Complement-Mediated Killing of *Escherichia coli*: Dissipation of Membrane Potential by a C9-Derived Peptide†

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ABSTRACT: The molecular mechanism of complement-mediated killing of Gram-negative bacteria has yet to be resolved, but it is generally accepted that assembly of the membrane attack complex (MAC) of complement on the outer bacterial membrane is a required step. We have now investigated the effect of the MAC and its precursor complex, C5b-8, on the membrane potential (ΔE_m) across the inner bacterial membrane. ΔE_m of whole cells was measured directly by using a lipophilic cation (tetraphenylphosphonium) that equilibrates with the potential or indirectly by measuring transport of solutes (proline and galactoside), which is dependent on ΔE_m. Our results indicate that the C5b-8 complex caused a *transient* collapse of ΔE_m in the absence of cell killing. Addition of C9 to allow formation of the MAC dissipated ΔE_m irreversibly, and the cells were killed. Since ΔE_m is generated across the inner membrane in Gram-negative bacteria, inner membrane vesicles were prepared and membrane potentials were generated either by adding D-lactate to energize the electron-transport chain or by creating a K^+ diffusion potential with valinomycin. C9 added in the absence of earlier acting complement proteins had no effect on ΔE_m of isolated, actively respiring vesicles or on K^+ diffusion potentials. In contrast, its C-terminal thrombin fragment (C9b), which has been shown earlier to contain the membrane-active domain of C9, efficiently collapsed ΔE_m in such vesicles. C9b did not require a specific receptor since it was effective on "right-side-out" and "inside-out" vesicles. These results are interpreted to indicate that a C9-derived fragment deenergizes cells and may be the causative agent for cell death.

Mechanisms of host resistance to infection may be divided conveniently into two categories: external physical barriers, such as skin and mucous membranes, and internal antibacterial systems. The latter may be subdivided into humoral and cellular systems, and many studies on host defense against bacterial infections have indicated important functions of serum complement in both processes. Cellular defense mechanisms against Gram-positive and Gram-negative bacteria are aided greatly by C3b-mediated opsonization followed by phagocytosis and intracellular killing. Direct humoral killing is mediated by the membrane attack complex (MAC)[1] of complement, but only Gram-negative organisms can be killed in this manner [for recent reviews, see Taylor & Kroll (1985) and Joiner et al. (1984)]. Although bacterial killing is often accompanied by lysis as a result of serum lysozyme gaining access to the peptidoglycan layer, cell killing does not necessarily depend on lysis (Inoue, 1971). In addition, marked synergism between low levels of free iron (maintained by partially saturated transferrin) and complement for killing of several bacterial strains has been reported; however, the exact mechanisms operating in this interplay between iron binding proteins, antibody, and complement require further clarification (Bullen, 1981).

The locus of the lethal event for MAC-mediated killing has not yet been determined, but it is generally accepted that assembly of the terminal complement proteins C5b-9 on the outer membrane is a required step. Thus far, the only report describing bacterial killing in the absence of C9 is that of Harriman et al. (1981). These authors showed slow killing of *Neisseria* in serum immunochemically depleted of C9, which was greatly enhanced upon reconstitution of the deficient serum with C9. However, assembly of the MAC on the outer membrane is not sufficient for killing as was demonstrated by Harriman et al. (1982) in a subsequent report. Using serum-resistant strains of *Neisseria gonorrhoeae*, these investigators provided clear evidence for the *stable* deposition of the MAC on the outer membrane on these resistant cells. Electron microscopic observations indicated formation of the classical complement lesion even though the cells were not killed. Others have shown that serum-resistant *Salmonella minnesota* escape complement attack by shedding the MAC from the outer membrane into the culture medium in the form of the inactive SC5b-9 complex (Joiner et al., 1982). The precise molecular mechanisms that allow resistant bacteria to escape complement killing are not known largely because the molecular mechanisms responsible for killing of sensitive bacteria are not understood.

Evidence that damage to the outer membrane by complement is not directly responsible for the lethal activity was provided by the studies of Feingold et al. (1968a). These

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[1] Abbreviations: EA, antibody-sensitized sheep erythrocytes; EAC1-8 and EAC1-9, EA carrying bound complement proteins C1 through C8 or C9, respectively; BAC1-8 and BAC1-9, antibody-sensitized bacteria carrying complement proteins C1 through C8 or C9, respectively; MAC, membrane attack complex of complement; R-9 serum, serum immunochemically depleted in C9; IM, inner bacterial membrane; OM, outer bacterial membrane; TMG, methyl thio-β-D-galactopyranoside; TPP^+, tetraphenylphosphonium; ANS, 8-anilino-1-naphthalenesulfonate; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Complement proteins are named in accordance with recommendations in *Bull. W.H.O.* (1968).

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investigators examined the effect of lysozyme-free serum on *Escherichia coli* in the presence of 0.6 M sucrose and found that cells plasmolyzed in this way are refractory to the bactericidal activity. The authors suggested that plasmolyzed cells escape killing because the inner membrane had retracted from the outer membrane and was, therefore, not damaged by complement although alkaline phosphatase, a periplasmic marker, was efficiently released. The notion that the inner membrane is the actual locus for MAC killing was again stated by Wright and Levine (1981), who further proposed that effective lesions are only formed at the adhesion zones between the outer and the inner membranes originally described by Bayer (1975). Their arguments are based largely on the observation that the kinetics of periplasmic enzyme release and intracellular cation release were virtually identical (at 37 °C) and that killing of cells carrying C5b-7 complexes by C8 plus C9 occurred in the absence of any new deposition of C5b-9 complexes on the inner membrane (Wright & Levine, 1981). Damage to the inner membrane was thought to cause dissipation of membrane potentials (ΔE_m) in an analogous fashion to membrane-active colicins such as E1, K, and I (Cramer et al., 1983). Such an explanation, however, is somewhat unsatisfactory because observations by Griffiths (1974) and by Taylor and Kroll (1983) indicate that electron transport inhibitors and uncouplers of oxidative phosphorylation, such as cyanide or dinitrophenol, actually *protect* cells from complement killing.

We have now reinvestigated the effects of the MAC on the membrane potential across the cytoplasmic membrane in whole cells and inner membrane vesicles. Our results indicate that C9-deficient (R-9) serum can collapse the cytoplasmic membrane potential and thereby inhibit membrane transport. However, such a transient collapse alone is insufficient to account for the bactericidal action of complement. In addition, we demonstrate that a C9-derived fragment is active in dissipating membrane potentials in actively respiring inner membrane vesicles. Parts of this work were presented earlier in preliminary form (Esser, 1980).

Materials and Methods

Chemicals. L-[U-^{14}C]Proline at a specific activity of 283 Ci/mol was purchased from Amersham, [^{14}C]methyl thio-β-D-galactopyranoside ([^{14}C]TMG) at a specific activity of 25 Ci/mol was from New England Nuclear, and [^{3}H]tetraphenylphosphonium bromide at a specific activity of 0.25 Ci/mol was kindly supplied by Dr. H. R. Kaback (Roche Institute). Nonradioactive proline and TMG were purchased from Sigma, tetraphenylphosphonium bromide was from K&K Laboratories, and ANS was from Molecular Probes, Inc.

Bacterial Strains. Escherichia coli K12, strains ML 308-225 and W1485, and B/SM, strain 1-1 and a phospholipase A deficient mutant D4 PLAL, were used in this work. The ML strain was supplied by H. R. Kaback, strain W1485 by R. D. Schreiber (Research Institute of Scripps Clinic), and strains 1-1 and its mutant D4 PLAL by K. Inoue (Osaka University Medical School). These strains had been used before in investigations on bactericidal effects of complement, and further details are given in earlier publications (Inoue et al., 1974; Schreiber et al., 1979; Tee & Scott, 1980). All strains were maintained on nutrient agar slants and grown at 37 °C with constant shaking either in trypticase soy broth (TSB) or in minimal medium A (Davies & Mingioli, 1950) containing 1% sodium succinate (hexahydrate) and 1% (w/v) glycerol as the sole carbon source.

Sera. Human sera, obtained from volunteers and from outdated blood supplied by the local blood center, were pooled and adsorbed with the respective *E. coli* strains. Sera immunochemically depleted of late-acting complement proteins (R sera) were prepared as described before (Dankert et al., 1985). Antibacterial sera were raised in rabbits following standard procedures.

Complement Proteins and Peptides. Human C9 was isolated from Cohn fraction III, and proteolysis with human α-thrombin was performed exactly as described by Dankert et al. (1985). C9a and C9b fragments were isolated by preparative SDS–PAGE as described by Shiver (1985) with a commercial apparatus (PrepGel, BRL Laboratories, Gaithersburg, MD).

Assays for Bactericidal and Bacteriolytic Activity. Aliquots of overnight cultures were incubated at 37 °C in test tubes containing 2 mL of medium A with continuous shaking, and growth was followed by monitoring the transmittance of the bacterial suspension at 650 nm. When the optical density reached a value of 0.4, corresponding to a cell density of about 1×10^9 cells/mL, 20 μL of specific antiserum were added, followed 20 min later by either 0.25 or 1.0 mL of whole human serum (WHS), or R-9, or by identical volumes of heated serum or of growth medium as controls. Again, 20 min later, aliquots were withdrawn, diluted in medium A, and plated to determine survival rates.

Transport Assays. Rates of transport of proline, TMG, or TPP^+, were determined with a filtration assay as described by Kaback (1974) with slight modifications. For TMG and proline transport studies, cells were cultured overnight, diluted with 0.1 M phosphate buffer to a cell density of about 2×10^9 cells/mL, and stored on ice until used. For TPP^+ uptake studies, late-log phase cells were first treated with EDTA as described by Schuldiner and Kaback (1975), resuspended in 0.1 M potassium phosphate buffer (pH 6.6) to a cell density of 2×10^9 cells/mL, and used immediately. Several 25-μL aliquots of normal or EDTA-treated cells were then mixed in small test tubes (12 × 75 mm) with an equal volume of 20 mM $MgSO_4$ and warmed to 37 °C in a heating and stirring metal block (ReactiTherm, Pierce Chemical Co.), 2 μL of 0.5 M DL-lactate was added, and then the transport substrate (time $t = 0$) was added to a final concentration of about 0.5 mM ($\approx 5 \times 10^4$ cpm) for TMG, 35 μM ($\approx 5 \times 10^4$ cpm) for proline, or 10 μM ($\approx 4 \times 10^4$ cpm) for TPP^+. At predetermined time intervals, the incubation mixture was diluted with 2 mL of 0.1 M LiCl and immediately filtered through polycarbonate filters of 0.4-μm pore size (Nucleopore) in a multiport filtration unit (Model FH 224V, Hoefer Scientific Instruments, San Francisco, CA) and washed with 2 mL of warm LiCl. The filters were dried on adsorbent paper at 45 °C and counted in 10 mL of scintillation cocktail (ACS, Amersham). For some transport experiments, bacterial cell intermediates were used. In these cases, aliquots from overnight cultures were grown to a density of about 2×10^9 cells/mL and incubated with specific antiserum (20 μL/mL of cell suspension) and with 1 mL of R-9 serum for 20 min to produce BAC1-8 cells. The cells were washed twice and processed for transport studies as described above.

Preparation of Membrane Vesicles. "Right-side-out" inner membrane (IM) vesicles were prepared by treating *E. coli* K12 cells with 10 mM EDTA and lysozyme (0.1 mg/mL) as described by Kaback (1971) and modified by Schuldiner and Kaback (1975); the plasmolysis buffer contained 35% (w/v) sucrose and 10 mM Tris (pH 8.0). Vesicles were resealed in 0.1 M Mes–10 mM KCl (pH 6.6) buffer, washed once in the same buffer, and pelleted. After gentle resuspension, vesicles were frozen and stored in liquid N_2 until used. "Inside-out"

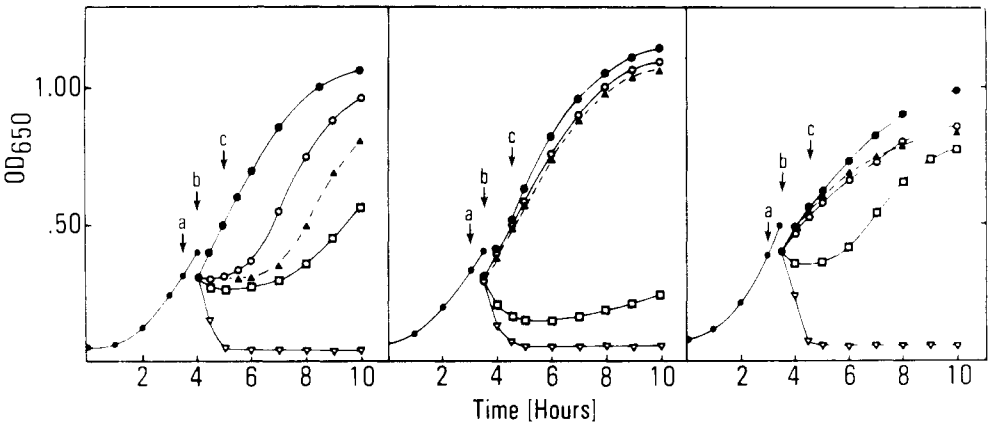

FIGURE 1: Effect of complement on bacterial growth curves. *E. coli* strains K12/W1485 (left panel), B/SM 1-1 (center panel), and ML/308-225 (right panel) were grown in 2 mL of medium A to a density of about 10^9 cells/mL ($OD_{650} \approx 0.4$) at which time 0.02 mL of specific antiserum was added (time point a); 20 min later (time point b), 1.0 mL of either buffer (●), heat-inactivated serum (○), whole human serum (▽), 4-fold-diluted serum (□), or R-9 serum (▲) was added, and after a further 20-min incubation (time point c), aliquots were withdrawn for plating assays.

membrane vesicles were prepared by sudden decompression of right-side-out vesicles in calcium-containing buffer as described by Reenstra et al. (1979) and modified by Dankert (1982).

Membrane Potential Measurements. Membrane potentials (ΔE_m) across IM vesicles were established in two different ways. IM vesicles were prepared in a potassium buffer (0.1 M KCl, 0.1 M MES, pH 6.6) and diluted 100-fold into a choline buffer (0.1 M choline chloride, 0.1 M MES, pH 6.6), and then either (i) valinomycin (1 nM) was added to create a potassium diffusion potential or (ii) ΔE_m was established by addition of D-lactate (10 mM) as substrate for the bacterial electron transport chain (Reeves et al., 1972). In each case a trans-negative potential is generated in right-side-out vesicles and, when D-lactate is used to generate ΔE_m, a trans-positive potential in inside-out vesicles. Trans-positive diffusion potentials across right-side-out vesicles were also produced by preparing IM vesicles in a choline-containing buffer and diluting them into potassium-containing buffer followed by addition of valinomycin. Changes in membrane potential were recorded by measuring the fluorescence intensity of a potential-sensitive dye, 8-anilino-1-naphthalenesulfonate (ANS), as described before (Dankert et al., 1982, 1985). An increase in fluorescence above base-line values reflects formation of a trans-positive potential, and a decrease in fluorescence below base-line values indicates formation of a trans-negative potential.

RESULTS

Bactericidal Activity of Human Sera. Since our goal was to dissect the effects of MAC assembly on bacterial viability and solute transport, it was necessary first to establish the effect of human serum and R-9 serum on the growth of the different bacterial strains and to establish the minimum dose of serum required for killing. We observed that at a density of 10^9 cells/mL all three strains were efficiently lysed by 1 mL of human serum as indicated by a rapid decrease in the absorbance at 650 nm (Figure 1). When viability was quantitated 20 min after addition of serum, about 4 log of cells was killed in each instance (Table I). A 4-fold lower amount of serum was somewhat effective against *E. coli* B/SM but only retarded the growth of the other two strains. Heat-inactivated serum and R-9 serum also proved to be somewhat bacteriostatic for strain W1485 and to a lesser extent for ML/308-225. For this reason, few experiments were carried out subsequently with *E. coli* strain K12/W1485. However, when R-9 was reconstituted with C9 that had been cleaved

Table I: Killing Activity of R-9 and Whole Human Serum

addition[a]	vol (mL)	survival of strain (%)[b] K12	B/SM 1-1	ML
buffer	1.0	100	100	100
heat-inactivated serum	1.0	98	100	98
whole human serum	1.0	0.01	0.01	0.01
whole human serum	0.25	0.15	0.17	0.20
R-9 serum	1.0	90	92	90
R-9 serum + $C9^c$	1.0	0.01	0.01	0.01
R-9 serum + $C9^n$	1.0	0.01	na[d]	na

[a] Additions were made to 2 mL of midlog cells (time point b in Figure 1). [b] Aliquots for plating were withdrawn 20 min after additions (time point c in Figure 1). [c] R-9 serum was reconstituted with physiological amounts of C9 or $C9^n$. [d] na = not assayed.

with human α-thrombin ($C9^n$), it was as effective as normal serum in killing the K12 strain (Table I) although "classical complement lesions", or poly-C9, are not formed in such a serum (Dankert & Esser, 1985).

Transport Studies. To appraise the effect of complement on the maintenance of a membrane potential (ΔE_m) across the inner membrane, we used three assays to determine ΔE_m. First, we measured the uptake of a permeant lipophilic cation, TPP^+, by cells that had been slightly permealized by treatment with EDTA. This ion equilibrates with the electrical potential across a hydrophobic barrier (Haydon & Hladky, 1972; Kaback, 1977) but will not pass through the outer bacterial membrane unless some of the lipopolysaccharide is removed by EDTA treatment. As shown in Figure 2A, TPP^+ is taken up rapidly by W1485; similar uptake was observed with strains B/SM and ML/308-25 [data not shown; see also Schuldiner & Kaback (1975)]. Incubation of antibody-sensitized cells with R-9 serum to assemble the C5b-8 complex on the cell surface abolished uptake of the ion in such cells over the same time span.

Transport of many nutrients is tightly coupled to the total electrochemical potential, or protonmotive force (Δp), in *E. coli*, and therefore, we decided to measure the effect of complement on transport of L-proline and of TMG, a nonmetabolizable lactose analogue. According to Mitchell and Moyle (1968), Δp is defined as

$$\Delta p = \Delta E_m + (2.3RT/F)\Delta pH$$

and as discussed by Kaback (1977), proline accumulation is, therefore, dependent on the pH of the outside solution. We have measured transport of L-proline at pH 6.6 (where $\Delta pH \approx 1$) and noticed rapid uptake of this amino acid in *E. coli* strain B/SM (Figure 2B). A few experiments were carried

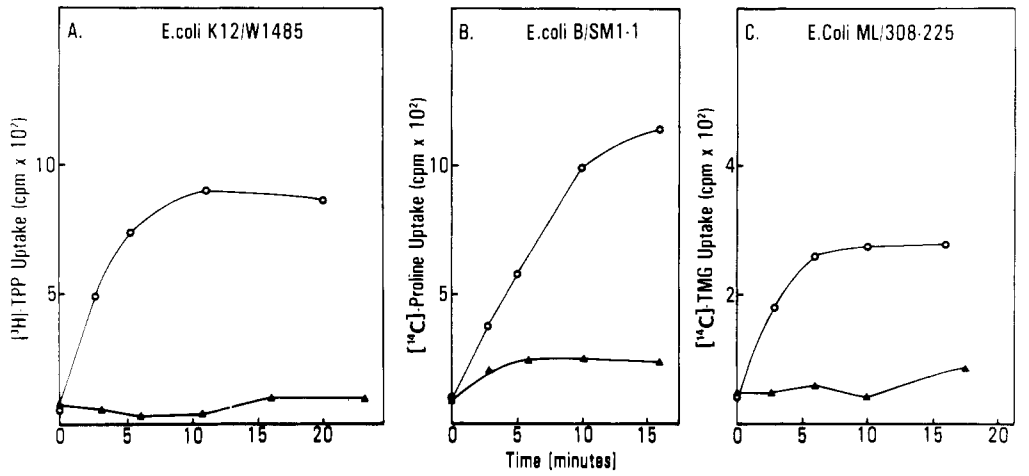

FIGURE 2: Transport of [^{3}H]tetraphenylphosphonium bromide (left panel), L-[U-^{14}C]proline (center panel), and [*methyl*-^{14}C]TMG (right panel) by different strains of *E. coli* at pH 6.6 in a phosphate–$MgSO_4$ buffer (○) or in the presence of R-9 serum (▲). Transport was measured by filtration at 37 °C (see Materials and Methods).

out with ML/308-225 at pH 7.6 (where $\Delta pH \approx 0$), and proline accumulation was also observed (data not shown). When R-9 serum was present during the assay, no accumulation of proline was observed over a 20-min time span (Figure 2B). Since proline is used for protein synthesis, uptake of this amino acid in actively growing cells may not always be quantitatively related to Δp. For this reason, we also measured uptake of TMG, a nonmetabolizable substrate, for which such complications should not exist. As shown in Figure 2C, TMG is also rapidly accumulated in bacteria, and such accumulation is not observed when R-9 serum is present during the assay. We have measured TMG transport only in ML/308-225 cells since this strain is already induced for galactoside transport and does not require preinduction.

The absence of a demonstrable membrane potential in the presence of R-9 serum was somewhat surprising since this deficient serum is not toxic to cells (see Figure 1). We considered the possibility that active transport was abolished for reasons other than dissipation of ΔE_m by the C5b-8 complex that is formed in R-9 serum, i.e., because of the action of unknown inhibitors. When BAC1-8 cells, which were washed free of serum, were tested for transport activities, it was evident that these cells also would not transport proline or TMG (data not shown). However, transport was only inhibited if such cells were tested soon after they were produced and washed free of serum. This observation was studied more closely in a kinetic assay where we measured the initial rate (i.e., accumulation after 1 min of incubation) of TMG transport of ML/308-225 cells in the presence of R-9 serum. As can be seen in Figure 3, uptake of TMG decreased immediately after addition of R-9 serum and was completely inhibited after 30 min but then started to recover after 1 h and was completely restored about 2.5 h later. In contrast, when C9 was added into the incubation mixture after inhibition of transport (t = 30 min), then TMG transport did not recover (Figure 3). Similar results were obtained when BAC1-8 cells were used; after an initial inhibition in the absence of C9, TMG transport increased again with time whereas in the presence of C9 no uptake was observed, and the cells were killed when assayed by plating (data not shown) although they were not lysed since lysozyme was not present when C9 was added.

Effect of C9 and C9-Derived Peptides on ΔE_m of Inner Membrane Vesicles. Since the experiments described above suggested that irreversible dissipation of the membrane potential was associated with the presence of C9, we searched for possible effects of C9 when added directly to vesicles

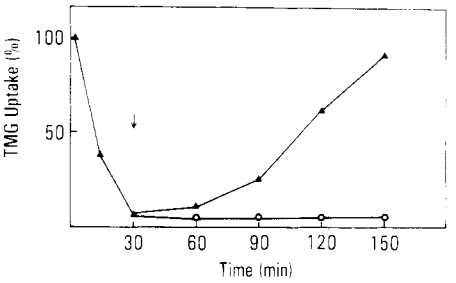

FIGURE 3: Time course of transport of [*methyl*-^{14}C]TMG by *E. coli* ML/308-225 after addition of R-9 serum (time = 0); 30 min into the incubation, either buffer (○) or C9 (▲) was added.

prepared from the inner membrane of *E. coli*. As reproduced in Figure 4, addition of valinomycin or D-lactate to right-side-out IM vesicles (Figure 4, top panel, traces A, B, D, and E) caused an immediate decrease in fluorescence (ΔE_m = trans negative), and when either was added to inside-out IM vesicles (Figure 4, bottom panel, traces A and B), an immediate increase in fluorescence resulted (ΔE_m = trans positive). Subsequent addition of C9 to IM vesicles caused no change in fluorescence (top panel, traces A and D; bottom panel, traces A and C); likewise, C9^n had no effect (data not shown). In contrast, when the purified C9b fragment was added, the fluorescence returned to base-line levels present before addition of valinomycin or D-lactate, signaling the dissipation of ΔE_m. The C9b peptide did not require a specific receptor or binding site since it was effective against both right-side-out and inside-out vesicles. Importantly, it collapsed membrane potentials independent of polarity and was even active in the absence of a potential since it prevented generation of a potential when added before D-lactate or valinomycin (Figure 4, top panel, traces C and F).

Discussion

Our results in this study clearly indicate that complete assembly of the membrane attack complex of complement on the surface of serum-sensitive *E. coli* causes immediate and irreversible dissipation of the membrane potential across the inner bacterial membrane. This conclusion is based on direct measurements of ΔE_m with lipophilic cations that equilibrate with the potential or measurements of proline and TMG transport, which depend on ΔE_m. Our observations, therefore, give some credence to the proposal of Wright and Levine (1981), who suggested that complement kills bacteria by dissipating membrane potentials or by equilibrating potassium

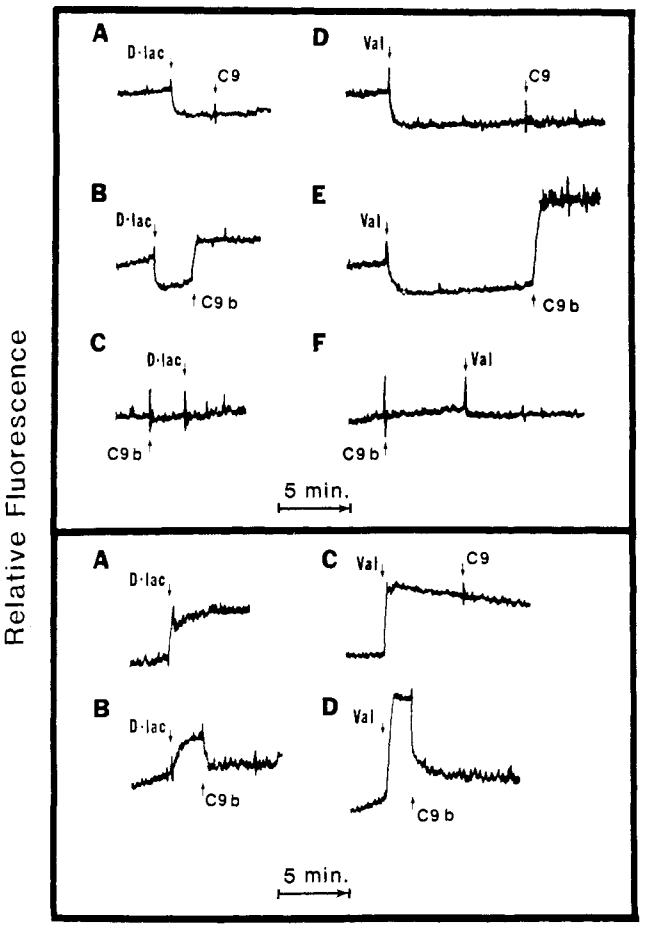

FIGURE 4: Effect of C9 and C9b on membrane potential of IM vesicles. Membrane potentials across right-side-out IM vesicles (top panel, traces A–F; bottom panel, traces C and D) were generated by addition of 10 mM D-lactate (D-Lac) or 1 nM valinomycin (Val) as described under Materials and Methods. ΔE_m was measured by monitoring the fluorescence of 10 μM ANS at 480 ± 4 nm (excitation = 380 ± 4 nm) with continuous stirring. A decrease in fluorescence reflects a trans-negative potential and an increase a trans-positive potential. Traces A and B, bottom panel, were obtained with inside-out vesicles. A total of 3 μg of C9 or 0.3 μg of C9b was added, and all measurements were done at 37 °C.

gradients. However, these investigators did not measure ΔE_m directly but rather relied on kinetic measurements of release of periplasmic and cytoplasmic markers.

Complete acceptance of this hypothesis is complicated by the fact that ΔE_m is also dissipated in bacteria incubated with R-9 serum, which is *nonlethal* as we have demonstrated here, and by the reports of Griffith (1974) and Taylor and Kroll (1983), who showed that uncouplers of oxidative phosphorylation and electron transport inhibitors, i.e., agents that lead to a collapse of ΔE_m, actually *protect* cells against complement attack. Furthermore, the results of Wright and Levine (1981) were questioned by Kroll and Taylor (Kroll et al., 1983; Taylor & Kroll, 1985), who could not correlate the rate of release of a cytoplasmic marker (Rb^+) with killing. There is, however, agreement that the early findings of Feingold et al. (1968b), who provided evidence for impairment of the IM, are substantially correct [for a more detailed discussion, see Taylor & Kroll (1985)]. How then can one reconcile the apparently conflicting observations (i) that R-9 serum causes IM damage as demonstrated by collapse of ΔE_m without cell killing, (ii) that creation of a nonenergized state through use of electron transport or phosphorylation inhibitors protects cells, and (iii) that impairment of IM integrity is directly associated with and may be required for killing?

First, our observation that R-9 serum causes a *transient* collapse of ΔE_m can be explained in two different ways. One possibility is that the C5b-8 complex is deposited on the surface at points of contact between the IM and the OM, also known as "Bayers junctions" (Bayer, 1975). This possibility was already considered by Feingold et al. (1968a) and subsequently by others (Martinez & Carroll, 1980; Wright & Levine, 1981) for the C5b-9 complex. Such adhesion points are considered to be transient or metastable (Lugtenberg & van Alphen, 1983), and the *transient* nature of the membrane potential collapse in R-9-treated cells would be consistent with deposition of C5b-8 complexes on transient adhesion zones. That the C5b-8 complex can impair membrane integrity is well-known from the observations of Stolfi (1968) and Götze et al. (1968) that EAC1-8 cells undergo slow hemolysis. Another possibility is that the remaining C9 in our immunochemically depleted R-9 serum causes damage of the IM (see below) and collapses the membrane potential. We did not detect hemolysis above background when undiluted R-9 serum was tested for C9 activity with EAC1-8 indicator cells as described previously (Dankert & Esser, 1985), but this test is not sufficiently sensitive to detect less than 1 pg of C9/mL. Assuming a concentration of 1 μM of C9 in normal serum and a reduction to about 1 pM in R-9 serum, we would still be adding about 10 C9 molecules per cell in the transport assays. This amount may be sufficient to dissipate ΔE_m but insufficient to reach a critical threshold required for killing. We are in the process of testing whether or not multiple passes of R-9 serum through our affinity column, which should reduce the C9 concentration even further, reduce the capacity of R-9 to inhibit transport.

Second, the protective effect of electron transport inhibitors and uncouplers is not unique; the same phenomenon is observed for colicin-mediated killing of bacteria (Reynolds & Reeves, 1969; Cramer et al., 1976). In this case, the explanation given is that "transmission" of the lytic "hit" on the IM requires an energized inner membrane. Obviously, one can invoke a similar mechanism for complement-mediated killing of bacteria. In the case of colicins, however, additional information is available that indicates that an active fragment of colicin reaches the inner membrane. Such information is missing for complement. Taylor and Kroll (1984) separated the OM from the IM and searched for complement-derived peptides in the IM but were not successful. It is of significance that the recoverability of the IM fraction was drastically reduced when serum-sensitive strains were used whereas it was normal for resistant strains. Loss of the IM fraction was not caused by phospholipase action nor by phase separation of proteins and lipids in the plane of the IM, and the authors suggested that complement attack caused changes in the physical properties of the IM phospholipids, preventing formation of vesicles.

We have addressed the possibility of an active complement fragment reaching the IM in a different way. Guided by our earlier observations that the C9b fragment of C9 can cause conductance changes across bilayer lipid membranes (BLM) and releases 6-carboxyfluorescein from lipid vesicles (Esser et al., 1984), we tested the effect of this fragment on the energy state of IM vesicles. Indeed, we found that C9b efficiently dissipates ΔE_m across such vesicles without requiring a specific receptor on the surface of the IM since it was effective on right-side-out and inside-out vesicles (Figure 4). This result provides the first experimental evidence that the mechanisms of bacterial killing by C9 and colicin may be similar. It is indeed remarkable how similar the two systems are: (1) both require a receptor on the OM, which for colicins can be the vitamin B12 receptor (*btu* B gene product) or the enterochelin

receptor (*ton* A and *feu* B gene products) or several others (Cramer et al., 1983) and for C9 is the C5b-8 complex (Müller-Eberhard, 1984); (2) an energized inner membrane is required for efficient killing, and uncouplers and inhibitors of cellular energy metabolism protect cells from colicin and complement killing; (3) fragments of colicin E1—the 20-kDa C-terminal tryptic fragment (Dankert et al., 1982)—and of C9—the C-terminal C9b fragment (this work)—cause dissipation of the membrane potential across the IM. Whether the actual killing mechanism of C9 is akin to colicin E1 or perhaps to colicin E2 or E3, i.e., inhibition of DNA synthesis or protein synthesis, respectively, is not known with certainty, although most recent data appear to indicate that cessation of macromolecular biosynthesis is a secondary event that reflects a running down of cellular activity in cells that have already been rendered nonviable (Taylor & Kroll, 1985). In addition to the similarities between colicin and complement killing listed above, there is also at least one notable difference. Whereas colicin E1 or its active fragment requires the presence of a trans-negative potential on IM vesicles to be effective (Cramer et al., 1983), C9b does not (Figure 4, top panel, traces C and F). Further suggestive evidence for the generation of a C9 fragment required for killing comes from the work of Taylor and Kroll (1984). These investigators noticed that C9 undergoes proteolytic cleavage on the OM of sensitive strains at a time coincident with initiation of bacterial viability loss but that it remains uncleaved on resistant strains.

A theory of complement-mediated bacterial killing that relies on the formation of an active fragment that deenergizes the cell has several advantages. First, it does not require that the MAC either spans the OM and straddles the periplasmic space in order to reach the IM or must be deposited on adhesion zones to contact the IM. It is very unlikely that the MAC would be able to form a stable channel that could traverse the complete double membrane system especially in light of our recent demonstration that a *tubular* MAC structure is not a prerequisite for hemolytic activity (Dankert & Esser, 1985) nor bacterial killing (this work) since $C9^n$ does not form such a structure. Binding to adhesion zones could result in impairment of the IM, but then one would expect the C5b-8 complex to be lethal, which is not the case for the three strains tested in this work but may be correct for serum-sensitive strains of *N. gonorroehae*, which are slowly killed in R-9 serum (Harriman et al., 1982). Second, if the "lethal unit" is a C9-derived peptide, then it is less difficult to reconcile the several different mechanisms that have been described for bacteria resistance to complement killing, i.e., increase in O antigen concentration on LPS and conversion to a smooth phenotype, shedding of C5b-9 complexes, and different binding of MAC proteins to IM constituents [for further details, see Joiner et al. (1984)]. Bacteria would have several means of escaping complement attack: stable binding of the amphipathic MAC could be prevented by increasing the *hydrophilic* OM barrier, i.e., core and O antigens; cleavage of C9 by an OM protease could be prevented either by loss of the enzyme or by protection of the cleavage site through shielding by other OM constituents; finally, activity of a peptide generated on the OM could be destroyed by proteolytic degradation in the periplasmic space before the fragment reaches the IM. All of these ideas are testable, and we have started to appraise some of these possibilities.

Previous hypotheses on killing of eukaryotic cells by complement have centered around the notion of colloid-osmotic lysis (Esser, 1982; Müller-Eberhard, 1984), and reactions invoking a primary dissipation of ΔE_m have received little support. However, recent experiments by Wiedmer and Sims (1985a,b) strongly indicate that such mechanisms may be operating. These investigators reported that in erythrocytes ΔE_m is irreversibly collapsed *before* hemolysis occurs. In contrast, assembly of the MAC on platelets causes a partial depolarization of ΔE_m, which spontaneously recovers to basal levels, and *no* lysis occurs. Inhibition of the Na^+/K^+ pump by ouabain inhibits repolarization of platelet membranes, indicating that these cells resist complement-mediated lysis by actively maintaining a normal membrane potential. Thus, even in eukaryotic cells dissipation of ΔE_m appears to be a required step for cytotoxicity. Whether such a function is mediated by the C5b-9 complex or by a MAC-derived C9 peptide awaits further investigation.

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Registry No. Complement C5b-8, 82903-91-1; complement C9, 80295-59-6; complement C9b, 83534-36-5; complement MAC, 82986-89-8.

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Two-Dimensional ^{1}H NMR Studies of Cytochrome *c*: Assignment of the N-Terminal Helix†

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ABSTRACT: The ^{1}H resonances of 11 sequential amino acids in the N-terminal helix of horse ferrocytochrome *c* were studied by two-dimensional nuclear magnetic resonance techniques. All the main-chain protons from Lys-5 through Ala-15 and many of the side-chain protons were assigned. *J*-Correlated spectroscopy (COSY) was used to distinguish protons on neighboring bonds and to recognize amino acid types. Nuclear Overhauser effect spectroscopy (NOESY) was used to define spatially contiguous protons and to determine amino acid sequence neighbors. The relayed coherence experiment (relay COSY) was used to resolve many ambiguities in intraresidue *J*-coupled connectivities and interresidue NOE connectivities. This required no explicit knowledge of the solution structure. The pattern of NOEs found is consistent with a regular α helix between glycine-6 and lysine-13; H bonding continues at least through alanine-15 [see Wand, A. J., Roder, H., & Englander, S. W. (1986) *Biochemistry* (following paper in this issue)]. Chain disorder occurs at the N-terminus. There is no indication of significant spin diffusion among the backbone amide and α-protons of this 12.4-kilodalton protein even at the longest NOE mixing time used (140 ms).

The primary problem encountered in ^{1}H NMR[1] studies of proteins is that of resolving individual resonances and assigning them to individual protons in the molecule. Even in the most recent past, considerable ingenuity has been required to assign a modest number of resonances in relatively small proteins. The conception (Jeener, 1971) and subsequent development of two-dimensional NMR techniques (Aue et al., 1976; Sorensen et al., 1983; Braunshweiler et al., 1983; Wider et al., 1984) has raised the possibility that ^{1}H NMR spectra of macromolecules may become amenable to complete analysis. The sequential assignment approach (Billeter et al., 1982; Wüthrich, 1983) provides a straightforward proton assignment methodology that has proven successful with several small proteins (Wagner & Wuthrich, 1982; Arseniev et al., 1982; Wemmer & Kallenbach, 1983; Keller et al., 1983; Stropp et al., 1983; Zuiderweg et al., 1983; Stassinopoulou et al., 1984). However, significant difficulties can be expected in the application of these techniques to the assignment of larger proteins (Wand & Englander, 1985). Here we describe an

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[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; COSY, *J*-correlated spectroscopy; NOESY, NOE correlated spectroscopy; ppm, parts per million.

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