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Does complement kill *E. coli* by producing transmural pores?

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SUMMARY

Three lines of evidence are presented to indicate that C5b-9 kills serum-sensitive *E. coli* K 12 cells by generating functional pores across the outer and inner bacterial membrane. First, viable cells carrying C5b–8 complexes are impermeable to *o*-nitrophenyl-β-D-galactoside (ONPG), but lose viability and become permeable to this marker upon post-treatment with purified C9 in the absence of lysozyme. Cells killed with colicin E1 or gentamicin are also impermeable to ONPG but take up the marker if they are post-treated with lysozyme-free serum. Second, killing by C5b–9 is highly effective, deposition of only a small number of complexes being lethal. This has been demonstrated in experiments where viable cells carrying 2000–4000 C5b–7 complexes per CFU were permitted to multiply in broth culture, and the daughter generations subsequently treated with purified C8 and C9. Fifty percent killing was observed in the fifth to sixth generation, corresponding to a dilution of C5b–7 complexes to 50–100 molecules/CFU. In the presence of 2 mM EDTA, further dilution of C5b–7 down to 8–30 complexes/CFU still caused 50% killing of daughter cells. Third, treatment of C5b–7 cells with purified CC8 and C9 results in the release of intracellular K^+, which commences immediately after addition of C8/C9. This was shown in experiments where C5b–7 cells were packed to high density in saline, post-treated with C8+C9, and K^+ directly measured in the cell supernatants. Based on these results, we propose that C5b–9 pores deposited in the outer bacterial membrane periodically fuse with the inner membrane, the transmural pores thus generated permitting rapid K^+ efflux, with cell death ensuing through the collapse of membrane potential.

INTRODUCTION

Although consensus is being reached that C5b–9 complexes form hydrophilic pores in cytoplasmic membranes of eukaryotic cells (Bhakdi & Tranum-Jensen, 1983; Mayer, 1984; Müller-Eberhard, 1984), it is controversial whether or not the complexes also form pores traversing the outer and inner membrane of serum-sensitive *E. coli*. Indeed, such a concept meets with apparent conceptual difficulties. The outer and inner membranes are believed to be physically separated by the peptidoglycan layer and periplasmic space. The total thickness of the cell wall measures at least 20 nm, whereas the hydrophobic, membrane-spanning portion of C5b-9 has been estimated to measure only 4–5 nm (Bhakdi & Tranum-Jensen, 1978, 1983). Zones of bioadhesion betwen the outer and inner membrane probably exist (Bayer, 1968), but nothing is known concerning their number, stability and dynamics of formation. The possibility that bioadhesion zones may represent the target for lethal attack by complement appeared to be indicated from early studies by Feingold and coworkers (Feingold, Goldman & Kuritz, 1968a, b). These investigators extended the observations of Spitznagel *et al.* (Spitznagel, 1966; Spitznagel & Wilson, 1966; Wilson & Spitznagel, 1968), who had demonstrated that C damages both the inner membranes of *E. coli*, by showing that plasmolysis in hypertonic sucrose protected the cells from serum bactericidal action. They also used ONPG influx measurements to demonstrate directly a permeability leak in the inner membrane of C-treated bacteria.

More recent data to support the pore concept of complement action in *E. coli* have been advanced by Martinez & Carrol (1980) and Wright & Levine (1981a, b). These authors demonstrated that ONPG influx into C-treated bacteria was independent of lysozyme (Martinez & Carrol, 1980) but dependent on C8 and C9 (Wright & Levine, 1981a, b). Considerations of the kinetics of membrane damage further led Wright & Levine to conclude that C5b–9 complexes damage both inner and outer membranes simultaneously via a two-hit mechanism, and transmural pore formation by C5b–9 complexes (possibly dimers) was proposed to represent the primary mode of C-mediated bacterial killing (Wright & Levine, 1981a, b).

Nevertheless, several aspects of bacterial killing by C remain

Abbreviations: C, complement; CFU, colony-forming units; HSA, human serum albumin; ONPG, *o*-nitrophenyl-β-D-galactoside; PBS, phosphate-buffered saline, pH 7·3; R8 or R9 serum, human serum selectively depleted of C8 or C9; VBS, veronal-buffered saline containing 0·15 mM Ca^{2+} and 0·5 mM Mg^{2+}; WHS_B bentonite-absorbed whole human serum.

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puzzling (Taylor, 1983). Unexplained are, for example, the apparent requirement for active metabolism by the bacteria under attack (Taylor & Kroll, 1983); the recovery of C5b–9 complexes exclusively in outer membrane fractions of *E. coli* (Kroll, Bhakdi & Taylor, 1983), and the apparent change in physical properties of the inner membrane, which leads to loss of its recovery after complement attack (Kroll *et al.*, 1983). In addition, interpretation of the rubidium-release data that formed one basis for the pore concept (Wright & Levine, 1981a, b) may be subject to some difficulties, since Kroll & Taylor reported a similar release of this marker with heat-inactivated serum (Taylor & Kroll, 1983). In this connection, further studies on the specificity of the permeability increase of the inner membrane towards ONPG would also seem desirable. Finally, Inoue *et al.* (1977) have presented some data indicating a requirement of very large numbers of C5b–9 complexes per cell (in order of 1500 lesions per bacteria) for killing to occur. If verified, this extreme inefficiency would place severe constraints on the pore model of C5b–9 bactericidal action.

In this study, we have sought to answer three questions. First, is the previously reported influx of ONPG into complement-treated bacteria specific for the action of a terminal complement component? Second, what is the minimum average number of C5b–9 complexes required to prevent bacterial colony formation? Third, does deposition of C5–9 cause K^+ release from bacterial cells? We wish to present data that collectively lead us to conclude that, in basic agreement with Wright & Levine (1981a, b), C5b–9 pores forming in the outer membrane are periodically 'hit' by functional bioadhesion zones, this causing K^+ efflux, membrane depolarization and cell death.

MATERIALS AND METHODS

E. coli K 12 strain W 3110 (Taylor & Kroll, 1983; Kroll *et al.*, 1983) was used in all experiments. ONPG influx was additionally studied in *E. coli* strain T 215, a galactoside-permease negative mutant kindly supplied by Dr P. Overath (Max Planck Institute, Tübingen, FRG). Human serum pooled from 20 healthy donors was depleted of C8 or C9 by passage over immunoaffinity columns at 4° as described elsewhere (Bhakdi & Tranum-Jensen, 1984, 1986). C8 and C9 were isolated from human serum according to published procedures (Biesecker & Müller-Eberhard, 1980; Steckel *et al.*, 1980), and C8 was radioionated to specific activities of $3–15 \times 10^9$ c.p.m./mg as described elsewhere (Bhakdi & Tranum-Jensen, 1986). Whole serum as well as R8 and R9 serum was depleted of lysozyme by absorption with bentonite (5 mg/ml) on ice for 10–15 min (Inai *et al.*, 1959; Kroll *et al.*, 1983; Taylor & Kroll, 1983). Colicin E1 was a kind gift from Dr W. Cramer (Dept. of Biological Sciences, Purdue University, West Lafayett, Ott).

ONPG influx measurements

E. coli T 215 was cultured in basal medium [composition: KH_2PO_4, 2 g/l; K_2HPO_4, 7 g/l; $(NH_4)_2SO_4$, 1 g/l; $MgCl_2 \cdot 6\,H_2O$, 0·1 g/l; vitamin-free casein hydrolysate, 2·5 g/l]. The cells were harvested in log-phase at a density of $2–3 \times 10^8$ CFU/ml. Synthesis of β-galactosidase was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside (Sigma, Munich, FRD) to the bacterial cultures 2 hr prior to harvesting (Pardee, Jacob & Monod, 1959). One volume of bacterial suspension was then washed twice in 50 mM sodium phosphate buffer, pH 7·2 (Eppendorf table-top centrifuge) and incubated with varying amounts of bentonite-absorbed WHS (WHS_B). Alternatively, bacteria were incubated with R9 serum (2–40% vol/vol final concentration) for 20 min, 37°, washed three times with 50 mM phosphate buffer, and resuspended to their original concentrations in this buffer. Purified C9 was then added to a final concentration of 70 μg/ml and the cells incubated at 37° for 20 min. Thereafter, 0·2 ml aliquots were removed for ONPG permeation tests (Dobrogosz, 1981). The aliquots were added to a mixture of prewarmed (30°) 50 mM phosphate buffer, pH 7·2 (4·1 ml) and 32 mM reduced glutathione (0·2 ml), and 0·5 ml of a 10 mM ONPG solution (dissolved in water) was added to initiate the reaction. After 15 min at 30°, the reactions were terminated by the addition of 1 ml of 1 M Na_2CO_3, and absorbance was measured at 435 nm. Total intracellular β-galactosidase was measured with freeze-thawed fractured T 215 cell suspensions.

In experiments designed to test the specificity of ONPG influx, *E. coli* were first incubated with colicin E1 (20 μg/ml) for 40 or 210 min at 37°. It is known that such treatment causes rapid K^+ efflux, collapse potential, and cell death (Feingold, 1970; Cramer, Dankert & Uratani, 1983). Total loss of viability was confirmed in our experiments by plating out 10 μl aliquots of the colicin-treated cells. ONPG permeation experiments were performed on the colicin E1-treated cells as described above. In addition, cells first killed by colicin E1 were post-treated with bentonite-absorbed whole human serum (WHS_B) or with R9 serum, and then analysed for ONPG uptake.

Another set of experiments was performed in which the *E. coli* were incubated with 50 μg/ml gentamicin for 3–5 hr at 37° and then similarly tested for ONPG uptake with and without post-treatment with WHS_B.

Killing efficiency of C5b–9

In the first series of experiments the average number of C5–7 complexes generated on the surface of *E. coli* after incubation with very high doses of R8 serum was determined. Cells were grown in TSB (Difco, Detroit, MI) to a density of $4–5 \times 10^8$ CFU/ml. Three ml were centrifuged and the cells washed twice in VBS, resuspended in 3 ml of this buffer, and incubated at 37° with 3–5 ml R8 serum for 20 min. The cells were then washed three times in VBS, resuspended in 8 ml TSB, and incubated at 32° in a water-bath. Four-hundred microlitre aliquots of bacterial culture were removed at regular intervals over a period of 150–210 min and given 120 ng C8 + 20,000 c.p.m. functionally active (bindable) ^{125}I-C8 + 30 μg purified C9. After 20 min at 37°, 10 μl aliquots were diluted and plated out for colony counting. Over 90% killing still occurred after 150 min of culture (see the Results section). The cells were centrifuged, washed three times in saline containing 1% HSA, and the total number of bound C8 molecules calculated from the amount of specifically bound radioactivity. Non-specific binding of ^{125}I-C8 was assessed by incubating *E. coli* cells not pretreated with R8 serum in the same manner. Non-specific binding was always less than 1·5% of total radioactivity.

In the second set of experiments, cells carrying C5b–7 were inoculated into a large volume of culture broth, so that the cells remained in the log-phase of growth throughout the experiments. To this end, 1 ml of washed suspended in VBS to 5×10^8 CFU/ml was treated with 1·5 ml R8 serum for 15 min at 37°. The cells were washed three times in VBS and resuspended in TSB at

starting densities of 10^3–10^4 CFU/ml. The cells were cultured at 32°, and 0·5 ml aliquots were removed at regular intervals over time-periods of 3·5–5 hr and given a mixture of C8 and C9 (50 μg/ml of each component final concentration) ±2 mM EDTA (final concentration) for 20 min at 37° min at 37°, and viability tests performed by colony counting. All counts were performed in triplicate.

Measurement of K^+ efflux

Cells carrying C5b–7 complexes were prepared by incubating 100 ml *E. coli* cell suspensions (1·5–3 × 10^8 CFU/ml) with 60 ml R8 serum for 15 min at 37°. After three washings with PBS, the cells were resuspended in 2 ml saline +1% glucose. Cells that received no R8 serum were processed in parallel as controls. C8 and C9 equilibrated in saline were then added to the cell suspensions at a final concentration of 100 μg/ml for each component. Three-hundred microlitre aliquots were withdrawn at varying time-intervals, centrifuged, and K^+ concentrations in the supernatants directly determined by flame photometry. One-hundred percent K^+ controls were obtained by treated bacteria with 4% SDS, boiling (for 15 seconds) and sonication.

RESULTS

Figure 1 depicts the results of experiments in which ONPG uptake into *E. coli* T 215 was determined after treatment with WHS_B or with R9 serum±C9 post-treatment. ONPG influx occurred in bacteria treated with WHS_B but not in cells that received R9 serum only. However, R9-treated cells took up the substrate molecule upon post-treatment with purified C9, and marker uptake was noted at R9 concentrations that, in conjunction with the C9 post-treatment, caused measurable loss of viability (Fig. 1). One-hundred percent killing was observed at serum concentrations between 5% and 10% (Fig. 1b). The extent of ONPG cleavage occurring within the 15 min period of incubation exhibited a dependence on serum concentrations, i.e. high serum doses induced more rapid diffusion of the substrate into the cells. We ascribe this finding to a dose-dependent increase in the number of lesions per cell. Very high doses of R9 serum (40% vol/vol) caused some loss of viability without inducing measurable ONPG influx. This putative C5b–8 effect may be causally linked to the tendency of C5b–8 complexes to aggregate (Bhakdi & Tranum-Jensen, 1984; Cheng, Wiedmer & Sims, 1985), but the phenomenon has not been studied further. Overall, the present data show a requirement for C9 in the induction of the permeability defect of the *E. coli* inner membrane towards ONPG. Since *E. coli* T 215 cells are entirely impermeable to ONPG in their viable state, uptake of this marker clearly stems from a dramatic, C9-dependent alteration in the permeability of the inner membrane towards the molecule. The above experiments were additionally performed with *E. coli* W 3110 cells with essentially the same results.

We then investigated the effects of colicin E1 and gentamicin on ONPG permeation. Neither agent was found to induce uptake of the marker into the non-viable cells (Table 1). Failure

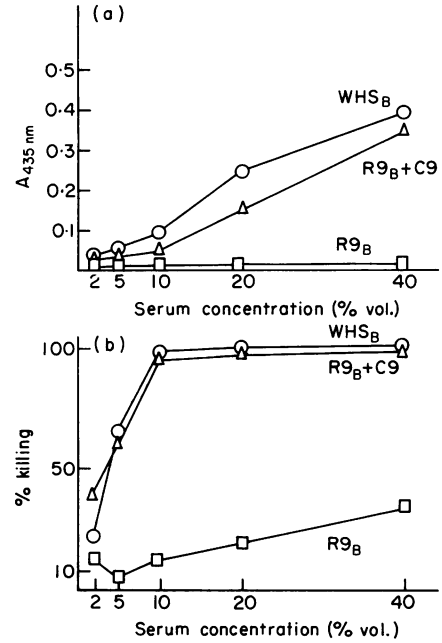

Figure 1. (a) Hydrolysis of ONPG by *E. coli* T 215 cells following treatment with bentonite-absorbed R9 serum ($R9_B$) alone, R9 serum followed by purified C9, or bentonite-absorbed whole human serum (WHS_B). The final concentration of serum used is given as the percentage volume. Whereas R9 serum alone induced no permeability defect in the inner membrane towards ONPG, hydrolysis of the marker was observed after post-treatment of the R9 cells with purified C9 (70 μg/ml), and the rate of ONPG hydrolysis was similar to that observed in cells treated with WHS_B. (b) Substantial killing of *E. coli* occurred at serum concentrations of 2% volume, and 100% killing was observed at serum concentrations between 5% and 10% volume. Only slight loss of viability was noted with high R9 concentrations.

Table 1. Hydrolysis of ONPG offered to *E. coli* K 12 W 3110 cells after treatment with colicin E1 or gentamicin, and after post-treatment with bentonite-absorbed WHS

Agent	Concentration	Incubation time	% of maximal ONPG hydrolysis	Killing (%)	% of maximal ONPG hydrolysis after post-treatment with 30% WHS_B
Buffer control	—	40 min	4·5	0	94
Colicin E1	20 μg/ml	40 min	3·8	93	89
Colicin E1	20 μg/ml	210 min	3·1	100	95
Gentamicin	50 μg/ml	60 min	0·8	95	91
Gentamicin	50 μg/ml	180 min	3·5	98	74

of ONPG to penetrate into *E. coli* after treatment with colicin E1 has been reported (Fields & Luria, 1969), and our results are thus confirmatory in this regard. However, when cells that had been incubated with either colicin E1 or gentamicin were post-treated with C5b–9 (recruited from WHS_B ONPG permeation was again observed (Table 1). These findings indicated that deposition of C5b–9 on *E. coli* with formation of transmural pores at very high serum doses not require a viable target organism.

Efficiency of C5b–9 bactericidal action

C5b–7 complexes attach firmly to cell membranes without exerting cytolytic or bactericidal effects, and retain their capacity to post-bind C8 and C9. This was exploited in experiments that permitted the minimal average number of C5b–9 complexes required to suppress formation to be reliably determined. The first set of experiments was performed in order to gain an estimate of the number of terminal complexes generated per CFU. Cells coated with C5b–7 were suspended to approximately 10^8 CFU/ml in culture broth, and the bacteria were allowed to multiply at 32° for 150 min. At various time-intervals (Fig. 2), 0·4 ml were removed and offered a mixture of 120 ng C8 ($\sim 5 \times 10^{11}$ molecules) +20,000 c.p.m. functionally active ^{125}I-C8+30 μg C9. After 20 min at 37°, the cells were pelleted, washed, and the specifically bound radioactivity determined. Ten μl aliquots were also appropriately diluted and plated out for colony countings.

Figure 2 depicts the results of an experiment. In all cases, post-treatment with C8 and C9 led to the quantitative killing of the bacteria and the daughter cells over the given time-period. The total number of bound C8 molecules was calculated from the amount of bound radioactivity, and it was typically found that a slight increase in C8 binding occurred during the first 30–60 min of culture. Thereafter, C8 binding decreased slightly to approach the original level at 120–150 min. Hence, there was no significant change in overall binding of C8, and this indicated that significant numbers of C5b–7 complexes were not removed from the bacterial cell surface during the process of cell division. By dividing the total number of bound C8 molecules with the number of CFU, we obtained estimates of the average number of bound C8 molecules per CFU (Fig. 2b). Under the given experimental conditions, the *E. coli* cells were found to carry between 2000 and 4000 C5b–7 complexes per CFU (on average) at the beginning of the culture. The number of C8 molecules/CFU sank to reach levels of around 300–400 at the end of the depicted experiment. This type of experiment led us to conclude that an initial number of 2000–4000 C8 binding sites (C5b–7 complexes)/CFU was a reasonable number upon which to base our further calculations.

The next set of experiments was conducted over a longer time-period, with the aim of determining the time-point at which approximately 50% killing of the daughter *E. coli* cells would occur. In these experiments, cells carrying C5b–7 were inoculated into a large volume of culture broth to yield starting densities of 10^3–10^4 CFU/ml, so that the bacteria remained in the log-phase of growth throughout the experiment. Aliquots were removed at various time-points, and the cells were treated

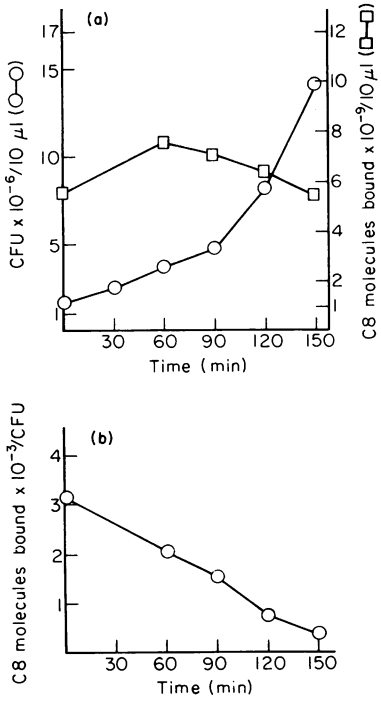

Figure. 2. *E. coli* W 3110 cells were treated with R8 serum to generate C5b–7 complexes, washed, and resuspended to approximately 10^8 CFU/ml in culture broth. Four-hundred μl aliquots were removed at the depicted time-intervals and offered 120 ng C8 + 20,000 c.p.m. active ^{125}I-C8 + 30 μg C9. After 20 min at 37°, the cells were washed and the number of bound C8 molecules calculated from the bound radioactivity (a). No gross variation in the total amount of bound C8 was observed. A plot of the mean number of C8 molecules bound per CFU was obtained by dividing the total bound C8 molecules by CFU (b).

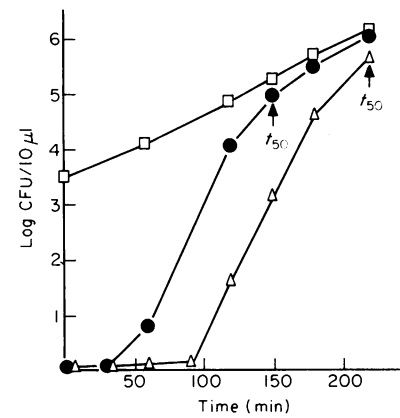

Figure. 3. Killing efficiency of C5b–9: C5b–7 dilution experiment. *E. coli* cells coated with 2000–4000 C5b–7 molecules/CFU were suspended in culture broth (32°) at a starting density of approximately 5×10^3 CFU/10 μl. Aliquots were removed at the depicted times and colony counts performed (□). Treatment of the cells with purified C8 + C9 caused loss of viability over an extended time-period (●), and 50% killing was still registered after approximately 150 min of culture (t_{50}). This corresponded to an approximately 40-fold dilution of C5b–7 complexes/CFU. If 2 mM EDTA was present during the post-incubation with C8 and C9, killing efficiency was apparently enhanced (△), and 50% killing occurred after a culture period of approximately 210 min, corresponding to a 250-fold dilution of C5b–7 complexes.

with a surplus of C8 + C9 (final concentrations of the individual components: 50–70 μg/ml) for 10 min at 37°. Viability counts obtained in these experiments ($n = 10$) indicated that the bactericidal potential of C5b–7 was retained down through many daughter cell generations. Typically, 50% bacterial killing was still registered in the fifth to sixth generation, corresponding to a mean dilution factor of 32–64 for the original C5b–7 complexes. Thus, a mean of 50–100 C5b–9 complexes generated on the surface of an *E. coli* cell sufficed to inhibit colony formation (Fig. 3).

The presence of 2 mM EDTA during the C8–C9 post-treatment apparently increased the efficiency of C5b–9 killing. In the presence of this chelator, which alone caused no killing of C5b–7 control cells at 37°, 50% bacterial survival was registered in the seventh to eighth generation, corresponding to a C5b–7 dilution factor of 125–250 and a mean of only 8–30 C5b–9 complexes/CFU (Fig. 3). The presence of 2 mM EDTA did not enhance the C8 uptake on the cells (data not shown).

C5b–9 dependent K^+ efflux from *E. coli*

E. coli cells carrying C5b–7 were suspended to densities of approximately 10^{10} cells/ml in saline/glucose. After the addition of C8 and C9, the release of K^+ could be directly measured in the cell supernatants. Control cells that carried no C5b–7, or cells that carried C5b–7 but receiving no C8 + C9, spontaneously released significant amounts of K^+ after 15–20 min, probably due to the extremely high cell density (Fig. 4). Concomitant with the spontaneous K release, gradual loss of viability and decrease in CFU was observed (not shown). However, the addition of C8 + C9 to C5b–7 cells caused a release of K^+ that commenced virtually instantaneously and was far above the spontaneous release (Fig. 4). After 15–20 min at 30°, over 50% release was always observed with C8/C9-treated cells, as opposed to less than 20% spontaneous release in the controls ($n = 4$ experiments). Thereafter, K release proceeded over a time-period of over 60 min, but no further divergence between the C5b–9 and control release curves could be noted.

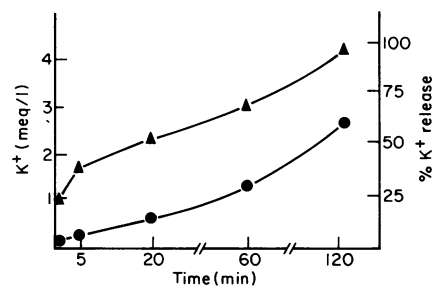

Figure 4. C5b–7 laden *E. coli* cells were packed to a density of approximately 10^{10} CFU/ml in saline and post-treated with purified C8 + C9 (100 μg/ml of each component, final concentration). Aliquots were removed at the given time and K^+ was determined in the cell supernatants by flame photometry (▲). Controls (●) received no C8/C9. The addition of C8 and C9 induced an immediate release of K^+ from a portion of the cells. Rather high spontaneous release of K^+ occurred in the controls after 20 min incubation (30°). K^+ release is expressed as absolute values (meq/l) to the left, and as a percentage of total K^+ to the right.

DISCUSSION

Several lines of evidence have in the past appeared compatible with the concept that complement kills serum-sensitive *E. coli* by generating transmural pores. Feingold *et al.* (1968a, b) demonstrated that plasmolysis of bacteria in hypertonic media protected the cells from complement killing. They also demonstrated that ONPG diffused into complement-killed cells, a finding that was subsequently confirmed and extended by Martinez & Carrol (1980) who showed that ONPG permeation did not require the presence of lysozyme. Wright & Levine (1981a, b) further refined the ONPG influx data by showing that bacteria remained viable and ONPG-impermeable at the C5b–7 stage, and that ONPG uptake occurred within minutes after C8/C9 binding to C5b–7 cells, paralleling loss of viability. They also presented data showing C5b–9-dependent release of Rb^+ from the cells. The interpretation of Rb^+ release data may, however, be difficult, since a high amount of unspecific release has been reported to occur spontaneously (Taylor, 1983).

In order to gain further insight into the mechanism of bacterial killing by C5b–9, we sought to answer three questions.

First, is ONPG uptake truly C9-specific? In an extension of Wright & Levine's experiments, we treated bacteria with C9-depleted serum and found that coating the cells with even very high amounts of C5b–8 did not induce a permeability increase of the inner membrane towards ONPG. Upon post-treatment of the washed cells with purified C9, however, ONPG uptake was immediately observed. Hence, disruption of the permeability barrier of the inner membrane for this marker was clearly C9-dependent.

To test further the specificity of ONPG uptake with respect to C9, cells were first killed to colicin E1 or gentamicin. We found that treatment with neither agent led to uptake of ONPG into the cells, and thus confirmed earlier data of Fields & Luria (1969) with regard to colicin E1. However, when cells killed with colicin E1 or gentamicin were post-treated with lysozyme-free serum, but not R9 serum, ONPG influx occurred. These findings corroborated the notion that diffusion of ONPG to the cytoplasm was indeed C9-dependent. They also indicated that the formation of the transmural defects did not basically require viable target organisms.

The second question pertained to the minimal number of C5b–9 lesions required to inhibit bacterial colony formation. Inoue *et al.* (1977) earlier estimated that a very large number of C5b–9 lesions (approximated at 1500 lesions/cell) was required to kill a cell. If confirmed, this might indicate either that the majority of complexes inserted in an abortive manner into the outer membrane, without 'hitting' the bioadhesion zones, or that killing required a cumulative effect of multiple C5b–9 complexes. The latter concept would tend to argue against the formation of discrete transmural pores. We generated C5b–7 complexes on cells, permitted the bacteria to multiply, and examined the effects of purified C8 and C9 given to the daughter generations. We found that multiplying bacteria could obviously not remove substantial amounts of C5b–7 from their surface, so that binding of C8 continued to occur hours after the initial deposition of C5b–7. Thus, the C5b–7 complexes remained quantitatively located in the outer membrane, i.e. shedding or translocation to the inner membrane was, at best, minimal. Killing by C8 and C9 continued to be remarkably efficient throughout many daughter generations, given that a

sufficient number of C5b–7 complexes were initially formed. The use of purified C8 and C9 components excluded a role for lysozyme in the killing process. Estimates of C8 binding using radiolabelled C8 tracer indicated that an average of 2000–4000 C5b–7 complexes were formed per CFU when the *E. coli* cells were treated with the very high R8 serum doses used in our experiments. These results are well compatible with the number of 900 complexes generated per CFU as reported by Joiner *et al.* (1985) with the use of 10% serum. Taking 2000–4000 C5b–7/CFU as a starting value for our calculations, we then determined the minimal average of C5b–9 lesions that was required to prevent colony formation. This number was surprisingly low, reproducibly in the range of 50–100 complexes/CFU. It was further diminished if 2 mM EDTA was present during post-incubation with C8 + C9. In this case, as few as 8–30 complexes/CFU sufficed to cause 50% killing. Hence, killing of *E. coli* K 12 cells by C5b–9 was obviously very efficient. The enhancing effect of EDTA remains unexplained at present.

Finally, experiments were performed to test directly whether C5b–9 caused K^+ efflux from bacteria. These studies appeared indicated in view of the possible interpretational difficulties of Rb^+ release data. By packing cells carrying C5b–7 to a sufficient high density in saline, we could directly measure K^+ efflux into the supernatant after treatment with purified C8 + C9. We found that, indeed, K^+ efflux commenced immediately after the addition of the C8/C9 components. We were unable to obtain satisfactory release data over prolonged time-periods due to the high bacground release that occurred after 15–20 min incubation. Nevertheless, the results clearly indicated that C8/C9 induced the immediate liberation of K^+ from a substantial portion of the bacterial cells. The fact that K^+ release was only partial within the first few minutes may have been due to the extremely high cell density in the given experiments.

If C5b–9 complexes do indeed generate transmural pores in serum-sensitive *E. coli*, the resulting K^+ efflux should be lethal because of collapse of membrane potential. How can the surprising efficiency of C5b–9 killing be accounted for? We speculate that bioadhesion zones continuously form in a transient, fluctuating manner, causing C5b–9 pores, which are primarily deposited in the outer membrane, to be periodically 'hit' by the inner membrane. The chance of a C5b–9 complex being 'hit' by a bioadhesion zone could depend on the density of complement complexes on the cell surface: hence, a cell carrying only 50–100 complexes may 'live' longer than one carrying 2000 complexes. Through cell division, daughter generations of cells carrying few C5b–9 lesions may even escape death. If this hypothesis is correct, inhibition of colony formation in our experiments might depend on two counteracting factors, i.e. the kinetics of formation of lethal zones of bioadhesion versus cell multiplication with the escape of daughter cells. The true number of C5b–9 complexes required to kill a serum-sensitive *E. coli* cell might thus actually be even lower that the numbers obtained in the present estimate.

In closing, it should be stressed that none of these studies has been directed at the question regarding the mechanisms of serum-resistance, which are probably manifold (Joiner & Frank, 1985), or at the problem of C9 multiplicity within C5b–9 complexes. It appears that a minimal average of $\geqslant 3{\cdot}3$ molecules C9 per C5b–8 complex is required to kill serum-sensitive Gram-negative bacteria (Joiner *et al.*, 1985), and all the experiments described in this study were performed in the presence of saturating amounts of C9.

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