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Multimeric complement component C9 is necessary for killing of *Escherichia coli* J5 by terminal attack complex C5b–9

[poly(C9)/complement-mediated bacterial killing]

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ABSTRACT **We studied the molecular composition of the complement C5b–9 complex required for optimal killing of *Escherichia coli* strain J5. J5 cells were incubated in 3.3%, 6.6%, or 10.0% C8-deficient serum previously absorbed to remove specific antibody and lysozyme. This resulted in the stable deposition after washing of 310, 560, and 890 C5b67 molecules per colony-forming unit, respectively, as determined by binding of ^{125}I-labeled C7. Organisms were then incubated with excess C8 and various amounts of ^{131}I-labeled C9. Plots of the logarithm (base 10) of *E. coli* J5 cells killed (log kill) vs. C9 input were sigmoidal, confirming the multihit nature of the lethal process. When C9 was supplied in excess, 3300, 5700, and 9600 molecules of C9 were bound per organism for cells bearing 310, 560, and 890 C5b–8 complexes, respectively, leading to C9-to-C7 ratios of 11.0:1, 10.8:1, and 11.4:1 and to log kill values of 1.3, 2.1, and 3.9. However, at low inputs of C9 that lead to C9-to-C7 ratios of <3.3:1, no killing occurred, and this was independent of the number of C5b–9 complexes bound. Formation of multimeric C9 at C9-to-C7 ratios permissive for killing was confirmed by electron microscopy and by binding of ^{125}I-labeled antibody with specificity for multimeric but not monomeric C9. These experiments are the first to demonstrate a biological function for C9 polymerization and suggest that multimeric C9 is necessary for optimal killing of *E. coli* J5 cells by C5b–9.**

Microbial killing is a critical function of the cytolytic C5b–9 terminal attack complex of complement. However, the molecular form of the C5b–9 complex necessary for optimal killing is unknown. It is reported that there is an absolute requirement for all five components (C5b, C6, C7, C8, and C9) of this lytic complex for killing of a number of Gram-negative bacteria (1–3). This is unlike the situation for complement-mediated erythrocyte lysis, in which a C5b–8 complex with no C9 is sufficient for lysis (4). Furthermore, the number of C5b–9 complexes that are necessary to kill a bacterium is apparently much larger than the number of C5b–9 required for erythrocyte lysis (ref. 5; unpublished observations). Bacterial killing follows a multihit dose–response curve (6), in comparison to the single-hit kinetics of erythrocyte lysis. Erythrocyte lysis requires the production of a transmembrane channel sufficient to allow only the passage of ions and monosaccharides (7), while bacterial killing is associated with partial or complete dissolution of the rigid Gram-negative outer membrane, a process reported to be more analogous to detergent lysis than to simple colloid osmotic lysis (8–10). These factors suggest the possibility that the form of the C5b–9 complex necessary for optimal bacterial killing may differ from the form necessary for erythrocyte lysis.

The molecular stoichiometry of C9 within the terminal complement complex is variable. The input of C9 influences the size of the transmembrane channel produced, with channel diameters varying from <14 Å up to 114 Å, depending on the dose of C9 (11–14). The physicochemical parameters of the C5b–9 complex bearing different C9 multiplicities also vary. Under conditions of limited C9, the complex is susceptible to partial proteolysis and can be completely dissociated with denaturing detergents. Interaction of excess C9 with the C5b–8 complex on a membrane surface (MC5b–8) results in polymerization of multiple molecules of C9 (15–18) into a $NaDodSO_4$- and protease-resistant (19) ringed cylinder [polymerized C9 or poly(C9)]. The necessity of large poly(C9) channels for lysis of erythrocytes is questionable because channels of smaller size have functional lytic activity. However, the possibility that either the large channels or the extensive membrane disruption (20) caused by poly(C9) are critical for effective cytolytic attack on biological membranes more complex than that of the erythrocyte has not been tested.

We investigated the molecular form of C5b–9 that was optimal for direct killing of a rough, complement-susceptible strain of *E. coli*. Our experiments indicate that at low surface densities of C5b–9, an average of three C9 molecules are required per C5b–9 complex for bacterial killing to occur. However, optimal killing requires higher C9 multiplicity. These experiments are the first to demonstrate a biological function for C9 polymerization.

MATERIALS AND METHODS

Buffers. The following buffers were used for these experiments: Hanks' balanced salt solution (HBSS) containing 0.15 mM $CaCl_2$ and 1 mM $MgCl_2$ ($HBSS^{2+}$); low-ionic-strength (μ = 0.060) dextrose with Veronal-buffered saline (VBS) containing 0.15 mM $CaCl_2$ and 1 mM $MgCl_2$ ($DGVBS^{2+}$); $DGVBS^{2+}$ containing 1 mg of bovine serum albumin per ml ($DGVBS^{2+}A$).

Bacteria. The J5 strain of *E. coli* was kindly provided by Elizabeth Ziegler (University of California, San Diego). Characteristics of this strain, which was derived from an isolate originally described by Elbein and Heath (21), are as described (22). This organism directly activates and is killed by the classical complement pathway in the absence of specific antibody (23).

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Abbreviations: anti-PC9, antibody with specificity for multimeric C9 neoantigens; C8D serum, serum deficient in C8; MC5b–8, C5b–8 complex on the membrane surface; poly(C9), polymerized C9; SC5b–9, fluid-phase C5b–9 complex containing S protein; cfu, colony-forming unit; log kill, logarithm (base 10) of *E. coli* J5 cells killed.

The organism was grown in trypticase soy broth with constant agitation at 37°C to an OD_{600} of 0.500, which corresponds to 6×10^8 organisms per ml. The organisms were then washed and suspended to the desired density in $HBSS^{2+}$.

Serum. Serum was obtained from seven normal volunteers, pooled, and frozen in aliquots at −70°C. Serum was also obtained from a patient with congenital absence of C8 (C8D serum). All serum was absorbed prior to use with 1×10^{10} *E. coli* J5 cells per ml at 0°C for 30 min in order to remove specific antibody. Pooled normal human serum and C8D serum were also depleted of lysozyme by addition of 10 μl of anti-lysozyme antiserum (Miles) per ml or by the addition of 50 μl of Sepharose CL-4B bearing anti-lysozyme IgG (kindly supplied by Raphael Martinez, University of California, Los Angeles) to each ml of serum at 0°C. Both procedures resulted in complete removal of lysozyme activity, as measured by the *Micrococcus lysodeicticus* assay (24).

Purification and Radiolabeling of Complement Components. Complement components C7 and C9 were purified to homogeneity with minor modifications of the procedure of Hammer *et al.* (25). Complement component C8 was isolated to functional purity and was >90% pure as judged by $NaDodSO_4$/PAGE.

C7 and C9 were labeled with $Na^{125}I$ or $Na^{131}I$ (Amersham) with Iodobeads (Pierce) (^{125}I- or ^{131}I-C7 and -C9). All components sustained a <25% loss of hemolytic activity with radiolabeling, measured as described (25).

Antiserum to Neoantigens Within Poly(C9). Antiserum was raised to neoantigens within poly(C9) and designated anti-PC9. Details of antibody preparation and characterization are reported elsewhere. Briefly, rabbits were immunized with heat-polymerized C9 prepared by a modification of the method described by Podack and Tschopp (20). Antiserum was sequentially absorbed with pooled normal human serum coupled to Sepharose CL-4B and with monomeric C9 coupled to Sepharose CL-4B. Absorbed antiserum reacted by ELISA and Ouchterlony techniques with purified MC5b–9, poly(C9), and SC5b–9, the fluid-phase C5b–9 complex containing S protein, but not with pooled normal human serum, monomeric C9, or other individual components of the C5b–9 complex. SC5b–9 contains two to three C9 molecules per complex. Therefore, reactivity of anti-PC9 with SC5b–9 indicates that the antibody can recognize multimeric C9 that has not polymerized into full rings. The IgG fraction of anti-poly(C9) antiserum (anti-PC9) was prepared (25) and was radiolabeled with $Na^{125}I$ by using Iodobeads (^{125}I-anti-PC9). In order to remove aggregates prior to use in binding assays, ^{125}I-anti-PC9 was centrifuged in an air-driven ultracentrifuge (Beckman) at 178,000 × *g* for 15 min at RT.

Serum Incubation/Bactericidal Assay. *E. coli* J5 cells at a final OD_{600} of 1.5 in $HBSS^{2+}$ were incubated in serum for 30 min at 37°C. Final serum concentrations of 10%, 6.6%, and 3.3% were chosen for experiments with C8D serum. After incubation in C8D serum, the organisms were washed twice in cold VBS and diluted to the appropriate concentration in VBS; then aliquots were placed into tubes for addition of ice-cold VBS, C8 alone, C9 alone, or C8 with various concentrations of C9. Samples were incubated for an additional 30 min at 37°C. Quantitation of viable bacterial colonies was performed as described (26). Bacterial killing in tubes containing C8 and C9 was expressed relative to colony counts in tubes lacking C8 or C8 and C9.

Binding of Radiolabeled C7 and C9. Specific binding of radiolabeled C7 and C9 to *E. coli* J5 was determined with a modification of a previously reported technique (26). ^{125}I-C7 was added to C8D serum prior to incubation with bacteria. Incubation conditions were as described above in the serum incubation/bactericidal assay. Organisms were washed twice after serum incubation, and radioactivity associated with the bacterial pellet after the second wash was determined. Cells bearing C5b–7 were incubated with C8 and labeled C9, and C9 binding was measured. The total number of molecules of C7 and C9 bound per organism were calculated as described earlier (26).

Binding of Anti-PC9 to J5 Cells. The binding of ^{125}I-anti-PC9 to J5 cells bearing C5b–9 was determined. Cells prepared with various inputs of unlabeled C9 were washed three times in $DGVBS^{2+}A$, then suspended in $DGVBS^{2+}A$ to $\approx 1 \times 10^6$ organisms per ml, and divided into 1-ml aliquots. ^{125}I-anti-PC9 at 80, 120, 160, 200, 240, 2000, or 4000 ng was added to cells at each C9 input. Aliquots were also prepared with ^{125}I-anti-PC9 and a 50-fold excess of unlabeled antibody for determination of nonspecifically bound ^{125}I-anti-PC9. All samples were incubated at RT for 30 min with periodic agitation; then 200-μl aliquots were removed, applied in triplicate to 1 ml of $DGVBS^{2+}A$ in microcentrifuge tubes, and centrifuged for 5 min at 12,500 × *g*. The supernatant was aspirated, and ^{125}I-anti-PC9 in the bacterial pellet was counted in a γ scintillation counter. The percentage of specific binding of ^{125}I-anti-PC9 was determined by subtracting the percentage of nonspecifically bound labeled antibody from the total percentage bound. The percentage of specific binding of ^{125}I-anti-PC9 on the saturable portion of the binding curve was thereby determined for each C9 input, and the molecules of ^{125}I-anti-PC9 bound was determined.

Electron Microscopy. Outer membranes from J5 cells bearing C5b–9 were examined by negative-stain transmission electron microscopy. Whole bacteria with bound C5b–9 were prepared exactly as described above for serum bactericidal assays. Controls included cells alone, cells incubated in C8D serum previously heated at 56°C for 30 min to inactivate complement, cells incubated in C8D serum without subsequent addition of C8 or C9, and cells incubated with C8 and C9 only. Samples were washed twice in $HBSS^{2+}$ and then subjected to lysis in a French pressure cell at 16,000 psi (1 psi = 6.9×10^3 Pa). Crude outer membranes were collected by centrifugation of French press lysates for 20 min at 178,000 in an air-driven ultracentrifuge (27). Membranes were applied onto collodion-coated grids by the agar diffusion method (28) and negatively stained with phosphotungstic acid; coded samples were examined by transmission electron microscopy.

RESULTS

Effect of C5b–7 Density and C9 Input on Killing of *E. coli* J5 Cells. Killing of *E. coli* J5 cells by C5b–9 was assessed as the input of C9 was varied (Fig. 1). Cells were incubated initially in either 3.3%, 6.6%, or 10.0% C8D serum, washed, and incubated with excess C8 (4000 units) and amounts of C9 varying from 1.25 to 160 units. Plots of the logarithm (base 10) of the *E. coli* J5 cells killed (log kill: logarithmic difference between cells incubated with C9 and those incubated with C8 alone) vs. the logarithm of C9 units added were sigmoidal in shape. Killing at low C9 inputs was more pronounced for cells incubated in 3.3% C8D serum than for bacteria incubated in 6.6% C8D, which in turn was more pronounced than for organisms receiving 10% C8D. In contrast, at inputs of C9 where a plateau was reached in killing, log kill was directly related to the concentration of C8D serum used in the initial incubation. These findings were not the result of kinetic differences, since the results were not altered significantly by extending the incubation period with C8 and C9 from 30 min to 90 min (data not shown). The results shown in Fig. 1 suggested that the stoichiometry of C9 binding to C5b–8 may be critical in determining the bactericidal efficiency of C5b–9. This possibility was pursued by performing direct measurements of terminal complement component binding to J5 cells.

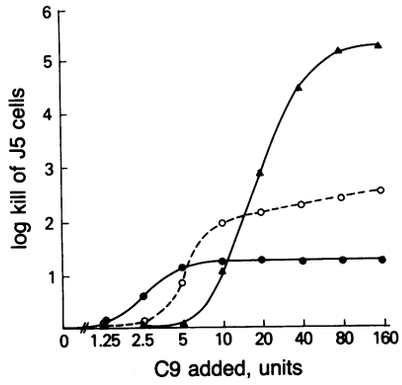

FIG. 1. The logarithm of C9 added vs. log kill of *E. coli* J5. *E. coli* J5 at a final OD_{600} of 1.5 was incubated in 3.3%, 6.6%, or 10% C8D serum for 30 min at 37°C to generate cells bearing 310 (●), 560 (○), or 890 (▲) C5b–7 complexes per cfu after washing. These cells were suspended to 1×10^7 cfu/ml, and aliquots were incubated with excess C8 and increasing amounts of C9 for an additional 30 min at 37°C; log kill was calculated as the logarithmic (base 10) difference between cells incubated with various C9 concentrations and those incubated with C8 alone. Results shown represent the mean for three experiments.

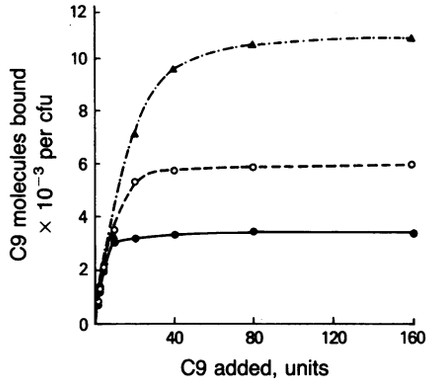

FIG. 2. Molecules of C9 bound per cfu of *E. coli* J5. *E. coli* J5 bearing 310 (●), 560 (○), or 890 (▲) C5b–7 complexes per cfu were prepared as described in the legend to Fig. 1. Then cells were incubated with C8 and increasing concentrations of either ^{131}I-C9 or ^{125}I-C9 for 30 min at 37°C, and the total number of bound C9 molecules was determined. Results shown represent the mean for three experiments.

Measurement of C5b–7 Deposition on J5 Cells. Deposition of C5b–7 was measured on J5 cells during incubation in 3.3%, 6.6%, and 10.0% C8D serum. The total number of C7 molecules specifically bound per colony-forming unit (cfu) after washing was assumed to represent the number of C5b–7 complexes per cfu. C5b–7 binding was linearly related to the dose of C8D serum (Table 1), with 310, 560, and 890 C5b–7 per cfu for 3.3%, 6.6%, and 10.0%, respectively.

Measurement of C9 Deposition on J5 Cells. Binding of radiolabeled C9 to J5 cells bearing different amounts of C5b–7 was measured. Deposition of C9 was determined with ^{125}I-C9 for organisms bearing unlabeled C5b–7 and with ^{131}I-C9 for organisms bearing C5b–7 labeled with ^{125}I-C7. Results were similar with both preparations of C9, but since they were more reproducible with ^{125}I-C9, this preparation was used for most experiments. C9 binding followed a monotonic pattern and was saturable (Fig. 2). Total C9 binding did not differ at C9 inputs of 10 units or less when cells bearing 310, 560, or 890 C5b–7 complexes were compared; at saturation, total C9 bound was directly related to the number of C5b–7 complexes bound.

C9-to-C7 Ratio vs. log Kill of J5 Cells. The ratio of C9 to C7 on J5 cells at various C9 inputs was calculated from the data in Fig. 2. The ratio was plotted vs. log kill at each C9 input (Fig. 3). At C9-to-C7 ratios of <3.3:1, regardless of the number of C5b–7 complexes per cfu, no killing of J5 was observed. Killing of J5, detected at C9-to-C7 ratios between 3.3:1 and 4:1, did not show differences for cells bearing 310, 560, or 890 C5b–7 per cfu. Killing with low C7 sites reached a plateau at a C9-to-C7 ratio of ≈6–8:1. The maximum achievable C9-to-C7 ratio was around 11:1, as reflected by the clustering of data points around this ratio at high C9 inputs in Fig. 3. Killing for organisms bearing 890 C5b–7 complexes per cfu reached a plateau only when this ratio was achieved. At the maximum attainable C9-to-C7 ratio, log kill correlated with the number of C5b–7 complexes per cell.

Table 1. Molecules of C5b–7 bound per organism to *E. coli* J5 cells during incubation in C8D serum

	C8D serum		
Exp.	3.3%	6.6%	10.0%
1	232	545	905
2	465	734	1199
3	234	401	521
4	ND	ND	937
Mean ± SEM	310 ± 77	560 ± 97	890 ± 140

E. coli J5 cells at a final OD_{600} of 1.5 in $HBSS^{2+}$ were incubated in 3.3%, 6.6%, and 10% C8D serum containing ^{125}I-C7 for 30 min at 37°C. Organisms were washed twice, and the number of C5b–7 molecules per organism was measured. ND, not done.

Binding of ^{125}I-Anti-PC9 and Demonstration of Poly(C9) by Electron Microscopy. We wished to confirm by separate methods that multimeric C9 on J5 cells was associated with killing. Two different techniques were used to establish formation of multimeric C9 on J5: (*i*) quantitative binding of an antibody with specificity for multimeric C9 and (*ii*) electron microscopic examination for poly(C9) complexes. Specific binding of ^{125}I-anti-PC9 to J5 cells was demonstrated at C9 inputs that led to significant killing and were calculated to give C9-to-C7 ratios of >4:1. At a C9 input (three units) giving a C9-to-C7 ratio of 1.3:1, or in the absence of C9, minimal specific binding of ^{125}I-anti-PC9 was detected (Fig. 4). Results are shown for organisms bearing 890 C5b–9 complexes per cfu only.

Negative-staining transmission electron microscopy was performed on outer membranes of J5 cells bearing C5b–9. Complement-derived ring lesions, thought to represent

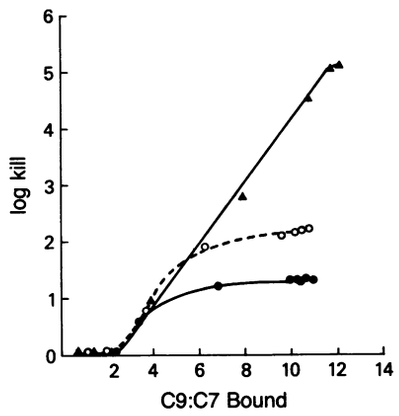

FIG. 3. C9-to-C7 ratio vs. log kill. The ratio of C9 to C7 on *E. coli* J5 bearing 310 (●), 560 (○), or 890 (▲) C5b–9 complexes per cfu was calculated based on the data in Fig. 2. The C9-to-C7 ratio was plotted vs. log kill for each of three different densities of C5b–9.

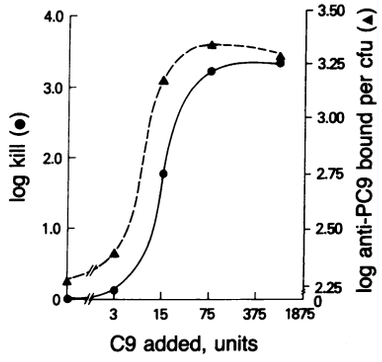

FIG. 4. Binding of anti-PC9 to *E. coli* J5 cells bearing C5b–9 complexes. Organisms bearing 890 C5b–7 complexes per cfu were incubated with excess C8 and increasing amounts of C9. Killing was measured, and the binding of ^{125}I-anti-PC9 molecules to the organisms was determined. Results shown represent the mean for three experiments.

poly(C9) containing 12 to 16 C9 per complex (13), were visualized only at C9 inputs leading to average C9-to-C7 ratios of 4:1 or greater (Fig. 5). At the lowest C9-to-C7 ratio (4:1) for which killing was detected (Fig. 5 *Left*), outer membrane fragments showed ring lesions with an average diameter of 8.82 ± 1.55 nm (mean ± SD, n = 23). At saturating inputs of C9 (Fig. 5 *Right*), the average lesion diameter was 10.84 ± 1.77 nm (n = 32, $P < 0.001$ in comparison to lesions in Fig. 5 *Left*). The number of complement-derived lesions visualized was dependent upon the C9 input, but the number and dimensions of the lesions were similar once saturating C9 inputs were achieved (not shown). However, adventitious ring structures indistinguishable from the complement C5b–9 complex were visualized in low numbers on all samples, including organisms never incubated in serum. These lesions are similar to those described by Swanson and Goldschneider (29) on cell walls on *Neisseria meningitidis* incubated in heated rat serum. At a C9 input leading to a C9-to-C7 ratio of 1.3:1 and no killing, the ring structures were not more numerous than those in controls (not shown).

DISCUSSION

Our experiments show that an average C9-to-C7 ratio of 3.3:1 or greater is necessary for killing of a rough strain of *E. coli* by C5b–9. This is in contrast to erythrocytes, where C5b–8 is known to be lytic and an absolute requirement for C9 has not been demonstrated. Optimal killing of bacteria requires C9-to-C7 ratios of 6:1 or greater. Formation of multimeric C9 at high inputs of C9 was demonstrated by deposition of radiolabeled terminal components, by binding of an antibody with specificity for multimeric C9 and by electron microscopy. Varying the number of bound C5b–7 complexes over a 3-fold range did not change the minimum C9-to-C7 ratio of 3.3:1 required to initiate killing at low C9 inputs. Thus, 5 units of C9 led to no killing of bacteria with 890 C5b–7 cfu per cell, but to a log kill value of >1 with 310 sites per cell. Importantly, the number of C5b67 complexes per cfu did correlate directly with killing at saturating C9 inputs.

A considerable number of reports have examined C9 binding to the C5b–8 complex on erythrocytes or lipid vesicles. The C9-to-C7 ratio of C5b–9 complexes formed on erythrocyte membranes or phospholipid vesicles is heterogeneous (7, 12, 16–19, 30–32). Therefore, determination of C9-to-C7 ratios with radiolabeled complement components provides an average value that may not, by itself, give information on the distribution of complexes bearing different C9-to-C7 ratios. To approach this problem, we performed several experiments. We found that fully closed rings of poly(C9) were visualized only at an average C9-to-C7 ratio of >4:1, suggesting that a C9-to-C7 ratio that leads to the formation of poly(C9) and bacterial death also leads to the appearance of ring lesions. Nonetheless, these ring lesions found at C9-to-C7 ratios of 4:1 were significantly smaller than the lesions visualized at saturating C9 inputs. To more definitively approach quantitation of C5b–9 heterogeneity, we examined the binding to J5 cells of antibody specific for neoantigenic determinants on poly(C9). Specific binding of anti-PC9 to J5 was detected only when a mean C9-to-C7 ratio of 4:1 or greater was achieved but was not detected at the lower ratios studied.

We have confirmed three previous reports indicating that C9 is required for bacterial killing when purified terminal complement components are used to form C5b–9 (1–3). We

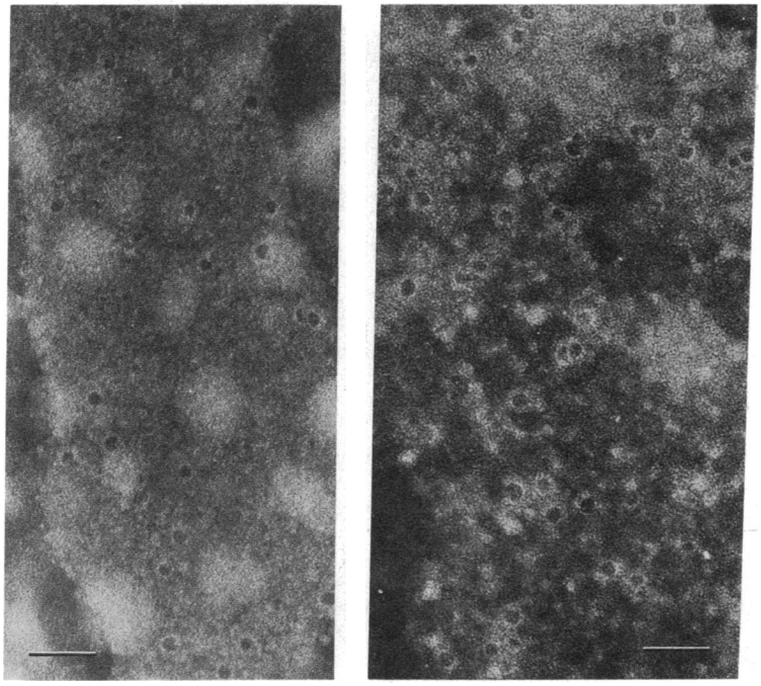

FIG. 5. Electron microscopy of *E. coli* J5 outer membranes bearing C5b–9. Outer membranes were prepared from organisms bearing C5b–9 complexes with different C9-to-C7 ratios. Outer membranes were examined by negative-stain transmission electron microscopy for the presence of ring lesions. The C9 inputs were 13.5 (*Left*) and 121.5 (*Right*) units, corresponding to C9-to-C7 ratios of 4.0:1 and 11.5:1 and log kill values of 0.92 and 3.23, respectively. (×175,500.) (Bar = 100 nm.)

also have found that to achieve killing of a rough *Re* mutant of *Salmonella minnesota* (Re 595) and a rough *Ra* mutant of *Salmonella typhimurium* (TV 119), the presence of C9 is an absolute requirement. In marked contrast to this body of evidence are two reports documenting slow killing of an *E. coli* isolate (33) and a serum-sensitive strain of *Neisseria gonorrhoeae* (34) in C9-deficient serum. It seems unlikely that this discrepancy represents simply a difference between the bacterial strains tested. A more plausible explanation is that an "extra factor" in serum acts in concert with C5b–8 to cause killing. In our experiments and in all previous reports in which purified terminal complement components were used to generate C5b–9, this "extra factor" would not be present at the time C5b–8 was formed. Several "extra factors" necessary for complement-mediated killing of rough Gram-negative organisms have been described (35–37), although most appear to act early in the complement cascade to facilitate activation. Another likely candidate for this "extra factor" is lysozyme, since lysozyme was not removed from the C9-deficient serum used in the experiments of Lint *et al.* (33) and Harriman *et al.* (34).

Our data do not allow us to distinguish whether the bactericidal activity of poly(C9) is a consequence of the large channel produced or is a consequence of the membrane disorganization engendered by poly(C9). It is reasonable to speculate that either consequence of poly(C9) formation may be required for effective disruption of the multilamellar, detergent-resistant cell wall of enteric Gram-negative bacteria. Alternatively, poly(C9) may be required to trigger a process of bacterial inner membrane dissolution (38, 39).

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