complement pathway (14), and human serum contains natural antibodies to many of these O-specific polysaccharides. It is possible that activation of complement by polysaccharide determinants in the LPS of S218 resulted in formation of a C5b-9 complex that was physically separated from the hydrophobic portion of the outer membrane. This may preclude insertion of C5b-9 into the hydrophobic domains. The relatively hydrophilic polysaccharide side chain might form a weak ionic binding site for the C5b-9 complex. Alternatively, the paucity of phospholipids in the outer membrane of smooth, gram-negative organisms (15), the low fluidity of such membranes (16), and the impermeability of these membranes to even very small hydro-

phobic molecules (17) suggest that a MAC formed even in close proximity to the core outer membrane determinants might be incapable of insertion.

These studies have explored the mechanism of resistance to serum killing of a gram-negative bacterium, *S. minnesota* S218. Our experiments show that complement components are efficiently consumed by *S. minnesota* S218, and these components are deposited on the bacterial surface. However, C5b-9 is released and is not bactericidal because the complex does not insert into hydrophobic regions in the outer membrane. Gram-negative organisms cause the majority of bacteremias in hospitalized patients, most of these blood culture isolates are serum resistant (18), and bacteremia with serum-resistant isolates is commonly associated with shock (19). It is possible that extensive generation of C3a and C5a by serum-resistant organisms, in the absence of bacterial killing, might be responsible for many of the biologic effects observed in gram-negative bacteremia.

Summary

The mechanism for consumption of terminal complement components and release of bound components from the surface of serum-resistant *Salmonella minnesota* S218 was studied. Consumption of C8 and C9 by S218 occurred through interaction with C5b67 on the bacterial surface because C8 and C9 were consumed when added to S218 organisms previously incubated in C8-deficient serum and washed to remove all but cell-bound C5b67. Rapid release of ^{125}I C5 and ^{125}I C7 from the membrane of S218 was dependent on binding of C8 because ^{125}I C5 and ^{125}I C7 deposition in C8D serum was stable and was twofold higher in C8D than in PNHS, and addition of purified C8 or C8 and C9 to S218 previously incubated in C8D serum caused rapid release of ^{125}I C5 and ^{125}I C7 from the organism. Analysis by sucrose density gradient ultracentrifugation of the fluid phase from the reaction of S218 and 10% PNHS revealed a peak consistent with SC5b-9, in which the C9:C7 ratio was 3.3:1, but the NaDOC extracted bound C5b-9 complex sedimented as a broad peak with C9:C7 of $<$1.2:1. Progressive elution of C5b67 and C5b-9 from S218 but not serum-sensitive *S. minnesota* Re595 was observed with incubation in buffers of increasing ionic strength. Greater than 90% of the bound counts of ^{125}I C5 or ^{125}I C9 were released from S218 by incubation in 0.1% trypsin, but only 57% of ^{125}I C5 and 25% of ^{125}I C9 were released by treatment of Re595 with trypsin. These results are consistent with the concept that C5b-9 forms on the surface of the serum-sensitive *S. minnesota* Re595 and the serum-resistant *S. minnesota* S218 in normal human serum, but the formed complex is released and is not bactericidal for S218 because it fails to insert into hydrophobic outer membrane domains.

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