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Killing of Gram-negative bacteria by complement

Fractionation of cell membranes after complement C5b-9 deposition on to the surface of *Salmonella minnesota* Re595

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The effect of C5b-9 deposition on the envelope of target Gram-negative bacteria was studied. In order to understand the changes occurring after complement deposition on the bacterial surface, the preparation of Gram-negative bacterial membranes by different methods involving the osmotic lysis of spheroplasts was investigated. Subsequent fractionation of the outer membrane (OM) and cytoplasmic membrane (CM) by sucrose-density-gradient centrifugation showed differences in the membrane profiles obtained. The results indicate that optimum separation of OM and CM components requires effective digestion of DNA in the total membrane preparation before density-gradient fractionation. *Salmonella minnesota* Re595 carrying the intermediate complement complex C5b-7 (BC1-7) or C5b-8 (BC1-8) were efficiently killed upon incubation with purified C8+C9 or C9 respectively. Human-α-thrombin-cleaved C9 ($C9^n$), which is unable to form tubular poly(C9), was shown to be more effective at killing than native C9. By using an optimized system for the separation of OM and CM, it was found that, subsequent to lethal complement attack, the CM could not be recovered when C9 was used as the terminal complement component, but was recovered with reduced yield when $C9^n$ replaced C9. The results show that inability to recover the CM on sucrose density gradients after complement attack may not be a consequence of an essential membrane damage event required for complement-mediated killing of Gram-negative bacteria.

INTRODUCTION

The complement system plays an important role in host defence against Gram-negative bacterial infection [for a review, see Taylor (1988)]. Activation of the complement system by susceptible Gram-negative bacteria leads to the generation of the terminal complement complex, C5b-9, on the bacterial surface, with subsequent loss of cell viability (Swanson & Goldschneider, 1969; Kroll *et al.*, 1984). When bacterial membranes are separated by sucrose-density-gradient centrifugation after complement-mediated killing in the absence of lysozyme, the C5b-9 complex is found exclusively in the outer membrane (OM) (Taylor & Kroll, 1984), even though killing is dependent on irreversible damage to the cytoplasmic membrane (CM) (Feingold *et al.*, 1968*a,b*; Wright & Levine, 1981; Taylor & Kroll, 1985). Stable formation of C5b-9 complexes on the OM appears to be a prerequisitie for killing (Inoue *et al.*, 1968; Joiner *et al.*, 1982*a,b*; Kroll *et al.*, 1984).

How does the C5b-9 complex exert its lethal effect at the CM from its location on the OM? Because of the dimensions of the lipid-binding domain on C5b-9 (Bhakdi & Tranum-Jensen, 1978), it is clear that the complexes are unable to form stable channels traversing both membranes. Formation of classical tubular complement lesions, probably containing at least eight C9 molecules (Podack *et al.*, 1982; Bhakdi *et al.*, 1987), is not required for killing, since it has been reported that an average of as few as three C9 molecules per C5b-8 complex are able to kill susceptible cells (Joiner *et. al.*, 1985; Bloch *et al.*, 1987). Further, human-α-thrombin-cleaved C9 ($C9^n$), which will bind to C5b-8 and polymerize to form string-like aggregates, but not closed rings (Dankert & Esser, 1985), is as effective as native C9 in killing susceptible *Escherichia coli* strains (Dankert & Esser, 1987).

It has been postulated that zones of bioadhesion between the OM and CM, described and characterized by Bayer (1968), are the targets for lethal attack by complement (Wright & Levine, 1981; Bhakdi *et al.*, 1987). Ishidate *et al.* (1986) have reported the apparent isolation of such attachment sites by sucrose-density-gradient centrifugation. However, it is known that different methods of cell disruption and lysate preparation result in different membrane profiles after sucrose-density-gradient centrifugation (Osborn *et al.*, 1972; Taylor & Kroll, 1984; Ishidate *et al.*, 1986).

In the present study, different methods for the prep-

Abbreviations used: complement proteins are named in accordance with the recommendations in Bull. W.H.O. (1968) **39**, 935–936; NHS-C8 and NHS-C9, normal human serum (NHS) depleted of C8 and C9 respectively. BC1-7 and BC1-8, bacteria incubated in NHS-C8 and NHS-C9 respectively; $C9^n$, human-α-thrombin-cleaved C9; CFU, colony-forming unit; CM, cytoplasmic membrane; OM, outer membrane; KDO, 3-deoxy-D-manno-octulosonic acid: GVB^{2+}, gelatin/veronal-buffered saline (Gewurz & Suyehira, 1980); PBS, phosphate-buffered saline (full composition given in text); LPS, lipopolysaccharide; BAS, Bentonite-absorbed serum.

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aration of Gram-negative membranes were investigated to study the effect of C5b-9 deposition on the bacterial surface, and an optimized method for the separation of CM and OM on sucrose density gradients was developed. The present paper also reports on the effects of C9 and $C9^n$ attack on *Salmonella minnesota* Re595 carrying C5b-8 complexes and the fate of the CM after complement attack.

MATERIALS AND METHODS

Chemicals and reagents

Egg-white lysozyme, DNAase 1 and human α-thrombin were purchased from Sigma, Poole, Dorset, U.K. All other chemicals were of the highest purity available from commercial sources.

Gelatin veronal-buffered saline containing calcium and magnesium ions (GVB^{2+}) was prepared according to the method of Gewurz & Suyehira (1980). PBS contained 0.15 M-NaCl, 2 mM-NaH_2PO_4 and 16 mM-Na_2HPO_4, pH 7.4.

Bacterial strains and media

E. coli 17 is a mutant strain derived from *E. coli* K12 which lacks cell-envelope-associated phospholipase A activity (Doi & Nojima, 1974) and was the gift of Dr. Shochichi Nojima, Teikyo University, Kanagawa, Japan. *E. coli* 225 was isolated from a patient with bacteraemia and was a gift from the Clinical Microbiology and Public Health Laboratory, Addenbrooke's Hospital, Cambridge, U.K. *S. minnesota* Re595, a heptose-less mutant containing only 3-deoxy-D-manno-octulosonic acid (KDO) in its lipopolysaccharide (LPS) core region, was obtained from Dr. Jacik Hawiger, Vanderbilt University, Nashville, TN, U.S.A. *E. coli* 17 and *S. minnesota* Re595 are serum-sensitive, and *E. coli* 225 is serum-resistant.

Sera

Lysozyme was removed from normal human serum (NHS) by Bentonite absorption, as described by Wardlaw (1962). Bentonite-absorbed serum (BAS) was depleted of C8 and C9 by the methods of Abraha *et al.* (1988) and Morgan *et al.* (1983) respectively. Sera were stored at −70 °C until required.

Complement proteins

C8 and C9 were purified from pooled NHS by immunoaffinity chromatography as described by Abraha *et al.* (1988) and Morgan *et al.* (1983). Thrombin-cleaved C9 ($C9^n$) was produced by incubating human α-thrombin with C9 at a ratio of 1:20 (w/w) in 10 mM-Tris/HCl (pH 7.4)/200 mM-NaCl for 5 h at 37 °C. All proteins were dialysed against GVB^{2+} and stored at −70 °C. Protein was determined according to the Lowry method with bovine serum albumin as standard, and by absorption at 280 nm.

Bacterial-killing assays

Formation of bacteria carrying intermediate complement complexes C5b-7 (BC1-7) and C5b-8 (BC1-8) was achieved essentially as described by Dankert & Esser (1987). Bacteria were grown to a concentration of approx. 4×10^8 colony-forming units (CFU)/ml, washed once in GVB^{2+} at room temperature, and resuspended to their original volume in GVB^{2+}. BC1-7 were formed by adding 0.2 vol. of NHS-C8 to the bacterial suspension, incubating at 37 °C for 40 min, and then washing the cells three times in GVB^{2+}. BC1-8 were formed similarly, but using NHS-C9. In comparing the effectiveness of C9 and $C9^n$ in bacterial killing, 2×10^7 CFU of BC1-7 were incubated with an excess of C8 (3 μg) and different concentrations of C9 or $C9^n$ in a total volume of 200 μl. Incubations were at 37 °C in a shaking water bath and, at intervals, 10 μl samples were removed and cell viability determined according to the method of Taylor & Kroll (1983).

Isolation of bacterial membranes

Bacterial membranes were isolated by a method based on that of Kroll *et al.* (1983). Bacteria were grown to approx 4×10^8 CFU/ml in Mueller–Hinton broth containing 0.33 μCi of [2-^{3}H]glycerol/ml at 37 °C on an orbital incubator; 25 ml aliquots were centrifuged at room temperature and the cells washed once with PBS. The cells were resuspended in 1 ml of 0.05 M-Hepes, pH 7.2, containing 0.75 M-sucrose and 200 μg of lysozyme/ml. Some suspensions were slowly diluted with 1 vol. of 3 mM-EDTA, pH 7.2, in which case the cells were previously resuspended in the sucrose solution containing 100 μg of lysozyme/ml. After incubation at room temperature for 10 min, the plasmolysed bacteria were pipetted slowly into 25 ml of gently stirred distilled water at room temperature, usually containing 250 μg of DNAase 1. After lysis, which was generally complete within a few seconds, membranes were collected by ultracentrifugation in a Beckman L5-65 ultracentrifuge (type 30 rotor, 80000 *g*, 60 min, 4 °C). Membranes were suspended in 0.25 ml of 0.05 M-Hepes, pH 7.2, and layered on to a 4.5 ml sucrose [20–60 % (w/w) in 0.05 M-Hepes, pH 7.2] density gradient. The gradients were centrifuged to equilibrium in a Beckman L5-65 (SW 50 swing-out rotor, 200000 *g*, 24 h, 4 °C) and fractionated by pumping from the bottom of the tube. Fractions (0.15 ml each) were collected, and the absorbance at 278 nm continuously monitored with an LKB SII Uvicord instrument. The density of each fraction was determined by refractometry using a sucrose standard curve as reference.

Analysis of membrane fractions

NADH oxidase activity was determined by method of Osborn *et al.* (1972). Radioactivity was measured by mixing 15 μl of the individual gradient fractions with 4 ml of LKB Optiphase scintillant and counting with a Packard 1500 Tri-Carb liquid-scintillation counter. The DNA content of fractions was determined by the fluorometric assay described by Hopcroft *et al.* (1985).

RESULTS

Bacterial killing by complement

Formation of BC1-7 and BC1-8 by incubation of *S. minnesota* Re595 in 20 % NHS-C8 or NHS-C9, followed by washing, did not result in any loss in viability. When BC1-8 were incubated with C9, the cells were rapidly killed, but C9 was found to be more efficient at killing when added with excess C8 to BC1-7 cells (Fig. 1). Addition of C8 and C9 alone to *S. minnesota* Re595 had no effect on cell viability. Similar data (not shown) were obtained for the serum-sensitive strain *E. coli* 17. The serum-resistant strain *E. coli* 225 was also incubated in

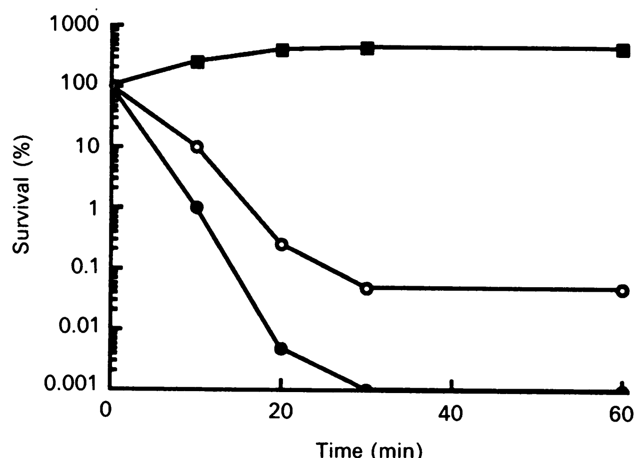

Fig. 1. Effect of C8 and C9 on the viability of *S. minnesota* Re595 carrying intermediate complement complexes

Excess C8 (15 μg/ml) and C9 (10 μg/ml) was incubated at 37 °C with *S. minnesota* Re595 (■) and BC1-7 (●); BC1-8 were incubated with only C9 (○). The concentration of cells was 1×10^8 CFU/ml. Samples were withdrawn at the indicated times and the cell viability was determined.

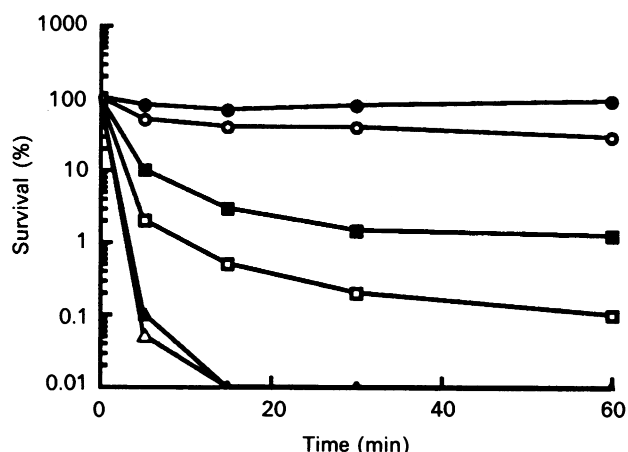

Fig. 2. Comparison of the effect of C9 and C9ⁿ on the viability of *S. minnesota* Re595 carrying intermediate complement complexes

BC1-7 (1×10^8 CFU/ml) were incubated at 37 °C with excess C8 (15 μg/ml) and either C9 at 0.066 μg/ml (●), 0.33 μg/ml (■) and 1.65 μg/ml (▲) or $C9^n$ at 0.066 μg/ml (○), 0.33 μg/ml (□) and 1.65 μg/ml (△). Samples were withdrawn at the indicated time intervals and cell viability was determined.

NHS-C8 and NHS-C9. After washing and addition of C8 and C9, no reduction in cell viability was detected (results not shown).

Fig. 2 shows that $C9^n$ is more effective than C9 in killing BC1-7 when added with excess C8. To investigate whether differences in killing kinetics between C9 and $C9^n$ were reflected by differences in membrane alterations associated with complement killing, bacterial membranes were isolated after C9- and $C9^n$-mediated killing.

Isolation of bacterial membranes

When attempting to isolate the CM and OM of Gram-negative bacteria both before and after complement attack, we found that different methods of membrane preparation produced variations in the profile of the membranes when subsequently separated by equilibrium sucrose-density-gradient centrifugation.

A total membrane fraction was obtained by ultracentrifugation of osmotically lysed spheroplasts, and conditions during preparation and lysis of spheroplasts were found to be critical. Only under the precise conditions described in the Materials and methods section does the OM appear to be permeable to lysozyme without EDTA treatment. Fig. 3 shows analyses of sucrose gradient fractions after centrifugation of total membrane preparations of *S. minnesota* Re595 through the gradient. The presence of DNAase and the absence of EDTA during membrane preparation resulted in the subsequent isolation of only two peaks after sucrose-density-gradient centrifugation (Fig. 3*d*). In accordance with previously published data on OM and CM densities (Osborn *et al.*, 1972; Taylor & Kroll, 1984) and the higher activity of NADH oxidase in the lighter peak, the dense and light peaks were identified as the OM and CM respectively. Very little DNA was detected throughout this gradient. When DNAase was omitted (Figs. 3*a* and 3*b*) or EDTA included (Fig. 3*c*) during membrane preparation, two major peaks identified as the OM and CM were recovered, but additional peaks of intermediate density were also recovered. These additional peaks were enriched in NADH oxidase activity compared with the major OM peak, indicating the presence of CM material, and were associated with high DNA concentrations [Fig. 3*a*(ii), 3*b*(ii) and 3*c*(ii)].

Because of the more complex membrane profiles obtained when using EDTA or omitting DNAase, preparation of membranes after complement attack was performed in the absence of EDTA and with DNAase present during osmotic lysis.

Effect of C9 and $C9^n$ on membrane integrity

Fig. 4 shows separation of bacterial membranes after treatment of BC1-7 derived from *S. minnesota* Re595 with excess of C8 and lethal concentrations of C9 and $C9^n$. Treatment of native *S. minnesota* Re595 with C8 and C9 or $C9^n$ had no effect on viability and subsequent membrane isolation produced a profile essentially identical with that in Fig. 3(*d*).

It has previously been shown that exposure of serum sensitive *E. coli* to lysozyme-free serum results in the loss of recovery of the CM on sucrose gradients which is concomitant with viability loss (Taylor & Kroll, 1985). A similar loss in membrane recovery was shown when BC1-7 cells derived from *S. minnesota* Re595 were treated with C8 and C9 (Fig. 4*a*). Exposure of BC1-7 to C8 and $C9^n$, which is more efficient at killing than C9, produced a considerable reduction in the recovery of the CM, but not its complete loss. The recovery of CM material was evident from CM-specific NADH oxidase activity as well as 280 nm-absorbing material and radiolabelled phospholipid (Fig. 4*b*).

Analysis of the OM peak revealed that the density of the OM increased more after C9-mediated killing than after $C9^n$-mediated killing. In both cases the density of the OM was higher than with untreated cells (Fig. 3*d*).

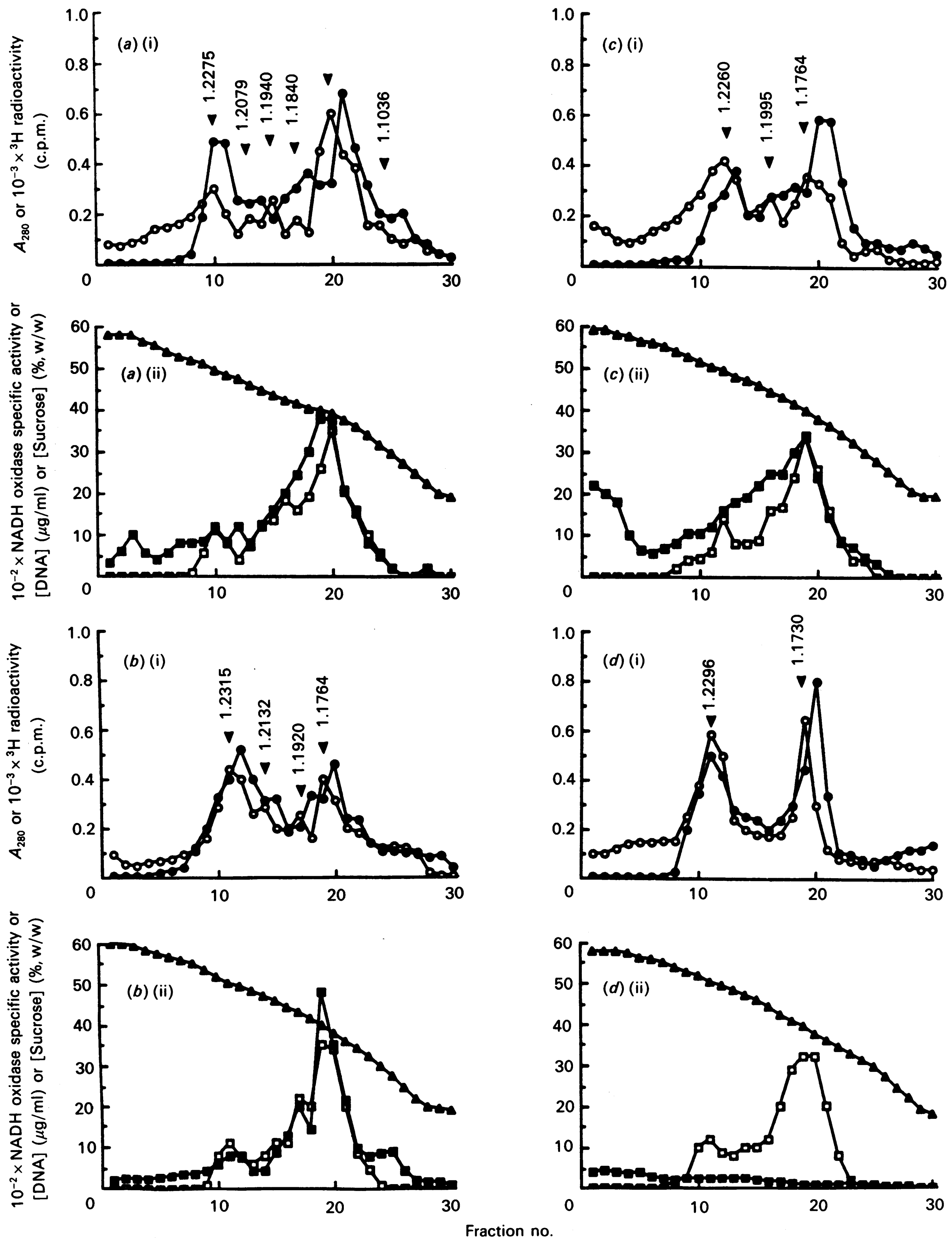

Fig. 3. Separation of membranes from [2-^{3}H]glycerol-labelled *S. minnesota* Re595 on linear 20–60%-(w/w)-sucrose density gradients

S. minnesota Re595 were plasmolysed in 0.75 M-sucrose containing 200 μg of lysozyme/ml (*a* and *d*), or 100 μg of lysozyme/ml to which 1 vol. of 3 mM-EDTA was added (*b* and *c*). Spheroplasts were then lysed by dilution into water (*a* and *b*), or water containing 10 μg of DNAase/ml (*c* and *d*). Total membrane fractions were collected by centrifugation and applied to sucrose density gradients. After centrifugation (Beckman SW 50 rotor, 200000 *g* 24 h, 4 °C) fractions were analysed for absorbance at 280 nm (○), ^{3}H radioactivity (●), DNA (■), NADH oxidase specific activity (□), and % sucrose (▲). Values above peaks represent densities in g/cm^3. NADH oxidase activity is expressed as change in absorbance/min per mg of protein. In each case (*a*–*d*) data from the same gradient are presented in two panels (i and ii) for clarity.

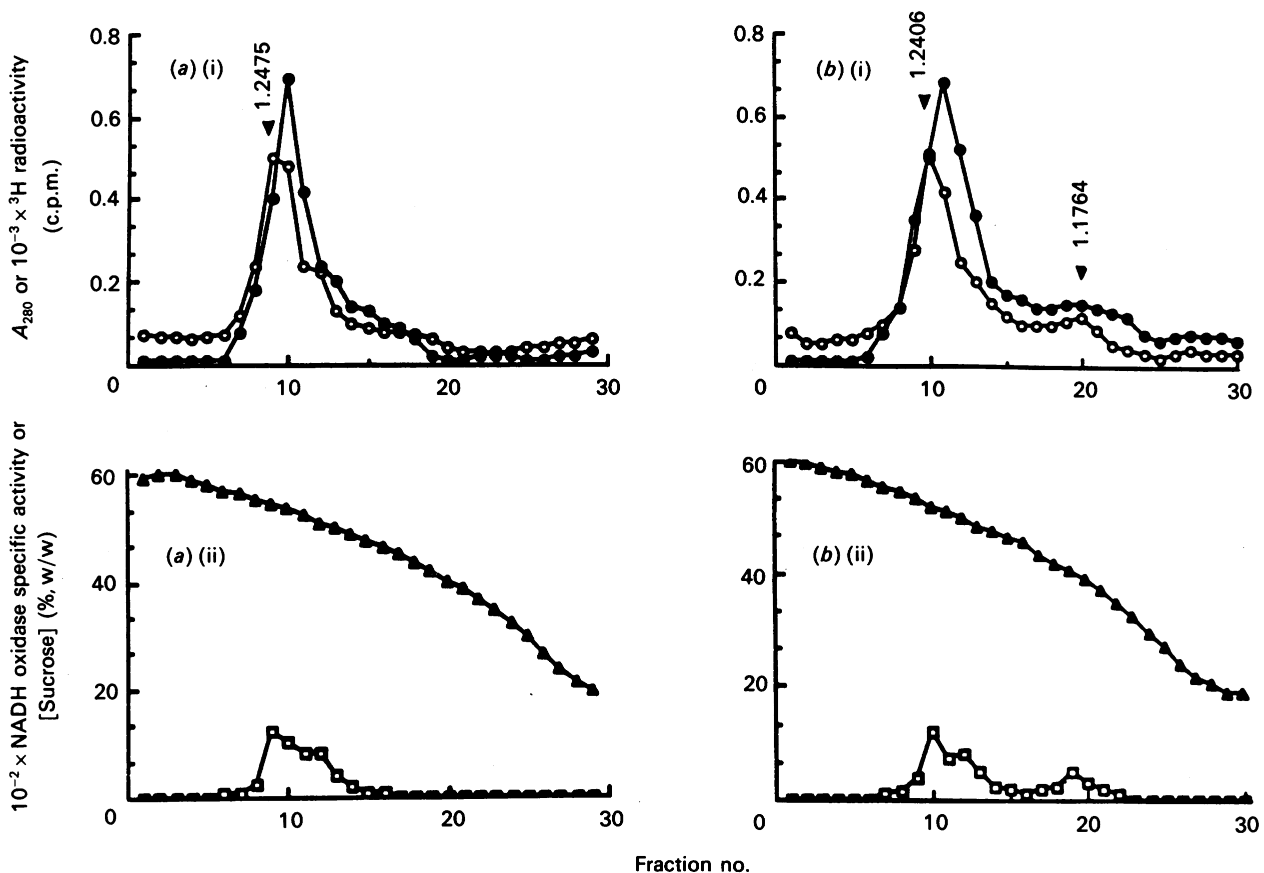

Fig. 4. Separation of membranes from [2-^{3}H]glycerol-labelled BC1-7 after C9- and C9^n-mediated killing

BC1-7 derived from *S. minnesota* Re595 (1×10^8 CFU/ml) were incubated at 37 °C for 15 min with excess C8 (15 μg/ml) and either C9 (2 μg/ml) (*a*) or C9^n (2 μg/ml) (*b*). Cells were washed in PBS and the membranes prepared, as described in the text, before fractionation on sucrose density gradients. Fractions were analysed for absorbance at 280 nm (○), ^{3}H radioactivity (●), NADH oxidase specific activity (□) and sucrose concentration (▲). Values above each peak represent densities in gm/cm^3. NADH oxidase activity is expressed as change in absorbance/min per mg of protein. In each case (*a*–*d*) data from the same gradient is presented in two panels (i and ii) for clarity.

DISCUSSION

Successful resolution of the membranes of *S. minnesota* Re595 into just two bands representing the OM and CM by sucrose-density-gradient centrifugation was found to be dependent on the presence of high concentrations of DNAase during osmotic lysis of plasmolysed cells. Omission of DNAase during lysis of spheroplasts or the use of EDTA during spheroplast formation was found to result in the isolation of additional bands to those identified as the OM and CM.

Earlier studies involving the separation of *S. typhimurium* membranes have reported the resolution of total membrane into four or more bands after sucrose-density-gradient centrifugation (Ishadate *et al.*, 1986; Jones & Osborn, 1977; Osborn *et al.*, 1972). It may be significant that these studies used only low DNAase concentrations or no DNAase during cell disruption. In the present study, analysis of additional bands with densities intermediate to the major OM and CM fractions revealed some characteristics of both membranes and were found to contain high concentrations of DNA. We therefore suggest that these additional bands result from DNA adhering to, and co-sedimenting, with membrane material. EDTA would chelate any available Mg^{2+} upon cell lysis, consequently inhibiting Mg^{2+}-dependent DNAase activity and thus accounting for additional bands when DNAase is used in the presence of EDTA. That DNA is found in the CM fractions is not surprising, since DNA is known to be attached to the CM at various points, and this feature is essential for the correct partitioning of replicating DNA during cell division (Dulbecco, 1980). Jones & Osborn (1977) reported difficulty in isolating the OM and CM from a heptose-less mutant of *S. typhimurium* after osmotic lysis of spheroplasts. It appears this is not a general feature of all such mutants, however (Lugtenberg & van Alphen, 1983), since *S. minnesota* Re595 is also a heptose-less mutant.

Zones of bioadhesion between the OM and CM have been proposed by Bayer (1968) and, although nothing is known about the nature of such putative attachment sites, they have been implicated in the synthesis and translocation of some OM macromolecules (Bayer, 1979; De Leij *et al.*, 1979, 1978; Smit & Nikaido, 1978). However, evidence for an alternative model for the biogenesis and translocation of OM proteins has also been described by Lugtenberg & Van Alphen (1983)

involving membrane blebbing and vesicular transport between the two membranes. Ishidate *et al.* (1986) have reported the isolation of zones of bioadhesion between the OM and CM by sucrose-density-gradient centrifugation. They identified and further purified a fraction of intermediate density possessing characteristics of both the OM and CM and having a labelling pattern in pulse–chase experiments consistent with it being an intermediate site in the movement of newly synthesized LPS from the CM to the OM. It should be noted, however, that no DNAase was used during membrane preparation and that no DNA determinations were undertaken on membrane fractions. The existence and transience of these zones of adhesion is an important question with respect to complement-mediated bacterial killing, since it has been suggested that the terminal complement complex exerts its lethal effect at the CM when it is attached to the OM at these sites (Wright & Levine, 1981; Bhakdi *et al.*, 1987).

In the present experiments, isolation of cell membranes from bacteria attacked by complement was routinely performed on BC1-7 cells incubated with C8 and C9. Addition of excess C8 and C9 (higher concentrations than normally present in 20% NHS) to BC1-7 resulted in more efficient killing of cells than addition of excess C9 to BC1-8. It is possible that the serum concentration of C8 limited the formation of complete C5b-8 complexes under the conditions of the present experiments and that the more efficient killing observed in the former case may simply reflect a greater proportion of C5b-8 complexes being formed from C5b-7 deposited on the cell surface. Killing of *S. minnesota* Re595 by C5b-9^n was more efficient than killing by C5b-9, which is in agreement with the data of Dankert & Esser (1987) for *E. coli* C600.

Taylor & Kroll (1985) previously demonstrated that OM fractions isolated from complement-sensitive *E. coli* by sucrose-density-gradient centrifugation contain C5b-9 when prepared from bacteria exposed for 10 min to lysozyme-free serum. Neither C3 nor C5b-9 was detected on the CM at any time during the bactericidal reaction, and after 30 min there was a complete loss of CM recoverability by sucrose-density-gradient centrifugation. In the present study we examined the loss of CM recoverability from *S. minnesota* Re595 after C5b-9 attack. The results suggest that effective bacterial killing by complement does not necessarily lead to the loss of CM recoverability, since efficient killing of *S. minnesota* Re595 by C5b-9^n is accompanied only by a decrease in the amount of CM material recovered and not its complete loss. In addition to differences in CM recoverability, C9- and $C9^n$-mediated killing resulted in differences in the densities of *S. minnesota* Re595 membranes when subsequently isolated by sucrose-density-gradient centrifugation.

The results of Dankert & Esser, (1986, 1987) suggested that the important event in loss of bacterial-cell viability after complement attack is the dissipation of the membrane potential across the CM. These authors have also suggested that killing involves the "expression of a membrane active product of C9" (Dankert & Esser, 1987). They demonstrated that C9 alone, when delivered directly to the periplasmic space, is able to effect a decrease in cell viability, but the mechanism of how C9 may achieve this is unknown. It is clear that damage to the CM is responsible for cell death in both C9- and $C9^n$-mediated killing, despite the fact that the membrane-attack complex is formed on the OM (Taylor & Kroll, 1985). The differences in the killing kinetics and differences in membrane characteristics after either C9- or $C9^n$-mediated killing raise the possibility that there may be some difference in the mechanism by which C9 and $C9^n$ exert their effect at or on the CM. However, the fact that C5b-9^n kills susceptible cells more efficiently than C5b-9 and that the CM can be recovered from sucrose gradients in the former case, demonstrates that complete loss of CM integrity may not be required for complement-mediated killing of Gram-negative bacteria.

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