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Transfer of Preformed Terminal C5b-9 Complement Complexes into the Outer Membrane of Viable Gram-Negative Bacteria: Effect on Viability and Integrity

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ABSTRACT: An efficient fusion system between Gram-negative bacteria and liposomes incorporating detergent-extracted C5b-9 complexes has been developed that allows delivery of preformed terminal complexes to the cell envelope (Tomlinson et al., 1989b). Fusion of *Salmonella minnesota* Re595 and *Escherichia coli* 17 with C5b-9-incorporated liposomes resulted in the transfer of 1900 C5b-9 complexes to each target bacterial cell. No loss in viability of bacteria was observed following fusion, even though the deposotion of 900 complexes onto the envelope following exposure to lysozyme-free serum effected a greater than 99% loss of viability. Increased sensitivity to antibiotics normally excluded from the cell by an integral outer membrane (OM), as well as the ability of the chromogenic substrate PADAC to gain access to periplasmically located β-lactamase, indicated that transferred C5b-9 complexes functioned as water-filled channels through the OM. A similar conclusion was drawn from measurements demonstrating the uptake by cells of the lipophilic cation tetraphenylphosphonium (bromide), a result further indicating that the membrane potential across the cytoplasmic membrane was maintained following C5b-9 transfer to the OM. Examination of *S. minnesota* Re595 by electron microscopy revealed no obvious difference between cells exposed to lethal concentrations of lysozyme-free serum and cells following fusion with C5b-9-incorporated liposomes. These data suggest either that there are critical sites in the OM to which liposome-delivered C5b-9 complexes are unable to gain access or that bacterial cell death is related to events occurring during polymerization of C9 on the cell surface.

The cell envelope of Gram-negative bacteria consists of three essential layers: the outer membrane that contains LPS[1] in the outer leaflet and forms the major permeability barrier of the cell, the peptidoglycan layer responsible for cell rigidity, and the cytoplasmic membrane, which possesses functions relating to the transport of nutrients, to oxidative phosphorylation, and to the synthesis of cell envelope and periplasmic macromolecules. Activation of the complement system by susceptible Gram-negative bacteria results in the generation and stable deposition into the OM of terminal C5b-9 complement complexes, events strongly correlated to complement-mediated bacterial cell death (Joiner et al., 1982a,b; Kroll et al., 1984; Taylor & Kroll, 1985). However, there is considerable evidence to suggest that bacterial killing is dependent upon perturbation of the CM [reviewed by Taylor (1983)], and the question as to how complement exerts its lethal effect on the CM from its initial location on the OM remains unanswered. C9 appears to be directly responsible for the cytotoxic effect of complement on Gram-negative bacteria, and the presence of multiple copies of C9 in individual C5b-9 complexes on the bacterial surface has been shown to

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[1] Abbreviations: C5b-9, terminal membrane attack complex of complement; BAS, bentonite-absorbed serum; NHS, normal human serum; NHS-C9, NHS depleted of C9; BC1-8, bacteria bearing complement proteins C1 to C8; CAPT buffer, 130 mM NaCl, 10 mM sodium acetate, 10 mM Na_2HPO_4, 5 mM Tris-HCl, pH 7.0; CFU, colony-forming unit; CM, cytoplasmic or inner membrane; OM, outer membrane; NaDOC, sodium deoxycholate; GVB^{2+}, veronal-buffered saline containing 0.1% gelatin, 0.15 mM $CaCl_2$, and 1.0 mM $MgCl_2$; $GGVB^{2+}$, GVB^{2+} containing 2 mg/mL glucose; LPS, lipopolysaccharide; PADAC, 7-(2-thienylacetamido)-3-[[2-[[4-(*N*,*N*-dimethylamino)phenyl]azo]pyridinio]-methyl]-3-cephem-4-carboxylic acid; TPP^+, tetraphenylphosphonium (bromide); Complement proteins are named in accordance with recommendations in *Bull. W. H. O.* (1968).

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be necessary for efficient killing (Joiner et al., 1985; Bloch et al., 1987; Bhakdi et al., 1987).

Zones of bioadhesion between the OM and CM (Bayer, 1968) have been proposed as sites at which C5b-9 insertion mediates bacterial cell death (Wright & Levine, 1981; Joiner et al., 1983; Born & Bhakdi, 1986). Bhakdi et al. (1987) suggested that formation of pores in the CM occurs when C5b-9 complexes in the OM are hit by transiently forming zones of bioadhesion. An alternative to zones of bioadhesion as a model for the translocation of newly synthesized macromolecules from the CM to the OM has been proposed by Lugtenberg and van Alphen (1983); in this model macromolecules are transported via vesicles that bleb off from the CM and fuse with the OM after traversing the periplasm. It has been suggested that C5b-9 insertion into the OM could interfere with such fusion but would not affect release of vesicles from the CM (Taylor & Kroll, 1985), thus causing destabilization of the CM. Two other possible mechanisms of complement-mediated CM damage are the introduction of a small unidentified cytotoxic serum component to the membrane via C5b-9 pores in the OM and the formation of a membrane-active product of C9 as a consequence of C9 binding to C5b-8 or of its subsequent polymerization. Although several C9 molecules per C5b-8 complex appear to be required for effective bacterial killing, it is not known whether the polymerization process itself plays a role in effecting cell death.

The question of how CM damage leads to bacterial cell death has also not been completely resolved. Intracellular ATP pools are depleted following exposure of susceptible *Escherichia coli* to lysozyme-free serum, and uncouplers and inhibitors of oxidative phosphorylation will protect cells from the lethal effects of complement (Taylor & Kroll, 1983), suggesting that complement may kill by causing dissipation of the electrochemical potential across the CM. In fact, strong evidence for irreversible dissipation of membrane potential has recently been presented by Dankert and Esser (1986). Following complement attack, the CM of susceptible cells becomes permeable to small molecules and cations such as *o*-nitrophenyl β-D-galactopyranoside (ONPG), [^{3}H]proline, and $^{86}Rb^+$ (Feingold et al., 1968; Martinez & Carroll, 1980; Wright & Levine, 1981; Kroll et al., 1983), and its recoverability on sucrose density gradients following ultracentrifugation is considerably reduced (Taylor & Kroll, 1985; Tomlinson et al., 1989a). Complement-induced changes to the CM are relatively discrete since the in situ CM lipid bilayer appears structurally intact when examined by electron microscopy (Wilson & Spitznagel, 1968; Davis et al., 1969) and cytoplasmic proteins such as β-galactosidase are not released during the killing reaction (Inoue et al., 1968; Wright & Levine, 1981; Taylor & Kroll, 1983).

In the present study preformed C5b-9 complexes have been introduced into the OM of viable Gram-negative bacteria, and their effect on the viability and integrity of bacteria, in the absence of any other serum components, has been determined.

Materials and Methods

Chemicals and Reagents. Human C7 and 7-(2-thienylacetamido)-3-[[2-[[4-(*N*,*N*-dimethylamino)phenyl]azo]-pyridinio]methyl]-3-cephem-4-carboxylic acid (PADAC) were purchased from Calbiochem, Nottingham, U.K. All radiolabeled reagents were purchased from Amersham Radiochemicals, Amersham, U.K. Nafcillin was obtained from Sigma, Poole, Dorset, U.K., and CGP-7040 was the gift of Dr. P. Traxler, Ciba-Geigy Pharmaceuticals, Basle, Switzerland. All other chemicals were obtained from usual sources and were of the highest purity commercially available.

Bacterial Strains and Media. Salmonella minnesota Re595 was derived from wild-type *S. minnesota* S218 by Dr. Jacik Hawiger, Vanderbilt University, Nashville, TN. *Escherichia coli* 17 is a double phospholipase A negative mutant of K-12 obtained from Dr. Shochichi Nojima, Teikyo University, Kanagawa, Japan. Both strains were grown at 37 °C with aeration in Mueller–Hinton broth and are serum sensitive. Introduction of the gene encoding β-lactamase into *E. coli* 17 was achieved by $CaCl_2$-induced transformation with plasmid pBR322 (Mandel & Higa, 1970). Selection of plasmid-containing clones was done on Mueller–Hinton agar plates containing 50 μg/mL ampicillin. *E. coli* 17(βL^+) were grown in Mueller–Hinton broth containing 50 μg/mL ampicillin.

Sera. Lysozyme-free serum (BAS) was prepared by bentonite absorption of normal human serum (NHS) as described by Wardlaw (1962). Depletion of C9 from NHS and BAS was achieved according to the method of Morgan et al. (1983). Sera were stored at −70 °C until required.

Complement Proteins. C9 was affinity purified from NHS by the method of Morgan et al. (1983). C7 and C9 were iodinated with ^{125}I and ^{131}I (Morgan et al., 1983) with incorporation of 0.8–1.4 and 2.0 I atoms per molecule, respectively. C5b-9 complexes were purified from human serum by incubation with rabbit erythrocytes, which resulted in complement activation and C5b-9 deposition onto the erythrocyte membranes. C5b-9 complexes were extracted and solubilized with NaDOC as described by Bhakdi and Tranum-Jensen (1982) and purified by Sepharose CL-4B column chromatography according to Biesecker et al. (1979). Purified C5b-9 containing iodinated C7 and C9 was prepared by addition of 4 and 1 μg of ^{125}I-C7 and ^{131}I-C9, respectively, to 200 mL of human serum before incubation with rabbit erythrocytes. Protein was determined as described by Lowry et al. (1951), with bovine serum albumin as standard. Determination of protein in detergent was made by measuring the absorbance at 280 nm with $E_{280nm,1\%,1cm} = 13$ [based on the mean value for purified terminal complement proteins (Berger & Hammer, 1988)].

Liposomes. Liposomes were prepared from phospholipids extracted from *S. minnesota* Re595 (Osborn et al., 1972) by dialysis removal of detergent from a phospholipid suspension in NaDOC (Tomlinson et al., 1989b). Adequate removal of NaDOC was essential because of the bactericidal effect of detergent on *S. minnesota* Re595, which accounted for the reported bacterial killing by C5b-9-incorporated liposomes in a preliminary study (Tomlinson et al., 1987). Qualitative and quantitative analyses of extracted phospholipids have been described (Tomlinson et al., 1989b). Liposomes incorporating C5b-9 complexes were prepared by mixing purified complexes in 2% (w/v) NaDOC with 2 mg/mL phospholipid in 2% (w/v) NaDOC before dialysis. Lipid:protein ratios of either 5:1, 15:1, or 30:1 (w/w) were used. Incorporation of C5b-9 was confirmed by dot-blot, rocket immunoelectrophoresis and electron microscopy of fractions following 10–45% (w/w) sucrose density gradient centrifugation (Bhakdi & Tranum-Jensen, 1980).

Cell–Liposome Fusion. Conditions for fusion between bacteria and liposomes were as previously described (Tomlinson et al., 1989b). For the transfer of maximum numbers of preformed C5b-9 complexes to the bacterial OM, cells were incubated with C5b-9-incorporated liposomes prepared from a 5:1 ratio of lipid:protein. Standard incubations (45 min, 37 °C) contained 4×10^8 CFU and a liposome preparation of 11 μg of phospholipid. This corresponded to an approximate

1:1 bacterial phospholipid:liposome phospholipid ratio (Neidhardt, 1987). As previously demonstrated, about 90% of liposome phospholipid is transferred to the cell envelope (Tomlinson et al., 1989b).

Termination of fusion by addition of EDTA (final concentration 25 mM) was followed by washing and resuspending of the cells to their original volume in CAPT buffer, pH 7 (Tomlinson et al., 1989b). Cells were then maintained at room temperature for 60–90 min before they were used in subsequent experiments to test cell envelope integrity. Cell viability was determined according to the method of Taylor and Kroll (1983).

Quantitation of Membrane-Bound C5b-9. Logarithmic or stationary-phase *S. minnesota* Re595 and *E. coli* 17 at a concentration of 4×10^8 CFU/mL in GVB^{2+} were incubated for 30 min at 37 °C with different concentrations of NHS-C9 supplemented with purified ^{125}I-C7 (4000 cpm/μL). The cells carrying C5b-8 (BC1–8) were then washed three times in GVB^{2+} and resuspended to their original volume, and bound ^{125}I radioactivity and cell viability were determined. BC1–8 were then incubated with purified C9 supplemented with ^{131}I-C9 (1400 cpm/μL) either at a concentration that would have been necessary to reconstitute the NHS-C9 to normal serum C9 levels (60 μg/mL) during preparation of BC1–8 cells or at a 5-fold excess (300 μg/mL) for 30 min at 37 °C. Cells were then washed three times in GVB^{2+}, and bound ^{125}I and ^{131}I radioactivity and cell viability were determined. Nonspecific binding of C7 and C9 was assessed by using NHS-C9 heated at 56 °C for 30 min in the first incubation.

Assessment of Cell Envelope Integrity. The use of PADAC, a chromogenic substrate for β-lactamase, in assessing OM integrity following complement attack has been described by Bhakdi et al. (1987). Access of PADAC to periplasmically located β-lactamase of *E. coli* 17(βL^+) was measured following liposome transfer of preformed C5b-9 to the OM by reduction in A_{580}.

The sensitivity of *S. minnesota* Re595 to nafcillin and the rifamycin antibiotic CGP-7040 was determined before and after transfer to preformed C5b-9 complexes to the OM. The mininum bactericidal concentration (MBC) of the antibiotics was defined as the concentration at which a greater than 90% loss in cell viability was observed and was determined after a 90-min incubation at 37 °C of log-phase cells with antibiotic; 4×10^7 CFU was inoculated into tubes containing serial dilutions of antibiotic in 200 μL of Mueller–Hinton broth, and after 90 min cell viability was determined as described. The MBC of both CGP-7040 and naficillin for *S. minnesota* Re595 was 100 μg/mL. Antibiotics were prepared in Mueller–Hinton broth from stock solutions of 50 mg/mL in DMSO. Following fusion of cells (4×10^7 CFU) with C5b-9-incorporated or -unincorporated liposomes, the cells were incubated in 200 μL of Mueller–Hinton broth containin subinhibitory concentrations of the antibiotics (90 min, 37 °C), and cell viability was determined. EDTA postfusion treatment of cells had a minimal effect on antibiotic sensitivity providing the cells were allowed a 90-min recovery period.

The rate of uptake of the lipophilic cation [^{14}C]tetraphenylphosphonium (bromide) (TPP^+) by *S. minnesota* Re595 and *E. coli* 17 was determined by the filtration assay of Kaback (1974) with the modifications of Dankert and Esser (1986). Following cell–liposome fusion and EDTA treatment, the cells (2×10^9) were washed and resuspended in 1 mL of 0.1 M phosphate buffer, pH 6.6. After 90 min at room temperature the cells were used to measure the uptake of TPP^+. EDTA postfusion treatment of cells did not affect TPP^+ uptake

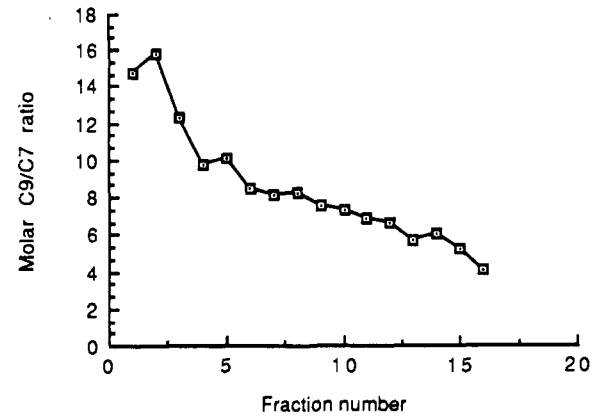

FIGURE 1: Molar ratio of C9 to C7 in eluted C5b-9-containing fractions following gel filtration chromatography. C5b-9 complexes deposited on rabbit erythrocyte membranes were extracted and solubilized in 10% NaDOC and applied to a Sepharose CL-4B column. Column fractions were assayed for ^{125}I-C7 and ^{131}I-C9. Calculations of molar C9 to C7 ratios were based on molecular masses for C7 and C9 of 120000 and 71000 daltons, respectively, and a serum concentration of 60 μg/mL for both proteins.

provided the cells were allowed a 90-min recovery period.

Electron Microscopy. Samples of liposome preparations and cells following fusion with liposomes were incubated with trypsin (final concentration 25 μg/mL) for 60 min at 37 °C before examination. Samples were applied to Formvar-coated copper grids and negatively stained with 2% phosphotungstate, pH 7. Specimens were examined with a Philips CM 10 electron microscope.

RESULTS

Purification of C5b-9. Following gel filtration of extracted iodinated C5b-9 complexes, fractions were analyzed for ^{125}I-C7 and ^{131}I-C9. The elution profile indicated purification of heterogeneous complexes; the calculated molar ratios of C9 to C7 in purified C5b-9 complexes ranged from 15 to 4 C9 molecules per C7 (Figure 1). SDS/urea–PAGE (Bhakdi et al., 1980) and subsequent immunoblotting of C5b-9 using goat polyclonal antisera to the terminal complement proteins (Taylor & Kroll, 1984) revealed the complexes to be of high purity. Isolated complexes containing eight or more C9 molecules per C7 were pooled, concentrated to 2 mg/mL by Amicon ultrafiltration (PM 30), and used for subsequent incorporation into liposomes. Incorporation of C5b-9 complexes into liposomes was previously demonstrated by rocket immunoelectrophoresis and by densitometric measurement (Tomlinson et al., 1989b). Figure 2 shows electron micrographs of C5b-9-incorporated liposomes, and the uptake of negative stain by the liposomes suggests functional incorporation of complexes.

Fusion of Liposomes with Bacteria. Fusion of liposomes prepared from phospholipid extracted from *S. minnesota* Re595 with *S. minnesota* Re595 or *E. coli* 17 resulted in the transfer of liposome phospholipid to the OM and its subsequent translocation to the CM. Additionally, fusion of C5b-9-incorporated liposomes with these bacteria resulted in the transfer and stable insertion of C5b-9 complexes into the OM; there was no association of transferred C5b-9 with the isolated CM (Tomlinson et al., 1989b). Figure 2C–E shows electron micrographs of *S. minnesota* Re595 following exposure to lysozyme-free serum and following fusion with C5b-9-incorporated and -unincorporated liposomes. The C5b-9 complexes seen on the cell envelope following lysozyme-free serum treatment show no obvious difference to those transferred by liposome fusion. Further electron microscope studies using

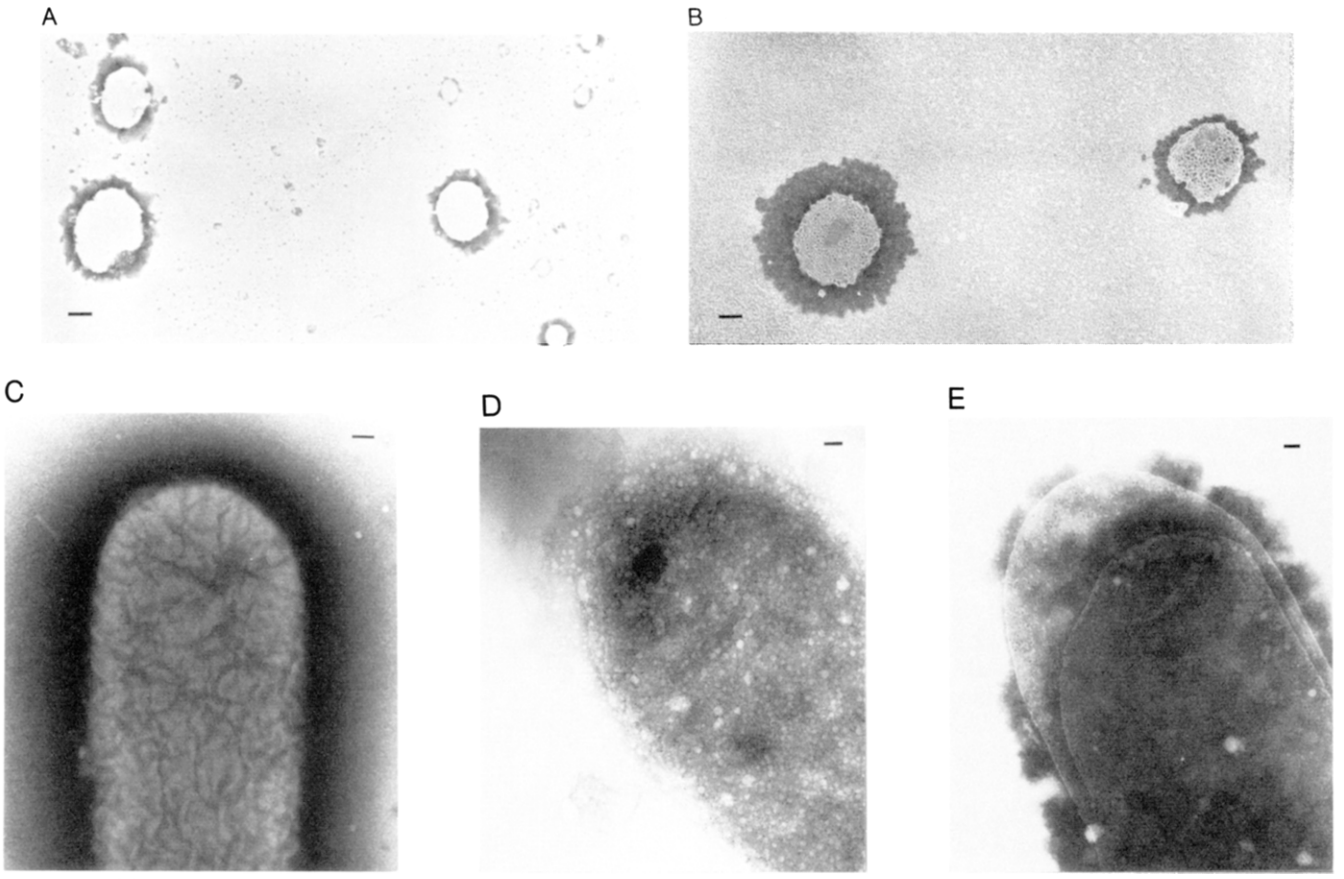

FIGURE 2: Electron micrographs of negatively stained unincorporated (A) and C5b-9-incorporated (B) liposomes and *S. minnesota* Re595 following fusion with unincorporated liposomes (C) and C5b-9-incorporated liposomes (D). (E) *S. minnesota* Re595 following exposure to lysozyme-free serum (45 min, 37 °C). Bacteria were treated with trypsin before preparation for electron microscopy. Scale bars represent 50 nm.

Table I: Fusion of *S. minnesota* Re595 with Liposomes[a]

liposome type[b]	% reduction in viable count	no. of C5b-9 complexes transferred per cell[c]
5:1	53	1900
15:1	46	470
PL	48	
PL (no Ca^{2+})	5	
no liposomes	0	

[a] Log-phase *S. minnesota* Re595 were fused with liposomes as described under Materials and Methods. Viability determinations were made after the EDTA recovery period. [b] Liposomes were prepared with 15:1 or 5:1 phospholipid:C5b-9 (w/w) ratios or from phospholipid alone (PL). Ca^{2+} was an essential buffer component for cell–liposome fusion. [c] Number of C5b-9 complexes transferred per cell calculated from cell-bound ^{125}I-C7 following fusion.

Table II: Exposure of *S. minnesota* Re595 Bearing C5b-8 to C9: Correlation between Viability Loss and C5b-9 Deposition on Log- and Stationary-Phase Cells

	log-phase cells		stationary-phase cells	
% serum[a]	% reduction in viable count	no. of C5b-9 complexes transferred per cell[b]	% reduction in viable count	no. of C5b-9 complexes transferred per cell
0	0	0	0	0
5	0	100	0	90
10	20	310	0	355
20	99	915	0	850
30	>99.9	1410	10	1230
50	>99.9	2450	50	2395

[a] Cells were exposed to NHS-C9, washed, and incubated with C9 at a concentration which would have been necessary to reconstitute the NHS-C9 used to normal serum C9 levels. [b] Number of C5b-9 complexes deposited per cell was calculated from the number of bound ^{125}I-C7 molecules after incubation ofBC1–8 with C9.

freeze–fracture techniques may yield more information regarding possible differences in the depth of C5b-9 insertion. The viability of *S. minnesota* Re595 following fusion with C5b-9-incorporated and -unincorporated liposomes is shown in Table I. Similar figures were obtained for *E. coli* 17 (data not shown). The numbers of C5b-9 complexes transferred to the cells shown in Table I represent the maximum values obtainable with the concentrations and conditions described. Reducing the liposome concentration resulted in the transfer of fewer C5b-9 complexes to the cells, and if the liposome concentration was increased, the resulting Ca^{2+}-induced aggregation could not be effectively reversed upon addition of EDTA. The reduction in viable count of around 50% following fusion was not attributable to transferred C5b-9 complexes since fusion with unincorporated liposomes resulted in similar decreases in viable count. It is possible that the reduced counts are due to aggregation, although in the absence of liposomes Ca^{2+}-induced aggregation of bacteria is effectively reversed with EDTA. C5b-9-incorporated liposomes were also prepared from complexes containing low C9 to C7 molar ratios (four to six C9 molecules per complex). There was similarly no reduction in viable count attributable to such complexes following fusion of these liposomes with *S. minnesota* Re595 and *E. coli* 17.

Quantitation of C5b-9 Deposition for Bacterial Killing. Table II shows the correlation between loss of viability of *S. minnesota* Re595 and number of C5b-9 complexes deposited per cell following incubation of cells carrying different numbers of C5b-8 complexes with C9. BC1–8 cells were incubated with

either an equivalent serum concentration of C9 (60 μg/mL) or a 5-fold excess; the calculated average numbers of C9 molecules per C5b-8 complex were 4.5 and 9.5, respectively. The resulting reduction in viable count was the same for both C9 concentrations. Log-phase cells required an average of 900 C5b-9 complexes per cell for effective killing (greater than 99% reduction in viability). By comparison, the delivery of up to 1900 preformed C5b-9 complexes per log-phase cell by liposome fusion produced no reduction in the viability of *S. minnesota* Re595 (Table I). Incubation of bacteria in either $GGVB^{2+}$ or Mueller–Hinton broth for up to 2 h after fusion did not result in any further decrease in viability (data not shown).

Incubation of log-phase *S. minnesota* Re595 or *E. coli* 17 in the absence of liposomes but under conditions for fusion did not affect the serum susceptibility of these cells: bacteria were incubated in CAPT buffer containing 25 mM Ca^{2+} at 37 °C with subsequent EDTA treatment (as for fusion). Cells were then exposed to 20% BAS, which resulted in a reduction in viability of greater than 99%. Similar results were obtained when 20% BAS was included during incubation with CAPT buffer and Ca^{2+}.

When *S. minnesota* Re595 enter stationary phase, they become much more refractory to the bactericidal action of complement (Table II). Their resistance does not appear to be due to exclusion of C5b-9 complexes from the bacterial surface since the number deposited per stationary-phase cell are sufficient for effective killing of log-phase cells. Calculations of C5b-9 numbers per cell for both log- and stationary-phase cells were made from ^{125}I-C7 and ^{131}I-C9 still bound after the incubation of BC1–8 cells with C9 (the second incubation). Following incubation of log-phase BC1–8 cells with C9 there was a reduction of approximately 10% in bound ^{125}I-C7 compared with a reduction of approximately 25% for stationary-phase cells. These results indicate that a proportion of C5b-8 complexes are less firmly bound to stationary-phase cells, although the number still firmly bound is still high enough to efficiently kill log-phase cells.

Incubation of bacteria in NHS-C9 heated at 56 °C for 30 min did not result in the deposition of C5b-8 on the cell surface, and subsequent addition of C9 had no effect on cell viability. ^{125}I-C7 and ^{131}I-C9 counts from experiments using heated NHS-C9 were used as background when numbers of cell-bound C7 and C9 molecules were calculated.

Integrity of the OM Following Transfer of Preformed C5b-9. The number of preformed C5b-9 complexes transferred to the bacterial OM by liposome fusion was greater than the minimum number necessary to effect killing by deposition following exposure to lysozyme-free serum. It was necessary therefore to determine whether the liposome-transferred C5b-9 complexes functioned as water-filled pores.

E. coli 17 cells containing plasmid pBR322 encoding periplasmically located β-lactamase [17(βL^+)] were fused with both unincorporated and C5b-9-incorporated liposomes. Following a recovery period the permeability of the OM of the fused cells to PADAC was measured by assessing its accessibility to periplasmic β-lactamase. Permeability changes of the OM to PADAC were similarly measured for BC1–8 cells derived from *E. coli* 17(βL^+) following incubation with C9. Incubation of 4×10^8 CFU in 40% NHS-C9 resulted in the deposition of approximately 1900 C5b-8 complexes per cell (a number similar to that transferred by liposomes under the conditions and concentrations described). Figure 3 shows that the C5b-9 complexes transferred to the OM from liposomes appear to be at least as effective at increasing OM

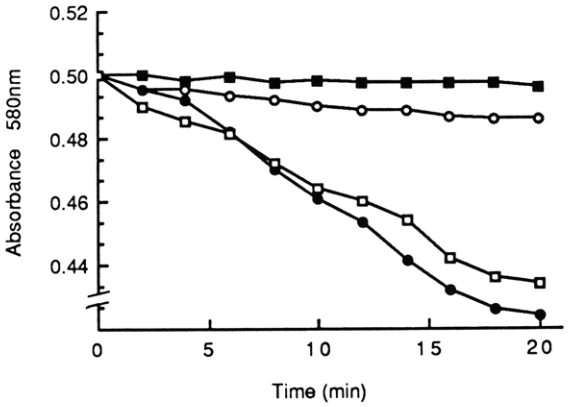

FIGURE 3: Cleavage of PADAC by periplasmic β-lactamase. *E. coli* 17(βL^+) were fused with C5b-9-incorporated liposomes [5:1 (w/w) phospholipid:C5b-9] (●) and unincorporated liposomes (○) under optimum conditions for phospholipid and protein transfer (Materials and Methods). Following fusion, cells were treated with EDTA, washed, and allowed a 90-min recovery period in EDTA-free buffer before the addition of PADAC. Cleavage of PADAC was monitored over 20 min by measuring the decrease in absorbance at 580 nm. PADAC was also added to *E. coli* 17(βL^+) following exposure to fusion conditions but without addition of liposomes (■) and to *E. coli* 17(βL^+) cells exposed to 40% lysozyme-free serum in place of liposomes (□).

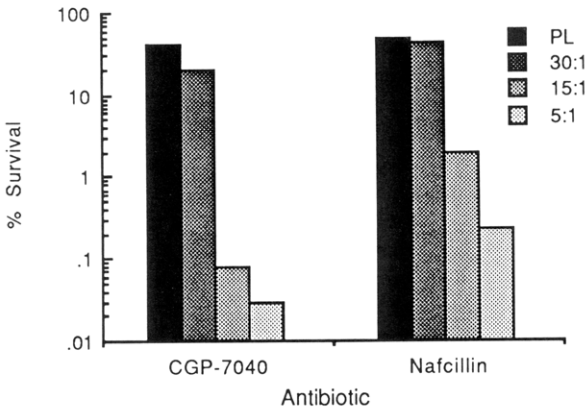

FIGURE 4: Exposure of *S. minnesota* Re595 following liposome fusion to subinhibitory concentrations of antibiotics. *S. minnesota* Re595 were fused under conditions optimum for phospholipid and protein transfer with unincorporated liposomes (PL) and C5b-9-incorporated liposomes containing either 30:1, 15:1, or 5:1 (w/w) phospholipid:C5b-9 ratios. Following a postfusion recovery period (see Materials and Methods), the cells were incubated for 90 min at 37 °C in either CGP-7040 (25 μg/mL) or nafcillin (25 μg/mL). 100% survival value was obtained from cells incubated under fusogenic conditions in the absence of liposomes.

permeability to PADAC as C5b-9 complexes deposited following bactericidal NHS-C9 plus C9 treatment. Phospholipid transfer was not responsible for the increase in OM permeability since fusion of unincorporated liposomes with the cells did not significantly alter the accessibility of PADAC to the periplasmic space. Providing *E. coli* 17(βL^+)–liposome fused cells were allowed to recover for a period of 90 min at room temperature postfusion, EDTA had no effect on OM permeability to PADAC. *S. minnesota* Re595 were not responsive to Ca^{2+} transformation under the conditions described.

The permeability barrier of the OM is responsible for conferring resistance to some Gram-negative bacteria against certain antibiotics (Nikaido & Nakae, 1979). To further study changes in OM permeability following liposome delivery of C5b-9, alterations in the sensitivity of *S. minnesota* Re595 and *E. coli* 17 to the antibiotics CGP-7040 and nafcillin were determined. Unless the OM is damaged, both *S. minnesota* Re595 and *E. coli* 17 display high levels of resistance to both

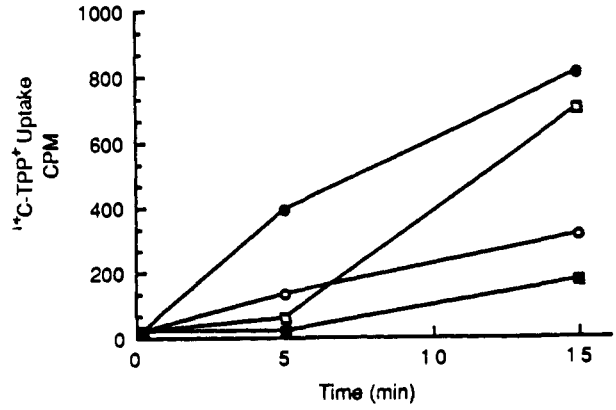

FIGURE 5: Uptake of [^{14}C]tetraphenylphosphonium (bromide) by *S. minnesota* Re595. Bacteria were incubated under fusogenic conditions (45 min, 37 °C) in the absence of liposomes (■), with unincorporated liposomes (○), and C5b-9-incorporated liposomes prepared with phospholipid:C5b-9 (w/w) ratios of 15:1 (□) and 5:1 (●). Following a postfusion recovery period, [^{14}C]TPP$^+$ was added to a sample and its uptake measured at 37 °C by a filtration assay (Materials and Methods) at the indicated time intervals.

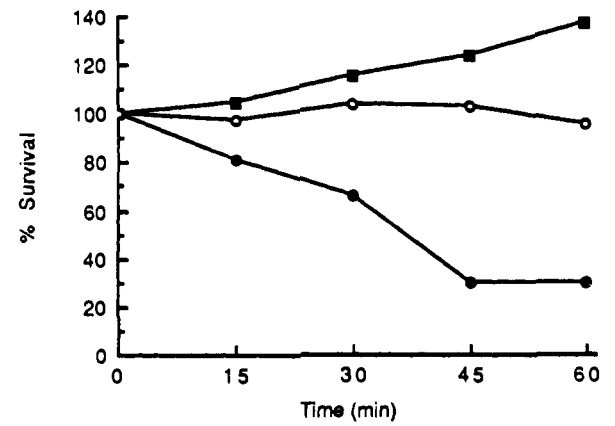

FIGURE 6: Survival of *S. minnesota* Re595 bearing liposome-transferred preformed C5b-9 complexes following exposure to various serum components. Following fusion of *S. minnesota* Re595 with C5b-9-incorporated liposomes [5:1 (w/w) phospholipid:C5b-9] and a recovery period, cells were incubated in GVB^{2+} at 37 °C with lysozyme (7.5 μg/mL) (●), purified C9 (90 μg/mL) (○), and MW 30 000 filtrate of lysozyme-free serum (50% in GVB^{2+}) (■). Samples were removed at the indicated time intervals, and viability was determined.

antibiotics (Figure 4). Following fusion of *S. minnesota* Re595 with C5b-9-incorporated liposomes, the cells become considerably more sensitive to CGP-7040 and nafcillin; increasing the number of C5b-9 complexes transferred results in an increase in the sensitivity of the cells to these antibiotics (Figure 4). Results obtained with *E. coli* 17 were similar except for a lower increase in sensitivity to both antibiotics following C5b-9 transfer (approximately 60% of those shown for *S. minnesota* Re595) (data not shown).

The lipophilic cation TPP$^+$ has been used to measure the membrane potential across the bacterial CM following complement attack (Dankert & Esser, 1986). Usually it is necessary to permeabilize the OM with EDTA to allow TPP$^+$ access to the CM, where its uptake is indicative of an electrochemical potential across the membrane. Figure 5 shows that, following fusion of *S minnesota* Re595 with C5b-9-incorporated but not C5b-9-unincorporated liposomes, TPP$^+$ is taken up by the cells without the need for EDTA treatment. This indicates that the transferred preformed C5b-9 complexes are responsible for allowing TPP$^+$ access to the CM but do not cause damage to the CM as measured by collapse of membrane potential. Permeabilization of the OM by EDTA as described by Schuldiner and Kaback (1975) resulted in the uptake of TPP$^+$ by normal *S. minnesota* Re595 cells but not by lysozyme-free serum killed cells (data not shown). Liposome-fused cells were treated with EDTA following fusion, but if the cells were subsequently given a 90-min recovery period in EDTA-free buffer, OM permeability to TPP$^+$ was not increased.

Because of the impaired permeability barrier function of the OM of Gram-negative bacteria due to C5b-9 insertion after exposure to serum, it is possible that a small unidentified component of serum is able to gain access and cause damage to the CM. Figure 6 shows that when *S. minnesota* Re595 carrying liposome-transferred preformed C5b-9 complexes were incubated with a MW 30 000 filtrate of lysozyme-free serum, there was no reduction in cell viability. Components other than lysozyme which may be removed from serum by bentonite absorption are clearly not necessary for bacterial killing since bentonite-absorbed serum is bactericidal. Addition of purified C9 also had no effect on the viability of these cells. However, incubation of these fused cells in 7.5 μg/mL lysozyme (serum concentration is 2–5 μg/mL) did result in a 70% reduction in viability after 60 min at 37 °C, although there is greater than 99.9% loss in viability following exposure to lysozyme-free serum.

Discussion

Heterogeneity of C5b-9 complexes with respect to the number of C9 molecules in each complex is well documented (Boyle et al., 1979; Ramm et al., 1983; Bhakdi & Tranum-Jensen, 1984; Tschopp et al., 1985), and it has been suggested that complexes containing eight or more C9 molecules exhibit the classical ring ultrastructure (Bhakdi et al., 1987). In the present study complexes containing from 4 to 15 C9 molecules were isolated. The molar ratio of C9 to C8 in NHS is 2, but by addition of an excess of NHS relative to activating target membrane (rabbit erythrocytes), complexes with higher C9 to C8 ratios were obtained since the number of C5b-8 sites per cell is restricted by alternative pathway activation (Tschopp et al., 1985). Tschopp et al. (1985) have reported the purification of C5b-9 exhibiting C9 heterogeneity, but in contrast to our results, they found that high molecular weight C5b-9 had a lower C9 to C7 ratio than low molecular weight C5b-9, which they attributed to aggregation. This difference is likely to reflect differences in conditions for C5b-9 formation and extraction.

Liposomes incorporating C5b-9 complexes were prepared from complexes expected to contain closed rings from C9/C7 molar ratio data. The transfer of these preformed C5b-9 complexes to the OM of *S. minnesota* Re595 and *E. coli* 17 did not result in any loss in viability, even though the deposition of fewer C5b-9 complexes per cell following exposure to lysozyme-free serum resulted in a greater than 99% loss in viability. These results suggest that the insertion of C5b-9 into the OM is not sufficient for bacterial killing, although it is recognized that stable C5b-9 insertion is a prerequisite for the killing of susceptible Gram-negative bacteria [reviewed by Taylor and Kroll (1985)]. There are a number of possible reasons for the inability of liposome-transferred, preformed C5b-9 complexes to kill *S. minnesota* Re595 and *E. coli* 17.

The OM of *E. coli* and *Salmonella* spp. is permeable to small hydrophilic molecules due to the presence of porins, which produce relatively nonspecific pores across the membrane (Benz, 1988); the rate of diffusion of solutes through *E. coli* porin channels is dependent on size, electrical charge, and hydrophobicity (Nikaido & Vaara, 1985). That transferred C5b-9 complexes are still able to function as large

water-filled pores, or at least as protein complexes capable of perturbing the permeability properties of the OM, is supported by data from three separate experiments. The increased sensitivity of *S. minnesota* Re595 and *E. coli* 17 carrying transferred C5b-9 complexes to the antibiotics CGP-7040 and nafcillin suggests that the transferred complexes are responsible for the ability of these antibiotics to traverse the OM. Nafcillin has a molecular mass of only 436 daltons but its hydrophobic character presumably prevents its passage through the porin channel (internal diameter about 1 nm), but not the larger C5b-9 channel (internal diameter about 10 nm). The molecular mass of CGP-7040 is 914 daltons, and in addition to considerations concerning its hydrophobic nature, its size would almost certainly prevent its passage through porin channels. The ability of PADAC and TPP^+ to gain access to the periplasmic space and CM, repectively, also indicates that the transferred C5b-9 complexes in the OM are functioning as water-filled channels, since the OM is normally impermeable to both. In addition to the concept of C5b-9 forming aqueous pores through membranes, there is evidence to suggest that C5b-9 causes a restructuring of the organization of lipids in its immediate vicinity, and thereby an increase in membrane permeability through a "leaky patch" (Esser et al., 1979; Esser, 1982; McCloskey et al., 1989). These models are not mutually exclusive.

Because TPP^+ will equilibrate with the electrochemical potential across the CM (Dankert & Esser, 1986), the uptake of TPP^+ by cells bearing preformed C5b-9 complexes also demonstrates that insertion of these complexes does not cause the dissipation of cytoplasmic membrane potential. The irreversible dissipation of membrane potential across the CM and the inability to recover the CM on sucrose density gradients are both associated with complement-mediated killing of *E. coli* following stable C5b-9 deposition on to the OM. It has been reported previously that the transfer of preformed C5b-9 to the OM does not result in the loss of CM recoverability (Tomlinson et al., 1989b). Goldman and Miller (1989) have reported the isolation of a CM fraction containing C5b-9 lesions from complement-susceptible, temperature-sensitive *S. typhimurium* mutants following exposure to serum; lysozyme was not removed from the serum and CM isolation necessitated the addition of a large excess of untreated carrier cells. It is possible that the subsequent identification of C5b-9 in CM fractions by Goldman and Miller (1989) was due to contamination with OM vesicles bearing C5b-9. Indeed, SDS–PAGE analysis of membrane fractions indicated large amounts of OM proteins in the isolated CM fraction.

It has been suggested that the closed-ring structure of C5b-9 which may be formed on the bacterial OM following serum exposure represents an inactive form of the complex (Dankert & Esser, 1987). The tubular form of poly(C9) is certainly not a requirement for effective killing of complement-sensitive cells since low numbers of C9 molecules per C5b-9 complex can cause viability loss (Joiner et al., 1985; Bloch et al., 1987; Bhakdi et al., 1987). Also, human α-thrombin-cleaved C9, which does not readily form tubular polymers (Dankert & Esser, 1985), is more effective at killing susceptible *E. coli* and *S. minnesota* than native C9 (Dankert & Esser, 1987; Tomlinson et al., 1989a). For this reason, liposomes incorporating C5b-9 complexes containing incomplete rings (low C9/C7 ratio) were fused with *S. minnesota* Re595, but the transfer of these complexes also failed to reduce bacterial viability.

Both Ca^{2+} and PO_4^- are required for efficient fusion between liposomes and bacteria and are known to have profound effects on the OM of *E. coli* and *S. typhimurium* (Brass, 1986; Nikaido & Vaara, 1987). The outer leaflet of the OM, thought to consist almost entirely of LPS, is stabilized by divalent cations, and LPS contains high-affinity binding sites for Ca^{2+} (Schindler & Osborn, 1979). At low temperatures (0–4 °C), the effect of Ca^{2+} on OM permeability allows passage of certain proteins across the membrane (Brass, 1986), and a temperature shift from 0 to 42 °C of Ca^{2+}-treated cells allows uptake of DNA (Mandel & Higa, 1970). Addition of PO_4^- at 0 °C also affects OM permeability, probably due to the formation and interaction of calcium phosphate complexes with LPS (Brass, 1986). Since these effects appear to be dependent on, or at least exaggerated by, temperatures around 0 °C, cells were always maintained at room temperature or above subsequent to cell–liposome fusion. Following fusion of cells and liposomes, it was necessary to add EDTA to reverse Ca^{2+}-induced aggregation. EDTA, particularly in Tris buffer, causes destabilization of the LPS monolayer and an increase in OM permeability. However, any effects on OM permeability by postfusion EDTA treatment were reversed following a recovery period in EDTA-free buffer at room temperature. It is unlikely that the inability of transferred preformed C5b-9 to kill *S. minnesota* Re595 or *E. coli* 17 is due to an effect of the buffers used for fusion since they had no effect on cell viability or on the susceptibility of these cells to serum.

Another possible reason for the inability of OM-located preformed C5b-9 to kill target bacteria is that an additional serum component is required. The possibility of a small membrane-active molecule normally present in serum exerting its lethal effect at the CM after gaining access to the membrane via the C5b-9 channel was excluded; incubation of *S. minnesota* Re595 bearing preformed C5b-9 with a low molecular weight filtrate of lysozyme-free serum did not result in any reduction in cell viability. C9 was similarly ineffective, but lysozyme was able to effect a moderate decrease in viability. Since native C9 is known to cause cell death of complement-susceptible *E. coli* when given access to the CM by osmotic shock (Dankert & Esser, 1987), it may be that C9 (MW 71 000), unlike lysozyme (MW 14 000), is unable to traverse the OM following C5b-9 insertion. However, the effect of the osmotic shock procedure on C9 and the bacterial membrane during C9 exposure is not known. Since exposure of rough strains of *E. coli* to lysozyme-free serum results in the release of periplasmic alkaline phosphatase (MW 80 000) (Wright & Levine, 1981; Taylor & Kroll, 1983), it is possible that C9 is able to traverse the OM but is not in a conformationally active form. However, these experiments do not exclude the possible involvement in bacterial killing of enzymes or bioactive peptides produced as a result of serum activation. It is also possible that other serum proteins involved in killing could be deposited on the bacterial surface during serum exposure, although Shreiber et al. (1979) have demonstrated that serum bactericidal activity against *E. coli* K12 can be generated from the 11 isolated proteins of the alternative pathway.

It is known that the formation and deposition of C5b-9 on the bacterial OM are a prerequisite for the destabilization of the CM and for killing. Our data suggest that C5b-9 deposition alone is not sufficient to cause cell death. Additional data supporting this supposition include the observation that complement-susceptible Gram-negative bacteria require an energy source for efficient killing to occur (Taylor & Kroll, 1983) and that bacteria in stationary phase are much more resistant to complement (Davis & Wedgewood, 1965; DeMatteo et al., 1981), an effect not related to the stable deposition of C5b-9 onto the OM (this paper). Further evidence

that C5b-9 perturbation of OM integrity is not related to the lethal event was provided by Kroll et al. (1983), who demonstrated that following exposure of susceptible bacteria to lysozyme-free serum the resulting kinetics of release of periplasmic alkaline phosphatase is unrelated to the rates of killing. A mechanism of complement killing related to an event occurring during the binding of C9 to C5b-8 and C9 polymerization cannot be ruled out. Dankert and Esser (1986, 1987) have provided indirect evidence for such a mechanism by demonstrating that addition of C9 to cells bearing C5b-8 collapses the cytoplasmic membrane potential and that the membrane potential of CM vesicles is dissipated by the α-thrombin-cleaved C9 fragment C9b, but not native C9. They postulated that a conformationally altered C9 molecule is created or a C9-derived peptide is generated following its interaction with the C5b-8 complex at the bacterial surface. Taylor and Kroll (1985) have reported the cleavage of C9 molecules bound to the OM at a point in time coincident with viability loss of susceptibile *E. coli*. However, no cleavage of C9 could be detected following exposure of colicin A protease negative mutants to serum, even though the cells were effectively killed.

Plasmolysis of complement-susceptible *E. coli* protects cells from the effects of C5b-9 in the OM (Feingold et al., 1968), and following transfer of C5b-9 to the OM by liposome delivery, it was possible that the increased phospholipid transferred to the OM may have had a similar protective effect by separating the two membranes. However, incubation of cells in nutrient media for up to 2 h after C5b-9 transfer did not affect cell viability, and also as previously reported (Tomlinson et al., 1989b), the bulk of the transferred phospholipid is translocated to the CM following liposome fusion.

The possible role of zones of bioadhesion between the CM and OM in the killing of susceptible cells by complement has been discussed (Wright & Levine, 1981; Joiner et al., 1983; Born & Bhakdi, 1986; Taylor & Kroll, 1985). Bhakdi et al. (1987) speculated that C5b-9 pores in the OM are transferred to the CM following the formation of transient attachment points between the membranes. Our data do not support this concept since there was no loss in cell viability and no detectable C9 in isolated CM following preformed C5b-9 transfer to the OM (Tomlinson et al., 1989b). An extension of this concept not previously considered and that would accommodate the present data is that only C5b-9 deposition at circumferential zones of cell envelope differentiation, the periseptal annuli (MacAlister et al., 1983), are lethal. Evidence suggests that these zones are not the same as the points of attachment described by Bayer (1968) and that events in the bacterial division cycle lead to the appearance and localization of these sites of cell division which contain the invaginated septum (Cook et al., 1986, 1987). Differences in OM composition at these specialized sites are likely, and the inability of liposomes to fuse with the OM at such regions would prevent the delivery of C5b-9 to these potentially critical sites. Periseptal annuli appear to form closed compartments and act as a permeability barrier to the lateral mobility of periplasmic macromolecules (Brass, 1986; Cook et al., 1986); they may also restrict lateral mobility of membrane components to ensure that necessary components remain in a specialized compartment (Brass, 1986). For this reason, C5b-9 complexes transferred to the OM by liposome fusion may not be able to gain access to this critical region. Deposition of C5b and subsequent C5b-7 insertion into the OM of dividing cells at these sites upon serum exposure would not face the same constraints as C5b-9 insertion by liposome delivery. The involvement of a polymerization product of C9, such as conformationally altered or cleaved C9 proposed by Dankert and Esser (1987), is not incompatible with our data; translocation of a C9-derived product could conceivably translocate from the OM to the CM via periseptal annuli. Since periseptal annuli are structures relating to division and growth of bacteria, this would also afford an explanation for the ability of complement-susceptible bacteria to withstand the lethal effects of serum when in stationary phase.

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Registry No. C5b-9, 82986-89-8; complement C9, 80295-59-6.

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