# Studies on the interaction of C1q, a subcomponent of the first component of complement, with porins from *Salmonella minnesota* incorporated into artificial membranes

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Purified outer membrane proteins (OMP) of *Salmonella minnesota*, Re-form, were incorporated into liposomes. These induced in macrophages a chemiluminescence signal identical to that of the intact Re-form. This signal was abolished by preincubation of porin-containing liposomes with purified C1q. Incorporation of isolated OMP into black lipid membranes (BLM) resulted in channel-formation which could not be inhibited by isolated C1q. Additionally, incubation of OMP-containing liposomes with BLM resulted in pore-formation within the BLM. This was amplified when lipid A was present within the liposomes. Preincubation of OMP-containing liposomes with purified C1q abolished pore-formation within the BLM.

C1q; Macrophage; Outer membrane protein; Liposome; Black lipid membrane

## 1. INTRODUCTION

Several strains of Gram-negative bacteria, such as *Klebsiella, E. coli, Shigella* and *Salmonella,* are killed by complement (C) after treatment with non-immune sera [1-3]. Studies on the interaction of C1, the first component of C, with serum-sensitive Re-strains of *S. minnesota* and *S. typhimurium* revealed that activation of the C-cascade is initiated by direct, antibody-independent binding of C1 via C1q to the bacteria [4,5]. Within the membrane of the serum-sensitive bacteria, the lipid A portion of lipopolysaccharides (LPS) as well as the porins (36/39 kDa) have been shown to be involved in direct binding of C1q [6-9].

Macrophages (Mϕ) have been shown to be a major site of C1q biosynthesis [10]. These cells, however, not only secrete C1q but also possess a membrane-associated form of C1q [11,12]. Recent experiments revealed that Mϕ are able to differentiate between those bacteria which are serum-sensitive and bind directly C1q and those bacteria which are serum-resistant and do not bind C1q. The serum-sensitive bacteria are bound to Mϕ membrane-associated C1q by LPS and/or porins. Binding of the bacteria to Mϕ was abolished when the bacteria were incubated with purified C1q before addition to Mϕ [13].

In this report we investigated the effect of C1q on isolated porins incorporated into artificial membranes such as black lipid membranes and liposomes. Black lipid membranes which contained porins showed a time-dependent increase in conductivity and ion transfer. Addition of purified C1q did not effect the number of formed channels. Liposomes containing porins added to black lipid membranes without porins resulted in pore formation within the black membrane and ion transfer. However, preincubation of such liposomes with purified C1q prevented conductivity and channel generation.

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## 2. MATERIALS AND METHODS

### 2.1. *Bacterial strain*

*Salmonella minnesota* (R595, SF 1167) were kindly provided by Dr G. Schmidt, Borstel, FRG.

### 2.2. *Isolation of outer membranes and purification of porins of* S. minnesota

This was performed as described previously [14]. In brief, bacteria were harvested in the late exponential phase of growth in nutrient broth (CM1; Oxoid Ltd.). Bacteria from a 2 litre culture were suspended in 30 ml 50 mM Hepes buffer, pH 7.4, containing 1 mg of DNase and RNase. After lysing the bacteria in a French Press, intact bacteria were separated by centrifugation (20 min, 5000 × *g*). The outer membrane was obtained after centrifugation of the supernatant (2 h, 36 000 × *g*), treatment of the pellet with 2% Triton X-100 in 20 mM Hepes, pH 7.4 (1 h, 4°C) and a second centrifugation (1 h, 36 000 × *g*). The membrane pellet was treated with lysozyme (1 mg/50 mg protein) and with papain (100 μg/50 mg protein) and the intact outer membrane proteins were solubilized with 20 mM Hepes, pH 8.2, containing 4 M urea and 1% *n*-octyl glucoside. The 36/39 kDa porin of *S. minnesota* was then purified by ion exchange chromatography (Mono-Q HR5/5, FPLC, Pharmacia, Freiburg) in the same buffer with an increasing salt gradient (40 to 770 mM NaCl).

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2.3. *Purification of C1q*

Human C1q was purified as described [15]. Euglobulins, precipitated from fresh serum, were redissolved in 50 mM Hepes, pH 7.8, 250 mM NaCl and applied to a Superose 6B column. Fractions corresponding to C1 hemolytic activity were further purified for C1q by chromatography on a cation exchanger (Mono-S) by fast protein liquid chromatography. The elution buffer was 20 mM Hepes, 10 mM EDTA, 10 mM 1,3-diaminopropane, pH 7.8 (60 to 300 mM NaCl).

2.4. *Lipids*

Lipid A of *S. minnesota*, R595, was obtained from List Biological Laboratories (Campbell, CA, USA). Diphytanoyllecithin (DPL) was purchased from Avanti Polar Lipids (Birmingham, AL, USA).

Other chemicals such as dimyristoylphosphatidylcholine and cholesterol were obtained from Sigma (Munich, FRG).

2.5. *Electrical measurements*

Optically black lipid bilayer membranes were obtained from a 1% solution of diphytanoyllecithin in *n*-decane/butanol (*n*-decane/butanol = 10:1 (v/v)). The chamber used was made of Teflon. The circular hole separating the two aqueous compartments of the teflon chamber had a diameter of 0.8 mm. For the electrical measurements Ag/AgCl electrodes were inserted into the aqueous solutions on both sides of the membranes. The current fluctuation experiments were performed using a Keithley 427 preamplifier. The amplified signal was monitored with a Gould OS 4040 oscilloscope and recorded with a tape recorder. Bilayer membranes were formed in 10 mM phosphate-buffered saline (PBS), pH 7.4, conductivity adjusted to 15 mS with NaCl. After the membranes had turned completely black, a voltage of 50 mV was applied. The membrane conductance was observed within 5–50 min. The temperature was kept at 25°C.

2.6. *Reconstitution of porins in lipid bilayer membranes*

An aqueous solution of porins and *n*-octyl glucoside (60 μg porins/40 μg *n*-octyl glucoside) was lyophylized, solubilized in 20 μl butanol and mixed with 40 μl of a 1% solution of diphytanoyllecithin in *n*-decane/butanol.

To incorporate lipid A into the BLM solution 50 μl of a 0.3% solution of lipid A in chloroform/*n*-decane/butanol (1:1:1) were mixed with 50 μl of the porin containing lipid matrix.

2.7. *Reconstitution of outer membrane vesicles*

Liposomes were prepared using a method modified from that of [16]. Dimyristoylphosphatidylcholine and cholesterol (molar ratio 2:1) were solved in chloroform. After removal of the solvent under a stream of $N_2$, 1.5 μmol of the dried liposomal lipids were resuspended in 0.2 ml aqueous solution containing $8 \times 10^{-4}$ μmol of isolated porins. The mixture was sonicated for 30 s and dried at 42°C. The protein/lipid mixture was then vortexed for 20 min in 0.2 ml 10 mM PBS (pH 7.4, $K$ = 15 mS, adjusted with NaCl) followed by brief sonication. The suspension was kept at 42°C for 30 min followed by slow cooling to room temperature.

To incorporate lipid A into liposomes 0.3 μmol lipid A diluted in chloroform was added to 1.5 μmol total liposomal lipid mixture. After removal of the solvent, liposomes were prepared according to the method described above. Lipid A or porins which were not incorporated into liposomes were separated by a PD-10 (Sephadex G25M) column (Pharmacia, Freiburg, FRG).

To bind C1q to porins which were incorporated into liposomes, OMP-containing liposomes were preincubated with human C1q for 2 h at 20°C. The molar ratio of C1q and OMP was 1:1, 1:5 and 1:10. C1q, which was not bound to porins, was separated by a PD-10 column.

2.8. *Macrophages (Mϕ)*

10–12-week-old Balb/C mice were injected intraperitoneally with 1 ml of thioglycolate broth (Oxoid Ltd.). 4 days later exudate cells were harvested by washing the peritoneal cavity of each mouse with 5 ml of 10 mM PBS, pH 7.4, collected by centrifugation (15 min, 250 × *g*) and suspended in CL-MEM-medium (Boehringer, Mannheim, FRG) to the desired cell concentration.

2.9. *Chemiluminescence*

Chemiluminescence responses of Mϕ were measured in a Bioluminat apparatus (Berthold Co., Wildbach, FRG). $1 \times 10^6$ Mϕ in 0.5 ml CL-MEM-medium were incubated with 0.1 ml of *S. minnesota*, Re-form ($6 \times 10^9$ cells/ml PBS) or 0.2 ml of liposomes (1.5 μmol liposomal lipids) and 0.1 ml of a 0.1% Luminol solution (5-amino-2,3-dihydro-1,4-phtalazinedione; Boehringer) over a 30 min or 60 min period. As controls PBS and phorbol myristate acetate were used.

## 3. RESULTS

### 3.1. *Binding of C1q to porin containing membranes does not affect the function of the porins*

To investigate the effect of isolated C1q on porins incorporated into black lipid membranes (BLM) lyophilized, isolated porins of *S. minnesota* were solvated in butanol and added to the lipid containing solution as described in section 2. This procedure was carried out to exclude any subsequent interaction of porins with C1q in the fluid phase. Fig. 1 shows the kinetics of channel formation of these separate experiments. The conductivity measured for one channel ranged between 2.4 and 2.8 nS. This is in agreement with systems using preformed BLMs to which solutions of porins were added [17].

Addition of purified C1q any time after reaching a plateau of channel formation (i.e. after 5 min) had no effect on channel numbers as measured by conductivity. To increase binding of C1q to porin containing BLM, lipid A was added together with porins. Addition of C1q to lipid A and porin containing BLM had no effect on the number of formed channels. Only in one experiment were all channels closed after addition of purified C1q. However, this effect was not reproducible.

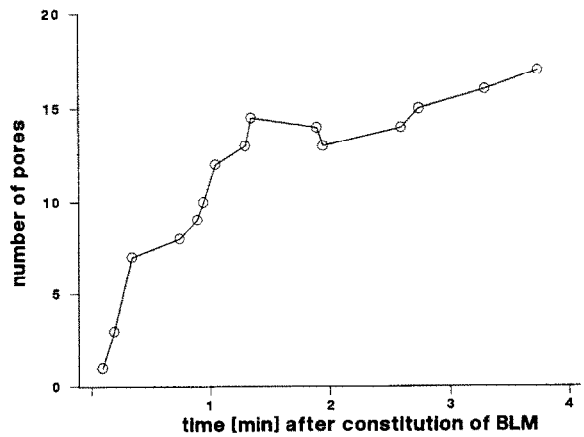


Fig. 1. Channel formation as a function of time (min) after constitution of porin-containing black lipid membrane.

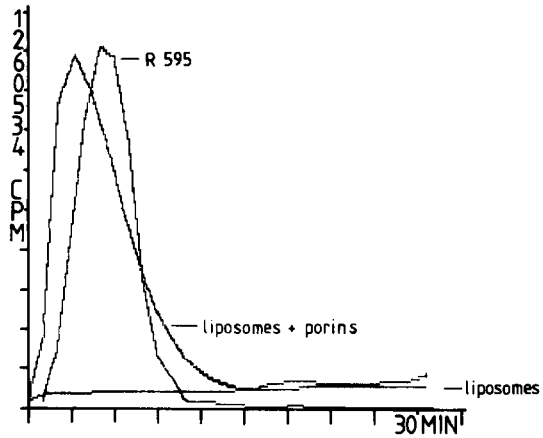


Fig. 2. Chemiluminescence signal (in cpm = counts per minute) of peritoneal Mϕ during phagocytosis of *S. minnesota*, R595, and liposome-containing porins.

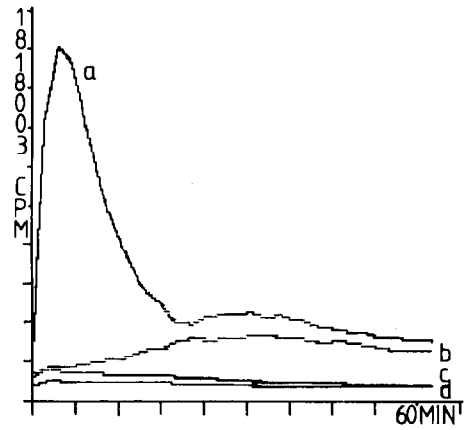


Fig. 3. Chemiluminescence signal (in cpm) of peritoneal Mϕ during phagocytosis of liposome-containing porins preincubated with different concentrations of purified C1q. (a) Control, ϕ C1q; (b) $8 \times 10^{-5}$ μmol C1q; (c) $4 \times 10^{-4}$ μmol C1q; (d) $8 \times 10^{-4}$ μmol C1q.

### 3.2. *Direct interaction of porin containing liposomes with macrophages*

To mimic the bacteria/Mϕ interaction, porins of the Re-form of *S. minnesota* were incorporated into liposomes as described in section 2. Addition of such liposomes to mouse peritoneal macrophages resulted in the same CL-signal as the intact bacteria (Fig. 2). Liposomes without porins as a control did not induce a CL-signal. Preincubation of liposomes containing porins with purified C1q abolished dose-dependently the CL-signal (Fig. 3). These experiments confirm the results obtained with intact C1q binding bacteria and support our previous interpretation that porins are one of the major components of the bacterial cell wall that bind to Mϕ via Mϕ membrane-associated C1q.

### 3.3. *C1q prevents binding of porin containing liposomes to black lipid membranes*

The next set of experiments (Fig. 4) demonstrates that porin-containing liposomes interact directly with porin-free BLM. The number of channels were markedly increased by incorporation of porins into the liposomes in the presence of lipid A. Preincubation of porin-containing liposomes with purified C1q (Fig. 4A) markedly reduced the numbers of channels and the kinetics of channel formation. The inhibitory effect of C1q on channel formation was amplified when the liposomes contained lipid A in addition to porins (Fig. 4B).

## 4. DISCUSSION

The goal of these studies is to extend our present understanding of the interaction of C1q with components of the cell wall of Gram-negative bacteria. With intact *Salmonella* strains it was found that lipid A of LPS and porins are involved in the direct antibody-independent activation of the classical C-cascade [8,9]. Macrophage membrane-associated C1q has been shown to be involved in binding of the serum-sensitive *Salmonella* strains via LPS and/or porins to Mϕ [13].

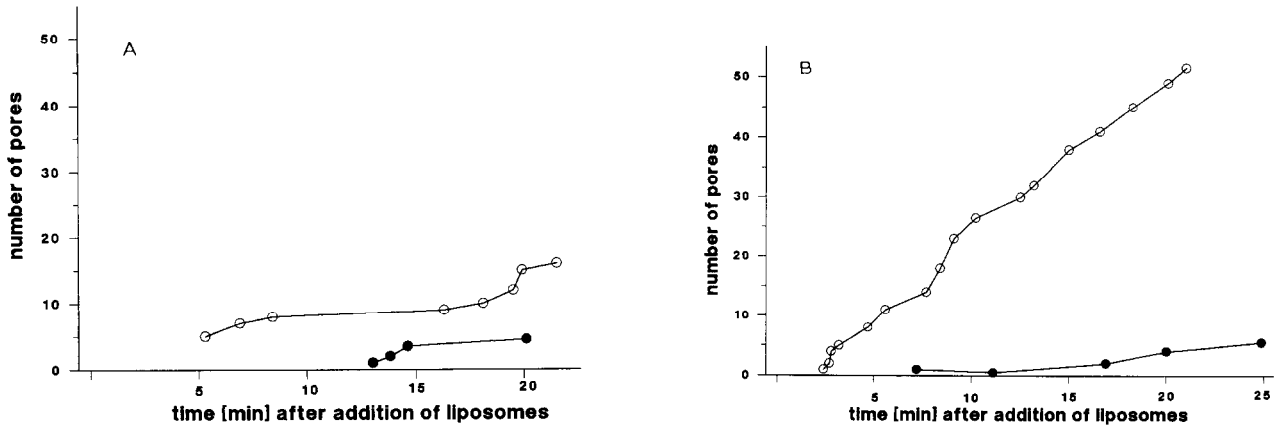


Fig. 4. Channel formation in preformed BLM after addition of liposome-containing porins (Fig. 4A; ○) or liposome-containing porins and lipid A (Fig. 4B, ○) to the aqueous phase. Fig. 4A, ●; 4B, ●: preincubation of liposomes with C1q.

In this report we were able to demonstrate, with porins incorporated in black lipid membranes (BLM), that purified C1q does not affect porin function (Fig. 1). However, C1q inhibits the interaction of porin-containing lipid membranes (liposomes) with other lipid bilayers (BLM) (Fig. 4). This inhibitory effect is amplified when besides porins lipid A is incorporated into the liposome membrane. This experimental model confirms that the bacterial cell wall constituents, porin and lipid A, are the acceptor molecules for C1q. This interpretation is further supported by the experiment showing that porin-containing liposomes induce in macrophages the same pattern of chemiluminescence as in intact bacteria and which is prevented by isolated C1q.

Although the artificial models of liposomes and BLMs are not comparable to the bacteria and macrophage system, the models indicate at least two different possible schemes for porin/C1q interaction: (i) C1q prevents the porin-mediated interaction of two lipid membranes by direct binding to the porin present in one membrane, (ii) C1q within a membrane such as macrophage membrane-associated C1q triggers binding of porin-bearing particles such as liposomes or intact bacteria to the Mϕ membrane.

Based on the experiments with artificial membranes, presented here, and on our earlier observations [13] with intact bacteria and macrophages, the following mechanism of interaction for macrophage and Gram-negative bacteria such as *Salmonella* is suggested: C1q binds directly to porins within the bacterial cell wall. This binding is amplified by lipid A. Binding of fluid phase C1q (e.g. in serum) to porins within the bacterial membrane leads to the activation of the C-cascade resulting in C-mediated killing of the bacteria. Porin-mediated binding of bacteria to C1q present in the macrophage membrane initiates uptake of the bacteria and the formation of a phagosome. Since the porins are associated with C1q they are either no longer able to insert into the membrane of the phagosome or the porin-mediated fusion of two membranes such as the phagosome and lysosome membranes is inhibited by C1q. Both possibilities prevent the phagosome/lysosome fusion resulting in survival of the bacteria within the cells. *Salmonella* have been described to survive in macrophages [13,18,19]. However, although the findings in this report regarding the interaction of porins with C1q support this concept, to substantiate such a hypothesis further experiments are required.

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