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Bacterial killing by complement

C9-mediated killing in the absence of C5b–8

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The ability of serum complement to kill Gram-negative bacteria requires assembly of the membrane attack complex (MAC) on the cell surface. The molecular events that lead to cell killing after MAC assembly are unknown. We have investigated the effect of C9 on bacterial survival in the presence and absence of its receptor, the C5b–8 complex, on the outer membrane. A fluorescence assay of the membrane potential across the inner bacterial membrane revealed that addition of C9 to cells bearing the preformed C5b–8 complex caused a rapid and complete dissipation of the membrane potential. No fluorescence change was observed in serum-resistant strains of *Escherichia coli*. Addition of trypsin, after C9 was bound to C5b–8, did not rescue the cells from the lethal effects of C9. Furthermore, assays of cell killing kinetics and C9 binding indicate that formation of tubular poly(C9) is not required for killing. When C9 was introduced into the periplasmic space in the absence of its receptor by means of an osmotic shock procedure, cell killing occurred. Other proteins, such as C8 or serum albumin, were not toxic, and C9 was ineffective against two resistant strains. The results presented here and previously [Dankert & Esser (1986) Biochemistry **25**, 1094–1100], when considered together, indicate that the 'lethal unit' in complement killing of some Gram-negative bacteria is a C9-derived product that acts by dissipation of cellular energy.

INTRODUCTION

The molecular mechanism of complement-mediated killing of Gram-negative bacteria requires that damage to the energy-transducing inner membrane (IM) results from the deposition of the membrane attack complex (MAC) on the bacterial outer membrane (OM). The MAC consists of the C5b–8 complex plus between 1 and 15 C9 molecules (Müller-Eberhard, 1984) that can form a tubular complex referred to as poly(C9) (Podack & Tschopp, 1982*a*). The postulate that the target for the lethal event is the bacterial IM was supported by the kinetic studies of Wright & Levine (1981*a*,*b*) which indicated coincident release of markers for OM damage (alkaline phosphatase) and IM damage (Rb^+). These authors speculated that the simultaneous release of material from both the periplasmic and the cytoplasmic space could be explained if the MAC were deposited on zones of adhesion (Bayer, 1975) between the IM and OM. However, it was reported later (Kroll *et al.*, 1983) that heat-inactivated serum could induce a rapid release of cytoplasmic Rb^+, indicating that this ion was not a useful analogue for K^+. Support for the data that the IM is the primary target for complement-mediated killing derives, however, from several studies on the role of cellular energy levels on bacterial survival of complement attack (Griffiths, 1974; Taylor & Kroll, 1983, 1984). It was found that inhibitors of oxidative phosphorylation, such as dinitrophenol and cyanide, which inhibit respiration in the IM, could actually protect bacteria from the lethal event of complement. This implies that energy input may be required for optimal complement activity. Furthermore, it is thought that the MAC is responsible for killing of bacteria, and that multiple C9 molecules are required for bacterial killing (Joiner *et al.*, 1985).

Our own studies on the bactericidal effect of complement have centred upon the lytic activity of the C9 molecule itself, and on the polymerization of the C9 molecule during MAC formation. We have shown that the *C*-terminal portion of the molecule, the C9b fragment, is able to dissipate the membrane potential of actively respiring isolated inner membrane vesicles of serum-sensitive *Escherichia coli* (Dankert & Esser, 1986). The membranolytic activity of this fragment was also demonstrated by its ability to form single ion-conducting channels in supported planar bilayers and to cause the release of dye molecules from liposomes (Shiver *et al.*, 1986).

In the present study, we demonstrate that a rapid inhibition of the membrane potential of the IM occurs upon addition of C9 to cells bearing the C5b–8 complex. Furthermore, we show that when C9 alone gains access to the periplasm of sensitive cells, a decrease in viability is detected. We also conclude that complement-mediated killing of bacteria is not coupled to the formation of

Abbreviations used: IM, bacterial inner membrane; OM, bacterial outer membrane; complement proteins are named in accordance with the recommendations in Bull. W.H.O. (1968) **39**, 935–936; BAC1–7, and BAC1–8, antibody-sensitized bacteria carrying complement proteins C1–C7, or C1–C8, respectively; $C9^n$, C9 cleaved by α-thrombin; MAC, membrane attack complex of complement; R–8, or R–9, serum immunochemically depleted of C8, or C9, respectively; NPN, *N*-phenyl-1-naphthylamine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TBS, Tris-buffered saline (10 mM-Tris/HCl/150 mM-NaCl, pH 7.2).

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SDS-resistant tubular poly(C9) and that the mechanism of cell killing involves the expression of a membrane-active product of the C9 molecule. To eliminate possible ambiguities we will follow the original nomenclature (Podack & Tschopp, 1982*a*) and refer to tubular polymeric C9 as poly(C9); thus, poly(C9) does not include other forms of polymeric C9, such as random or string-like aggregates, or partially closed rings.

MATERIALS AND METHODS

Chemicals

EDTA, NaCl, Tris and Tryptic Soy Agar (Difco) were purchased from Fisher Scientific. Acrylamide and SDS were obtained from Serva Fine Biochemicals. The fluorescent dye NPN was supplied by Molecular Probes. $Na^{125}I$ was purchased from New England Nuclear and the iodination reagent (Iodo-Beads) from Pierce Chemical Co. All other reagents and media were purchased from Sigma.

Bacterial strains and media

Escherichia coli strains C600, JC411, LP1395, C14 and *Pasteurella hemolytica* (biotype A, serotype 1) were used in this study. The C600 and JC411 strains were supplied by W. A. Cramer (Purdue University), the LP1395 and C14 strains by P. W. Taylor (Ciba-Geigy Research Centre, Horsham, U.K.), and the *P. hemolytica* strain by M. Lawman (University of Florida). Cells were grown at 37 °C in L-broth (containing casamino acids, 3 g/l; yeast extract, 5 g/l; NaCl, 5 g/l; potassium succinate, 10 g/l; pH 7.0) with constant shaking to mid-exponential phase (approx. 5×10^8 cells/ml). The cells were washed one to three times with TBS and resuspended to the desired density.

Sera

Human sera were obtained from Civitan Regional Blood Center, Gainesville, FL, U.S.A. Sera deficient in human C8 or C9 were prepared by immunoaffinity adsorption as outlined (Dankert *et al.*, 1985).

Proteins

Human C8 and C9 were purified as described (Dankert *et al.*, 1985). Human α-thrombin was a gift from Dr. John Fenton (Albany), 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one ('TPCK')-treated trypsin was bought from Worthington, and soybean trypsin inhibitor from Sigma.

Radioiodination

Proteins were iodinated with $Na^{125}I$ (Amersham) by using the Iodo-Bead (Pierce Chemical Co.) method as suggested by the manufacturer. Specific radioactivity was usually 1–2 Ci/g and the labelled C9 remained better than 85% haemolytically active, in accordance with reports by others (Sims, 1983; Stewart *et al.*, 1984).

Bacterial killing assays

Cells were grown in L-broth, washed, and resuspended in TBS containing 0.05 mM-$CaCl_2$ to approx. 1×10^9 cells/ml, serum was added (1:10 final dilution), and the cells were incubated at 37 °C with constant shaking. At various times, cell samples were withdrawn, diluted into 0.9% NaCl, plated on 4% tryptic soy agar and viability was determined 16–18 h later by colony counting. For the formation of bacterial cells carrying intermediate complement complexes, the cells were incubated in R–8 to form BAC1–7, or in R–9 to form BAC1–8, respectively. Cells were then washed three times in TBS and stored on ice until use, but not for longer than 30 min.

The effect of trypsin digestion on complement-mediated killing was determined by diluting cells either into 0.9% NaCl that contained in addition 0.25 mg of trypsin/ml, or trypsin (0.25 mg/ml) was added to BAC1–8 intermediates just before addition of C9, and then processing continued as described. Such high concentrations of trypsin are very effective in destroying the haemolytic activity of C9 and in preventing poly(C9) formation (J. R. Dankert, unpublished work). The effectiveness of $C9^n$ in bacterial killing was determined by reconstituting R–9 with $C9^n$ (final concentration 60 μg/ml) that contained trace-labelled ^{125}I-$C9^n$.

Uptake and polymerization of C9 on bacteria

C9 binding to bacterial cell walls and formation of poly(C9) was determined by using a slight modification of a procedure described by Tschopp *et al.* (1985) for measuring poly(C9) formation. In brief, cells were periodically removed from the incubation mixture, washed three times in TBS and the pellet was solubilized in 4% (w/v) SDS/50% (v/v) glycerol at 37 °C for 15 min before application to the gel. Gel electrophoresis was performed according to Laemmli (1970) under non-reducing conditions on linear acrylamide gradient (2–20%) gels. The gels were fixed in 50% methanol/10% acetic acid and processed for radioautography using Kodak XAR-5 X-ray film. The relative amounts of SDS-resistant C9 were determined by scanning radioautography films on a laser densitometer (model SL-504-XL; Biomed Instruments) and determining the area under each peak.

NPN fluorescence assay

Fluorometry experiments were performed with an Aminco–Bowman spectrofluorometer (modified with a temperature-regulated stirred cuvette) according to the method of Phillips & Cramer (1973). Briefly, 50 μl of the appropriate cell suspension was added to 200 μl of TBS in a stirred cuvette at 37 °C (final cell concentration approx. 5×10^8 cells/ml). NPN dissolved in methanol was added to a final concentration of 2 μM and the fluorescence was monitored at 420 ± 5 nM (excitation 360 ± 5 nM). The final methanol concentration did not exceed 0.2%, and the total dilution caused by reagent additions was always less than 1%.

Osmotic shock procedure

Cells were grown and washed as described above, and the cooled (4 °C) cells were rapidly injected (to yield a final concentration of approx. 1×10^9 cells/ml) into TBS containing 1.0 M-sucrose and 20 mM-EDTA at 37 °C (Phillips & Cramer, 1973). Proteins to be 'shocked' into the periplasmic space, e.g. C9, C8 or bovine serum albumin, were present at a concentration of 15 μg/ml. The cells were then allowed to incubate for 3 min at 37 °C before dilution into 0.9% NaCl and plating for viability determinations. For some control experiments the protein to be shocked into the periplasm was added 3 min after the plasmolysis was initiated, then allowed to

incubate for a further 3 min, and the suspension was processed as before.

RESULTS

Relative sensitivities of bacterial strains to complement attack

The bacterial strains used in this study have been divided into three groups based on the relative sensitivity of each strain to complement attack, as shown in Table 1. These three groups are sensitive, moderately sensitive, and resistant. The resistant strains show no loss in viability when cells (10^9/ml) are incubated for 1 h in 50% serum, in fact viability increases. The moderately sensitive *P. hemolytica* strain suffers a 40% loss in viability under these conditions. The sensitive strains show a 4–5 log loss in viability under the conditions described above, and at least a 2 log decrease when a 10% serum concentration is used. Serum depleted of C9 (R–9) showed no killing activity against the resistant and moderately sensitive strains, but could effect a small (10–30%) loss in viability for the C600 strain (results not shown). R–9 serum reconstituted with thrombin-cleaved C9 ($C9^n$) was as effective as normal serum under these conditions.

Kinetics of bacterial killing and poly(C9) formation

To investigate the role of C9 in the killing process, and the involvement of the polymerization of C9 into the SDS-resistant tubular form of the molecule, formation of a high-M_r form of C9 was assessed by SDS/polyacrylamide-gel electrophoresis and radioautography. As shown in Fig. 1, ^{125}I-C9 molecules are recovered from washed bacterial envelopes in low-M_r (C9) and high-M_r (MAC) forms. Although relatively little C9 has been taken up at the 10 min point, the 30 and 60 min time points show a large increase in SDS-resistant C9 polymers. When the time course of bacterial viability is compared with the formation of high-M_r C9 it is seen (Fig. 2) that poly(C9) formation occurs long after 99% of the cells have been rendered non-viable. When uncouplers of oxidative phosphorylation, such as CCCP, are present during complement attack an increase in cell survival is observed (Fig. 3). Although the relative amount of poly(C9) formed in the presence and absence of CCCP appears to be similar in radioautographs, scanning densitometry of the negatives (results not shown) reveals that the amount of poly(C9) formed is increased by 50% in the presence of the uncoupler even though cell killing has decreased by more than 90%. Further investigation into the relationship between poly(C9) formation and cell killing was performed with thrombin-cleaved C9, $C9^n$, which has been shown by us to be a form that does not readily form tubular poly($C9^n$) (Dankert & Esser,

Table 1. Serum sensitivity of bacterial strains

Cells were incubated in either buffer or 50% (v/v) human serum for 60 min at 37 °C, diluted, plated on 4% tryptic soy agar, incubated at 37 °C for 16–18 h and colonies were counted. Numbers of colony forming units are ±10% (quadruplicate assays).

Strain	Colony forming units in: Buffer	Colony forming units in: 50% serum	Classification
E. coli C600	1.4×10^9	5×10^4	Sensitive
E. coli JC411	1.1×10^9	6×10^5	Sensitive
P. hemolytica	1.9×10^9	1×10^9	Moderately sensitive
E. coli LP1395	1.0×10^9	1.1×10^9	Resistant
E. coli C14	1.1×10^9	1.2×10^9	Resistant

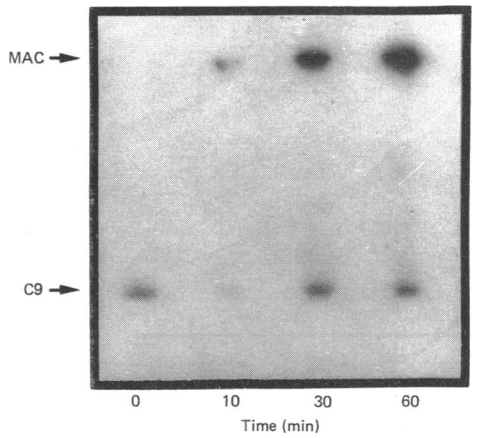

Fig. 1. Uptake and polymerization of C9 on bacterial envelopes

The *E. coli* strain C600 was grown to mid-exponential phase, washed twice in TBS and resuspended to 5×10^8 cells/ml. Serum, trace-labelled with ^{125}I-C9, was added to a 30% final concentration, and the mixture was incubated at 37 °C. At the designated time points, aliquots were withdrawn and washed twice in TBS. The washed cell pellet was solubilized, and applied to a 2–20% linear acrylamide gradient gel as described in the Materials and methods section. Monomeric and polymeric C9 was visualized by radioautography.

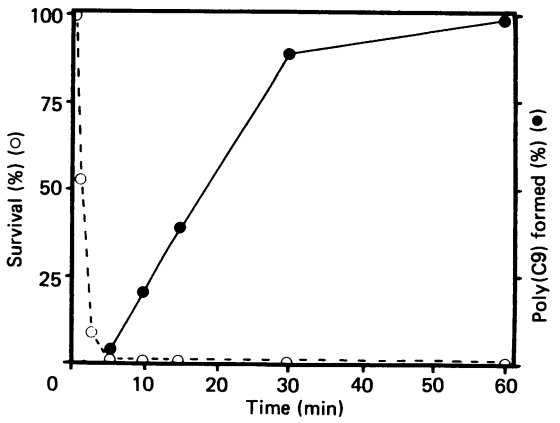

Fig. 2. Time course of loss of viability and of production of poly(C9)

Cells (C600) were grown, washed, and incubated with trace-labelled (^{125}I-C9) serum as described in Fig. 1. At the indicated time points, aliquots were withdrawn for both the viability assay and for radioautography. Viability is plotted as survival (%) of the starting population (○) Poly(C9) formation (●) was quantified by densitometry of the radioautographs as described in the Materials and methods section, and the amount of poly(C9) formed at 60 min was set to 100%.

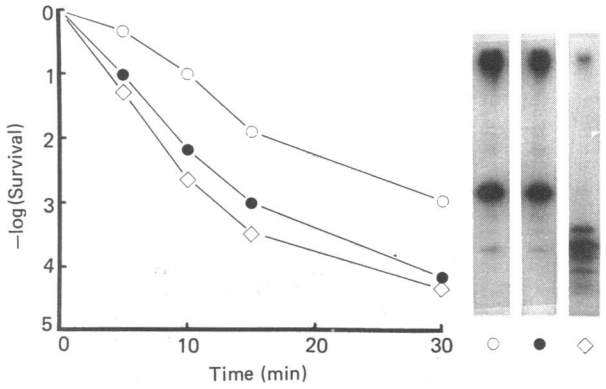

Fig. 3. Effect of uncoupling oxidative phosphorylation and of C9^n on viability

Cells (C600) were grown and washed as described (Fig. 1) and subjected to complement attack using serum depleted of C9 (R–9) that was reconstituted with (*a*) C9 (60 μg/ml) and CCCP (2 μM) (○), (*b*) with C9 (60 μg/ml) (●), or (*c*) with C9^n (60 μg/ml) (◇). Reconstituted R-9 was trace-labelled with ^{125}I-C9 to allow visualization of monomeric and polymeric C9. Samples for radioautography were taken at the 30 min time point.

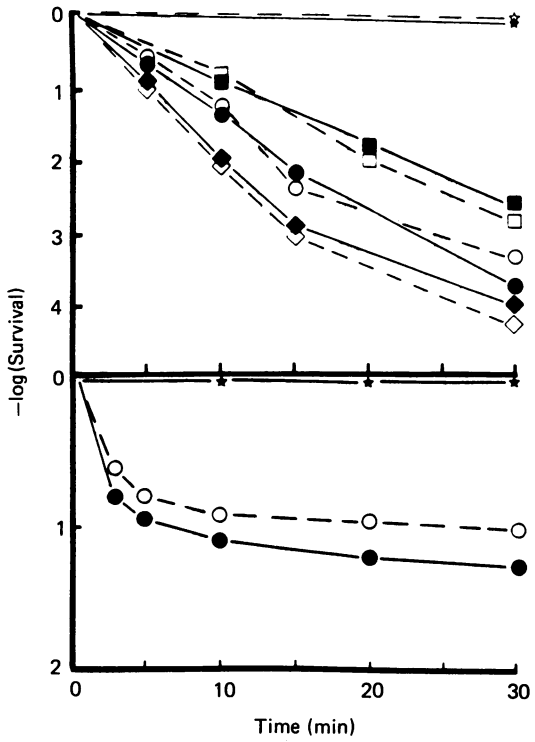

Fig. 4. Effect of trypsin on rate of cell killing

Upper panel. Cells (C600) were incubated with serum at 37 °C (◇, ◆), 31 °C (○, ●), 23 °C (□, ■) or 4 °C (☆, ★) and aliquots were withdrawn at the indicated time points. One aliquot was diluted 100-fold into saline (open symbols), the other into saline containing trypsin (200 μg/ml (closed symbols). Lower panel. BAC1–8 cells (5×10^8 cells/ml), prepared as described in the Materials and methods section, were incubated at 37 °C in TBS (○, ●) and at time = 0, C9 (10 μg/ml) was added, or trypsin (200 μg/ml) was added just before C9 (★) and aliquots were withdrawn at the indicated time points for dilution into saline (open symbols) or into saline containing trypsin (200 μg/ml) (closed symbols). All cells were incubated further for 3 min at 23 °C and further dilutions were then completed for plating.

1985). Although C9^n is more effective than C9 in killing bacteria, little (approx. 5%) high-M_r C9^n is produced after 30 min (Fig. 3). Because cell viability is assayed from plate counts 12–16 h after complement attack, it could be argued that poly(C9) formation occurring after withdrawal of a cell sample and dilution into saline was actually responsible for cell killing. To ensure that the cell viability assay represents the actual time course of killing, cells were diluted into saline containing trypsin (0.25 mg/ml) to degrade monomeric C9 and prevent poly(C9) formation. As seen in Fig. 4 (upper panel), when cells are diluted into trypsin, the rate of killing is unchanged. When BAC1–8 cells are incubated with C9, a similar time course of killing is observed and again the presence of trypsin in the diluent has no effect (Fig. 4, lower panel). In contrast, when trypsin is added just prior to the addition of C9, cell killing is abolished, thus demonstrating that the functional domain of C9 is protected against trypsin attack immediately after its binding to the C5b–8 complex.

In order to investigate further the time course of the effects of complement on bacterial cells, a fluorescence assay was utilized. The neutral lipophilic dye NPN is an indicator of cellular energy levels in *E. coli* (Phillips & Cramer, 1973), and has been used to assay for killing of *E. coli* by certain colicins (Cramer *et al.*, 1983). Upon de-energization of the IM, a structural change is transmitted to the OM of the cell which allows the dye to penetrate into the lipophilic regions of the OM. This change in the environment of the dye increases its fluorescence (Helgerson & Cramer, 1977), but NPN does not directly measure the membrane potential across the IM as do other voltage-sensitive dyes (Waggoner, 1979). Fig. 5 shows the changes in fluorescence of NPN-treated *E. coli*. Trace (*a*) demonstrates the increase in fluorescence upon dissipation of the membrane potential across the IM by the uncoupler CCCP. When BACl–8 cells are used and NPN fluorescence of these cells is monitored, the addition of C9 causes a rapid rise in dye fluorescence that is complete within 25–30 s (trace *b*), but addition of C9 alone in the absence of its receptor complex C5b–8 has no effect (trace *c*). Of significance is the fact that addition of C9 to BAC1–8 prepared from the resistant LP1395 strain caused no increase in fluorescence (trace *c*) whereas addition of CCCP to such complement-resistant cells de-energizes the cells and causes the expected fluorescence change (trace *d*). Similar results were seen when C9^n was used in place of C9 in any of the fluorescence assays described or when KCl instead of NaCl was used in the buffers (results not shown).

These studies taken together indicated that loss of cell viability is tightly coupled to the binding of C9 to the C5b–8 site on the bacterial surface and may be concomitant with de-energization of the IM. In order to investigate the role of the C5b–8 complex as a receptor for the C9 molecule and the role of C9 in membrane potential dissipation, a series of experiments was performed that allowed access of C9 to the periplasmic space of bacteria. For these experiments cells were subjected to osmotic shock by injecting them into concentrated (1 M) sucrose after their OM had been

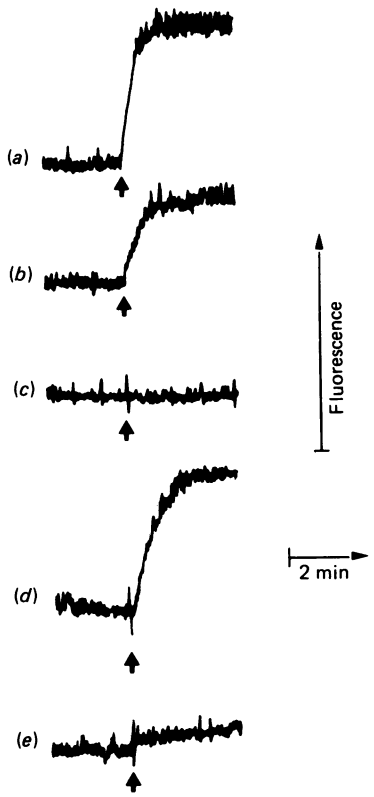

Fig. 5. Effect of C9 and CCCP on cellular energy of bacteria

The lipophilic dye NPN was used to monitor cellular energy levels in normal cells, BAC1–7, and BAC1–8. BAC1–7 and BAC1–8 cells were formed as described in the Materials and methods section. NPN fluorescence (420 ± 5 nm) was monitored continuously at 37 °C, and additions were made to the cuvette at the indicated arrows. Trace (*a*), C600 and addition of 2 μM-CCCP; Trace (*b*), BAC1–8 (C600) and addition of C9 (2 μg/ml); Trace (*c*), BAC1–7 (C600) and addition of C9 (2 μg/ml); trace (*d*), LP1395 and addition of 2 μM-CCCP; trace (*e*), BAC1–8 (LP1395) and addition of C9 (2 μg/ml).

permeabilized by incubation in EDTA. This abrupt change in osmolarity leads to removal of water from the cytoplasm, a lowering of the cytoplasmic volume and an increase in the periplasmic volume. Molecules present in the medium are drawn through gaps created by EDTA in the OM and enter the periplasm. Including C9 in the surrounding medium caused a decrease in cell viability (Table 2) but no significant loss in viability could be detected if C8 or bovine serum albumin were shocked into the periplasmic space. In addition, if C9 was added 3 min after plasmolysis, no decrease in viability was detected. This indicates that C9 must be present during the plasmolysis to allow the molecule to be effectively 'pulled through' the EDTA-disrupted OM. Omitting EDTA in the procedure prevented disruption of the OM and consequently no extracellular molecules can enter the cell and no loss in viability was observed. When thrombin-cleaved C9 ($C9^n$) was added during the shock procedure, killing was just as effective (Table 2) as with C9.

Studies with the moderately sensitive *P. hemolytica* strain or with the two serum-resistant strains of *E. coli* (LP1395 and C14) indicate that serum sensitivity correlates well with sensitivity to the C9 shock procedure (Table 2). Although resistant bacteria may be sensitive to the shock procedure itself, additional killing by C9 was not observed.

DISCUSSION

An understanding of the mechanism of bacterial resistance to complement-mediated killing requires that the process of cell killing is understood first (Esser, 1982; Taylor & Kroll, 1985). Although it is generally agreed that assembly of the MAC on the OM is required, and that the final target is the IM, the nature of the lethal entity is unknown. Our current results indicate that C9 is directly involved in the killing process in *E. coli*. A fluorescence assay was utilized here to investigate the time course of cellular damage by whole complement and specifically by C9. The neutral dye NPN does not easily enter the hydrophilic OM because of its hydrophobicity, but is taken up upon re-energization of the IM (Phillips & Cramer, 1973). It has been used by others to monitor colicin-mediated killing of bacteria (Cramer *et al.*, 1983). When C9 is added to serum-sensitive cells bearing the C5b-8 complex, a rapid (approx. 12–15 s) and complete increase in NPN fluorescence occurs. This surprisingly fast effect of C9 must also take into account the times required for (i) C9 binding to C5b–8 to complete the C5b–9 complex, (ii) translocation of the effect of the formed complex to the IM, (iii) the actual de-energization of the IM, (iv) the resultant induction of a structural change in the OM, and, finally, (v) the change in fluorescence of the dye molecule. Because no change in NPN fluorescence is observed when C9 binds to the C5b–8 complex on resistant *E. coli* LP1395 it is highly unlikely that binding of NPN to the C5b–9 complex is responsible for the fluorescence change. Since treatment of bacteria with EDTA also leads to an increase in NPN fluorescence (Helgerson & Cramer, 1977) the question can be asked whether the rise in NPN fluorescence upon addition of C9 to BAC1–8 signals the dissipation of the membrane potential across the IM or a structural change similar to the ones induced by EDTA without any changes in membrane potential. We discount the second possibility for the following reasons. First, we have demonstrated previously (Dankert & Esser, 1986) that C9 addition leads to irreversible dissipation of the membrane potential in whole cells as measured by three standard procedures that monitor potential-driven transport processes. Second, if the NPN fluorescence change were caused by a structural change in the OM brought about by the assembly of the MAC then the assembly of this complex should also cause a fluorescence change in resistant bacteria which, however, was not observed. Thus, as in the case of colicin-mediated killing, this dye also appears to be useful in studies on complement-mediated membrane potential dissipation.

The very fast dissipation of the membrane potential by C9 is in accord with the inability of trypsin to rescue bacteria from the lethal effects of C9, and both results indicate that the binding of C9 to the C5b–8 complex must be tightly coupled with damage to the IM. We have previously demonstrated that the *C*-terminal fragment (C9b) of C9 dissipated the membrane potential of actively respiring inner membrane vesicles of serum-sensitive *E. coli* (Dankert & Esser, 1986), and reported on its ability to cause the efflux of ions from liposomes and

Table 2. Effect of osmotic shock on bacterial strains

For experimental details see the text. Abbreviation: BSA, bovine serum albumin.

Time of addition	Strain	Addition: Buffer	BSA	C8	C9	$C9^n$	Survival (%)
During shock	*E. coli* C600	+	−	−	−	−	80
		−	+	−	−	−	82
		−	−	+	−	−	78
		−	−	−	+	−	15
		−	−	−	−	+	17
	E. coli JC411	+	−	−	−	−	11
		−	+	−	−	−	10
		−	−	−	+	−	< 2
	P. hemolytica	+	−	−	−	−	100
		−	+	−	−	−	100
		−	−	−	+	−	55
	E. coli LP1395	+	−	−	−	−	83
		−	+	−	−	−	82
		−	−	−	+	−	80
	E. coli C14	+	−	−	−	−	85
		−	+	−	−	−	85
		−	−	−	+	−	83
After shock	*E. coli* C600	+	−	−	−	−	85
		−	+	−	−	−	85
		−	−	+	−	−	82
		−	−	−	+	−	85

to form ion-conducting channels in supported lipid bilayers (Shiver *et al.*, 1986). The intact C9 molecule, however, had no effect upon respiration of bacterial vesicles.

From studies on colicin-mediated killing of bacteria it is known that one effective way to kill Gram-negative bacteria involves dissipation of the membrane potential. One single molecule of colicin E1 kills a cell by depolarization of the IM (Cramer *et al.*, 1983). Futhermore, colicin E1 contains a membrane-active domain (Dankert *et al.*, 1982) that can be liberated by proteinase digestion and the resulting fragment produces ion-conducting channels in planar lipid bilayers (Bullock *et al.*, 1982). Thus, the possibility exists that a similar mechanism is utilized by complement. Colicin E1 utilizes the vitamin B12 receptor (Dimasi *et al.*, 1973) in the OM to reach the IM. Receptorless mutants have been isolated that are not affected by the colicin because the molecule cannot bind to the cell (Davies & Reeves, 1975); however, such resistant mutants can be killed by osmotic shock in the presence of the colicin (Tilby *et al.*, 1978; Ohno-Iwashita & Imahori, 1981). It has been proposed (Stock *et al.*, 1977; Tilby *et al.*, 1978) that toxin molecules are drawn into the rapidly expanding periplasmic space because of the osmotic imbalance.

We have used this approach in the present study to introduce C9 into the periplasm of the cell and to determine its effect on cell viability. Our results indicate that C9 in the absence of any other complement component is able to cause cell death. Assuming that the maximal periplasmic space after osmotic shock is 100% of the cell volume, and using the known concentrations of cells and C9 in the reaction volume, we estimate that as few as eight molecules taken up per cell can cause cell death. It should be noted that efforts to quantify more precisely the number of C9 molecules per cell are complicated because of the unusually high binding of radioiodinated C9 to untreated cells which makes it difficult to estimate very small numbers of molecules per cell. The inability of C9 to affect osmotically shocked but serum-resistant cells in the same way indicates that resistance does not involve changes in binding of C9 to C5b–8 on the OM, or insertion of C9 into the OM, because these cells are refractory to C9 even after it gains access to the periplasmic space and the IM. Of course, this finding does not eliminate the possibility that other resistant bacteria are protected from complement attack because of different mechanisms, such as inefficient binding of the MAC to the OM or shedding of the MAC (Joiner *et al.*, 1982).

We do not know yet which form of C9 delivers the lethal hit to the IM; however, it is extremely unlikely that it is poly(C9). First, our time course studies, in the presence of trypsin, indicate that loss of viability and tubular poly(C9) formation are not coupled events. Secondly, $C9^n$ is more efficient than C9 in killing bacteria although it does not readily form tubular poly($C9^n$), whereas conditions that favour poly(C9) formation, such as uncoupling of oxidative phosphorylation, decrease cell killing. Thirdly, monomeric C9 in the absence of its receptor, the C5b–8 complex, is lethal if it gains access to the periplasmic space and the IM. The inability of trypsin to interfere with killing, and the rapid dissipation of cellular energy by C9 as measured by NPN fluorescence, suggest that the 'lethal C9 unit' is produced quickly, and reaches the IM very rapidly. Because of our earlier observations (Dankert & Esser, 1986) that C9 addition to BAC1–8 collapses the membrane potential of whole cells and that C9b can dissipate the membrane potential of IM vesicles but that C9 cannot, we postulate that either a conformationally altered C9 molecule is created or a peptide is generated from C9 immediately

after its binding to the C5b–8 complex on the OM. Such a peptide could then translocate across the periplasm and reach the IM, or it could move laterally in the plane of the OM and reach the IM at adhesion zones. The latter process would be expected to be more efficient and much faster than the former, but both could happen simultaneously. This hypothesis is in accord with earlier observations of Feingold *et al.* (1968) who observed that killing of bacteria that were first plasmolysed and then exposed to antibody and whole serum is delayed but not eliminated. Under such conditions the IM retracts, adhesion points are supposedly broken, and a lethal product must then translocate across the periplasm to reach the IM. Likewise, it is consistent with earlier observations indicating that the rate of killing of stationary cells is much slower than of exponential-phase cells. Stationary cells have few or no adhesion zones (Lugtenberg & van Alphen, 1983).

Resistance to such a killing mechanism could evolve in several ways. C9 could be prevented from expressing its lethal activity by intrinsic OM constituents, or by formation of poly(C9), since this complex is known to be very resistant to proteolytic degradation (Podack & Tschopp, 1982*b*). If C9 is processed by an effector intrinsic to the target cell, such an effector could be missing from resistant cells. Obviously, it will be necessary now to elucidate the exact structure of the bactericidal C9-derived product and its mode of production.

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