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Pore Formation by Complement in the Outer Membrane of Gram-Negative Bacteria Studied with Asymmetric Planar Lipopolysaccharide/Phospholipid Bilayers

Guido Schröder, Klaus Brandenburg, Lore Brade, and Ulrich Seydel
Forschungsinstitut Borstel, D-2061 Borstel, Federal Republic of Germany

Summary. The interaction of complement with an asymmetric planar lipopolysaccharide/phospholipid bilayer system as a model for the lipid matrix of the outer membrane of Gram-negative bacteria has been studied. The addition of whole human serum to the aqueous solution at the lipopolysaccharide side of the asymmetric membrane resulted in a rapid increase of the bilayer conductance in discrete steps, indicating the formation of transmembrane pores, which were not observed in the case of pure phospholipid membranes. The amplitudes of the discrete conductance steps varied over a range of more than one order of magnitude. The mean single step conductance was (0.39 ± 0.24) nS for a subphase containing (in mM): 100 KCl, 5 $MgCl_2$ and 5 HEPES buffer. The steps were grouped into bursts of typically 9 ± 3 events per burst and the conductance change within one burst was (8.25 ± 4.00) nS.

The pore-forming activity of serum at the asymmetric membrane system was independent of the presence of specific antibodies against the lipopolysaccharide but was dependent on calcium ions. Furthermore, the pore-forming activity required complement component C9.

A model for the mode of pore formation by complement is proposed: The complement pore is generated in discrete steps by insertion of C9 monomers into the membrane and their irreversible aggregation to water-filled channels with a diameter of approximately 7 nm assuming a circular geometry.

Key Words outer membrane · planar lipid bilayer · lipopolysaccharide · complement · pore formation · membrane reconstitution

Introduction

Activation of both the classical and alternative complement pathway to completion results in the assembly of C5b-9 complexes that have the potential to insert into membranes of eucaryotic cells and form pores (Bhakdi & Tranum-Jensen, 1983; Müller-Eberhard, 1985; Bhakdi & Tranum-Jensen, 1987). Fully assembled complement lesions appear in the electron microscope as hollow cylinders with a diameter of approximately 10 nm and a length of 15 nm (Tranum-Jensen et al., 1978, Tranum-Jensen & Bhakdi, 1983). Component C9 assumes a dominant role in formation of the membrane-embedded cylinder (Tschopp, Engel & Podack, 1984).

Although the observation that fresh serum is capable of killing certain types of bacteria led to the discovery of the complement system, the mechanisms of bacterial killing by complement as well as the mechanisms of resistance against complement, which is an attribute of many Gram-negative strains, are poorly understood or discussed controversially. Of particular importance is the question of the possible role of lipopolysaccharide (LPS), the major amphiphilic component of the outer membrane of Gram-negative bacteria, as a physical barrier against terminal complement complex insertion (Joiner et al., 1982*a,b*).

Since complement acts primarily on the bacterial surface, it is clearly necessary to study the interaction processes occurring at the outermost layer, which is in the case of Gram-negative bacteria the outer membrane. Planar bilayers are a powerful tool to study functional properties of pore-forming proteins via measurement of conductance changes. Only few investigations into the conductance changes induced by complement as measured in planar phospholipid (PL) bilayers (del Castillo et al., 1966; Barfort, Arquilla & Vogelhut, 1968; Wobschall & McKeon, 1975; Michaels et al., 1976; Benz et al. 1986) as well as in patch-clamped cells (Jackson, Stephens & Lecar, 1981) have been reported, and the results of these studies concerning pore size, its stability, and also requirements of complement components for the induction of conductance changes differ considerably. Moreover, as these investigations were exclusively performed with phospholipid membrane systems, they are not unreservedly transferable to the membrane system of Gram-negative bacteria, which has a quite different

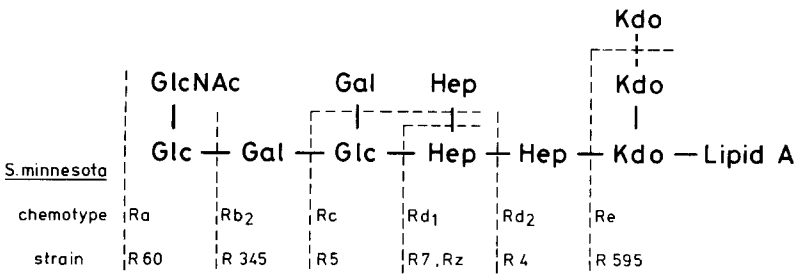

Fig. 1. Schematic structure of the core oligosaccharide of *S. minnesota* LPS. Phosphate groups are omitted. Dashed lines indicate lipopolysaccharides as found in core-defective mutants, the chemotype being labeled Ra to Re. The terms R60 to R595 define the respective strains of *S. minnesota*. The wild-type (S-form) LPS additionally contains the covalently linked O-specific chain. *Abbreviations: Kdo* = 3-deoxy-D-manno-2-octulosonic acid, *Hep* = L-glycero-manno-heptose, *Glc* = D-glucose, *Gal* = D-galactose, *GlcNAc* = D-N-acetyl-glucosamine

membrane architecture and composition (Nikaido & Vaara, 1985; Rietschel et al., 1987).

In a recent study (Seydel, Schröder & Brandenburg, 1989) we reported on the successful reconstitution of the lipid matrix of the outer membrane of Gram-negative bacteria as an asymmetric planar lipid bilayer system, built up on one side exclusively from rough mutant LPS and on the other side from a PL-mixture resembling the natural phospholipid composition as determined for *Salmonella typhimurium*.

We have now investigated the interaction of complement with voltage-clamped planar LPS/PL bilayer membranes. Evidence will be provided that the interaction with serum leads to the assembly of water-filled pores and that the assembly of these pores proceeds within the membrane in discrete steps via insertion and irreversible aggregation of C9. The pores are heterogeneous with respect to their size, presumably due to their differing content of C9 molecules.

Materials and Methods

Lipids

The deep rough mutant LPS of *Salmonella minnesota* strain R595 was used in most experiments. The structure of the core region of various LPS from rough mutants of *S. minnesota* is shown in Fig. 1. The R595 LPS consists of the lipid portion of LPS, lipid A, and an α-2.4-linked disaccharide of 3-deoxy-D-manno-2-octulosonic acid (Kdo). In some experiments, Rd1 and Rc LPS from *S. minnesota* strains Rz and R5, respectively, and Re LPS from *Escherichia coli* strain F515 were additionally used. LPS was extracted by the phenol/chloroform/petrolether method (Galanos, Lüderitz & Westphal, 1969), electrodialyzed (Galanos & Lüderitz, 1975) and lyophilized.

Phosphatidylethanolamine (PE) from *E. coli* (type V), phosphatidylglycerol (PG) from egg yolk lecithin (sodium salt), cardiolipin (CL) from bovine heart (sodium salt), phosphatidylcholine (PC) from bovine brain (type III-B) and phosphatidylserine (PS) from bovine brain were purchased from Sigma (Deisenhofen, FRG) and used without further purification.

Sera, Complement Products, and Serological Assays

C9 deficient human serum (C9defHS) and purified C9 (purity 95%) were obtained from Sigma. Whole human serum (WHS) was tested for the presence of antibodies, using the passive hemolysis test (Brade et al., 1987). The antibodies were absorbed from the serum with Re LPS-coated sheep red blood cells (SRBC, 100 μl packed cells per 1 ml undiluted serum) at 4°C (Rozalski et al., 1989). The complement activities of the different sera used as complement source were tested in the direct hemolysis test performed as follows. SRBC were coated with amboceptor, a rabbit antiserum which is directed against SRBC (Amboceptor 6000, purchased from Behring, Hamburg, FRG). 200 μl of Packed SRBC were coated with 35 μl of amboceptor, washed three times in phosphate-buffered saline, finally suspended in veronal-buffered saline to 0.5%. Twofold serial dilutions of the sera in veronal-buffered saline were prepared in wells of microtiter plates (50 μl per well), 50 μl of amboceptor-coated SRBC were then added. The plates were incubated for 1 hr at 37°C. The bottom of the plates were examined for hemolysis determining the 50% lysis as endpoint titer.

Asymmetric Planar Bilayers and Electrical Measurements

To simulate the composition of the PL moiety of the inner leaflet of the outer membrane as determined from *S. typhimurium* (Osborn et al., 1972), PE, PG and CL were mixed in a molar ratio of 81 : 17 : 2 (PE : PG : CL) and dissolved in chloroform at a final concentration of 2 mg/ml. LPS, which forms the lipid matrix of the outer leaflet of the outer membrane, was dissolved in chloroform/methanol (9 : 1 by volume) by heating the suspension to 80°C for a few minutes.

The asymmetric planar LPS/PL bilayers were prepared essentially by the technique of Montal and Mueller (1972) in which planar membranes are formed by the apposition of two lipid monolayers spread on an aqueous subphase from the respective lipid solutions.

For the aqueous subphase, standard buffer solutions (5 mM HEPES and 5 mM $MgCL_2$, for membrane stabilization) with

different concentrations of KCl at pH 7 (KOH) were used. The electrical conductivity of each buffer solution was measured with a LF 610E conductometer (WTW, Weilheim, FRG) (cell constant of 1 cm^{-1}).

Our apparatus for membrane formation consists of two Teflon compartments of 1.5 ml-volume each which are separated by a hexadecane-pretreated 12.5-μm thick Teflon foil (Angst und Pfister, Zürich, CH) with a small aperture of typically 200-μm diameter.

For electrical measurements, planar membranes were voltage clamped via a pair of Ag/AgCl electrodes (type E255 purchased from In Vivo Metric Systems, Healdsburg, CA) which were connected to a voltage source (Enertec Schlumberger, St. Etienne, France) and a current-voltage (I/V) converter based on an OPA 111 (Burr Brown, Filderstadt, FRG) operational amplifier with a feedback resistance of $4.25 \times 10^8\ \Omega$ and a feedback capacitance of 27 pF. Voltage signs refer to the electrode virtually grounded by the operational amplifier. Current is positive when the cations flow into the virtually grounded compartment.

Bilayer formation was observed by continuously monitoring membrane capacitance. For the conductance measurements a constant potential was applied to one electrode. The output of the I/V converter was passed through a 4-pole low-pass Bessel filter (Ithaca, NY), the corner frequency of which was adjusted to 10 Hz (−3 dB). After further amplification by factors of 1, 10 or 100, signals were digitized at a sample rate of 20 Hz with a PCI-20089W-1 analog input board (Burr Brown) plugged into an AT-compatible microcomputer system. All experiments were performed at 37°C. Sera and complement components were added always as aliquots of 10 μl to the LPS side of the membranes which was virtually grounded by the operational amplifier. After addition, the bathing solutions were stirred vigorously for 30 sec by means of magnetic bars.

Results

The addition of whole human serum (WHS) to the aqueous phase at the LPS side of voltage-clamped asymmetric planar LPS/PL bilayers resulted in a rapid macroscopic increase of the transmembrane current as shown in Fig. 2. The changes were first detectable after a period of approximately 1 min after the serum application, and then accelerated until the membrane finally disrupted (typically within a period of 3 min). In experiments with other Re-type LPS (F515 from *E. coli*) and with LPS having a more complete inner core region (Rd1, Rc from *S. minnesota*) essentially the same findings were made as compared to the experiments with R595 LPS. The time lag between addition of serum and changes of current was presumably due to the time required for diffusion of serum components across unstirred layers in front of planar membranes and for the activation of the complement cascade.

No changes of the transmembrane current were detected when WHS was applied to symmetric membranes built-up exclusively from the PL mixture or from a mixture of PC and PS in a molar ratio of 4 : 1 (*see* Fig. 2).

The following experiments were performed with the asymmetric LPS/PL membranes. At low serum concentration (final dilution about 1 : 1500) and high sensitivity of the recording system, discrete current steps were detected (*see* Fig. 3) which frequently showed a marked grouping into bursts, i.e., periods of rapidly following discrete steps alternated with nearly silent periods during which the mean current did not change significantly.

Typically two basic patterns of the development of the conductance in discrete steps within the bursts were observed (Fig. 4*a* and *b*). The most frequently observed pattern (type A, Fig. 4*a*) showed consecutively following steps with unsystematically varying amplitudes and the other (type B, Fig. 4*b*) systematically increasing current amplitudes. From the height of each discrete step, conductance values can be calculated, which result for traces of type A in the cumulative histogram shown in Fig. 5, obtained from 15 experiments and a total number of 159 single events. The histogram shows that the amplitudes of the discrete steps varied over a range of more than one order of magnitude (minimum 60 pS, maximum 1.5 nS). The mean amplitude was (0.39 ± 0.24) nS measured at +20 mV transmembrane voltage and for a bathing solution containing 100 mM KCl, 5 mM HEPES and 5 mM $MgCl_2$ at pH 7 (KOH). Due to the considerable noise of the current signal after a small number of steps, it is inevitable that events with small amplitudes remain undetected. Thus, the decline of the amplitude distribution for small amplitudes and its maximum might reflect rather the lower detection probability for steps with small amplitude than a characteristic of the true distribution.

To test the possible significance of antibodies against Re-type LPS in this system, antibodies were absorbed from WHS with Re LPS-coated sheep erythrocytes. In experiments with this absorbed serum no significant differences as compared with the activity of the WHS before absorbtion were detected (*data not shown*).

For elucidating the pathway on which the activation is initiated, experiments were performed in the presence of 5 mM EGTA, a specific calcium-chelating agent that selectively blocks the classical pathway while permitting activation on the alternative pathway. It was found that presence of EGTA completely abrogated the pore-forming activity of WHS, indicating a predominant involvement of the classical pathway.

Experiments with C9-deficient serum were performed to demonstrate that the observed conductance changes are caused by complement. A typical experiment is shown in Fig. 6. The addition of C9defHS (final dilution 1 : 150) caused no changes, even at prolonged observation times (up to 15 min).

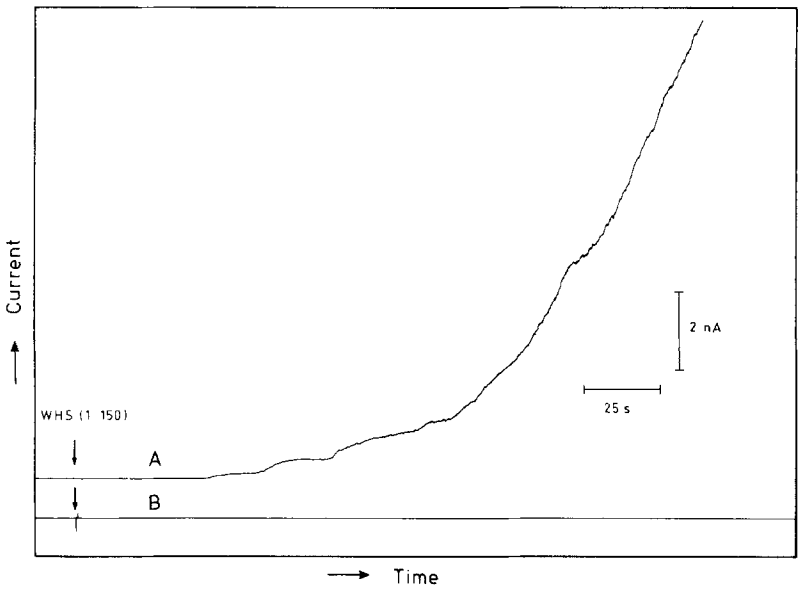

Fig. 2. Trace *A*: Macroscopic current response of an asymmetric LPS/PL bilayer upon addition (marked by an arrow) of 10 μl of WHS to the aqueous solution at the LPS side of the membrane (final dilution of serum 1 : 150). Transmembrane voltage was clamped to +20 mV. Temperature was 37°C and the bathing solution contained 100 mM KCl, 5 mM $MgCl_2$, 5 mM HEPES at pH 7 (KOH). The membrane broke at the end of the trace. Trace *B*: Same as for trace *A* but for symmetric PL bilayer

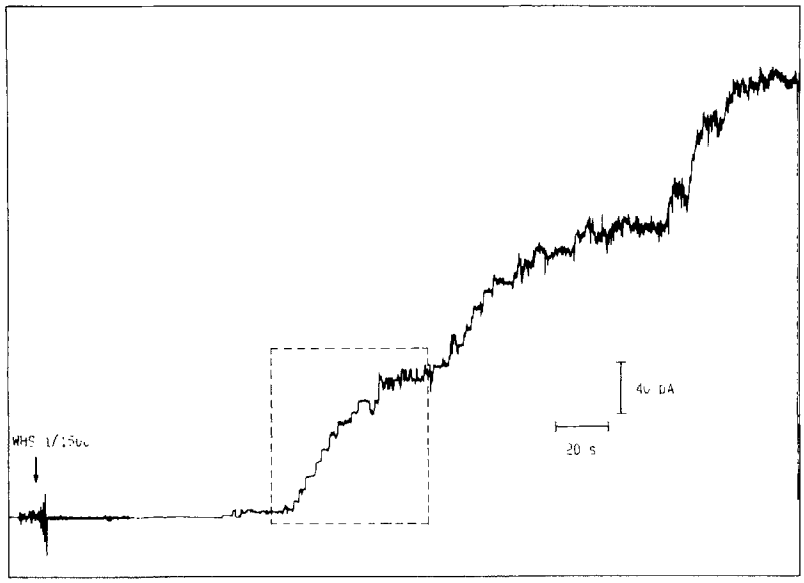

Fig. 3. Microscopic current steps for asymmetric LPS/PL membranes under conditions as in Fig. 2 but at one order of magnitude lower serum concentration (final dilution 1 : 1500). Initial noise after application originates from magnetic stirrer. Enlargement of marked area is shown in Fig. 4*a*

The subsequent addition of purified C9 led again to changes as observed for WHS, i.e., a rapid increase of the transmembrane current in discrete steps. However, as can also be seen from Fig. 6, the grouping of the discrete steps into bursts was more expressed under these experimental conditions than in the experiments with WHS at the same final dilution of 1 : 150 (*see* Fig. 2). At a 10-fold higher dilution of the WHS (*see* Fig. 3) the grouping into bursts, again, compared well with the behavior depicted in Fig. 6. This may be explained by the much lower complement activity of the C9defHS after addition of isolated C9 with a titer of only 1 : 20 as compared to that of WHS which had a titer of 1 : 80 as determined in the direct hemolysis test. From repeated experiments it was found that each burst contained a mean of 9 ± 3 events (minimum 5 and maximum 13 in 30 single evaluations). Between such bursts the current remained nearly constant. The change of the current signal within one burst corresponded to a conductance change of (8.3 ± 4.0) nS (minimum 2.5 nS, maximum 20.0 nS in 30 evaluations). Both, the evaluation of the conductance change within one burst and the counting of the number of single steps within the bursts, were performed only for bursts well separated from others. Addition of purified C9 alone did not cause any changes of the transmembrane current.

To evaluate further how the ions move within the complement-generated lesions, we tested the in-

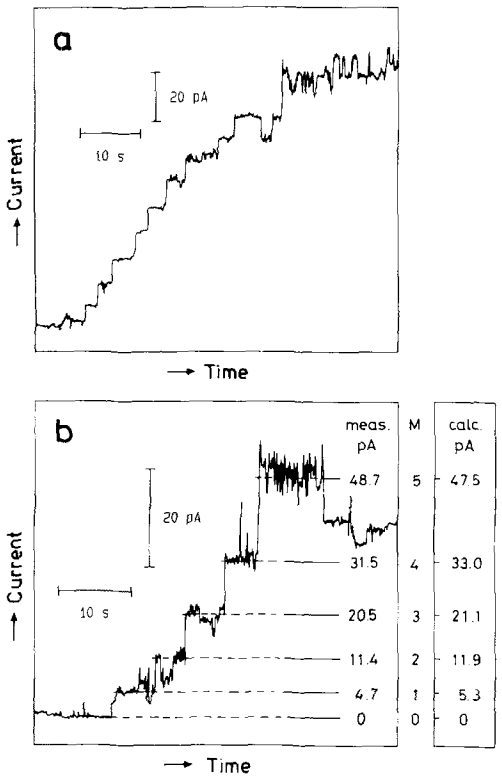

Fig. 4. Sequences of microscopic current steps after addition of WHS under experimental conditions as in Fig. 3 with (*a*) unsystematically varying amplitudes of the current steps and (*b*) systematically increasing current amplitudes. The numbers in the inserted columns in *b* give a comparison between the measured currents and those calculated according to the proposed aggregation model (*see* Discussion)

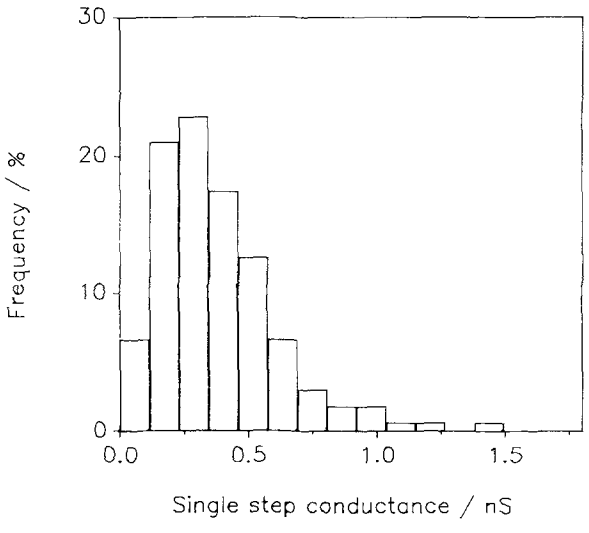

Fig. 5. Cumulative histogram of single conductance steps measured under conditions as in Fig. 3

fluence of the specific conductivity of the bathing solution and of the transmembrane voltage on the amplitude of the discrete conductance steps. In Fig. 7, the mean amplitude of the current steps induced by WHS in the asymmetric LPS/PL bilayers is ploted *versus* the specific conductivity of the membrane bathing solution (KCl concentration varying in the range of 10 to 200 mM). For the calculation of the regression curve a linear relation between single step conductance and specific conductivity of the bathing solution was assumed. The increase of single step conductance with increasing electrical conductivity of the bathing solution indicates that the complement lesion is water filled.

The following protocol was applied to obtain a steady-state current-voltage relationship for the LPS/PL bilayers under the influence of complement. In the experiments with low serum doses (dilution about 1 : 1500) the first burst of events was generally followed by a silent period of sufficient duration to apply a sequence of long-lasting voltage pulses ($\geq$15 sec) with increasing amplitude and to measure the corresponding current. A typical current-voltage curve obtained by this procedure is shown in Fig. 8. In the range between approximately -80 to $+80$ mV the relationship was linear. For positive voltages above 80 mV, the *I/V* curves showed a clear tendency towards a superlinear behavior. At negative voltages below approximately -80 mV discrete transitions between various conductance levels have been observed, the upper limits of which showed a similar superlinear behavior as observed for high positive voltages. However, this behavior possibly does not reflect the characteristic of the channel itself but evolves from the general instability of the membranes under the influence of serum, especially at high clamp voltages.

In experiments with a 10-fold KCL gradient (10 mM KCl at the LPS side (virtually grounded), 100 KCl at the PL side) a reversal potential of -55 ± 2 mV was measured, corresponding to a potential of -27.5 mV for the measurement with calomel electrodes with saturated KCl bridges (Vassilev & Tien, 1985). This indicated a cation selectivity of the conductance pathways induced by complement. Applying the Goldman-Hodgkin-Katz equation (Schultz, 1980), a permeability ratio P_{K^+}/P_{Cl^-} of approximately 3.8 was obtained.

Discussion

The asymmetric planar LPS/PL bilayer system used in this investigation allowed us to study, in a very direct way via conductance measurements, the effects of complement activation on a membrane target that mimicked the lipid matrix of the outer mem-

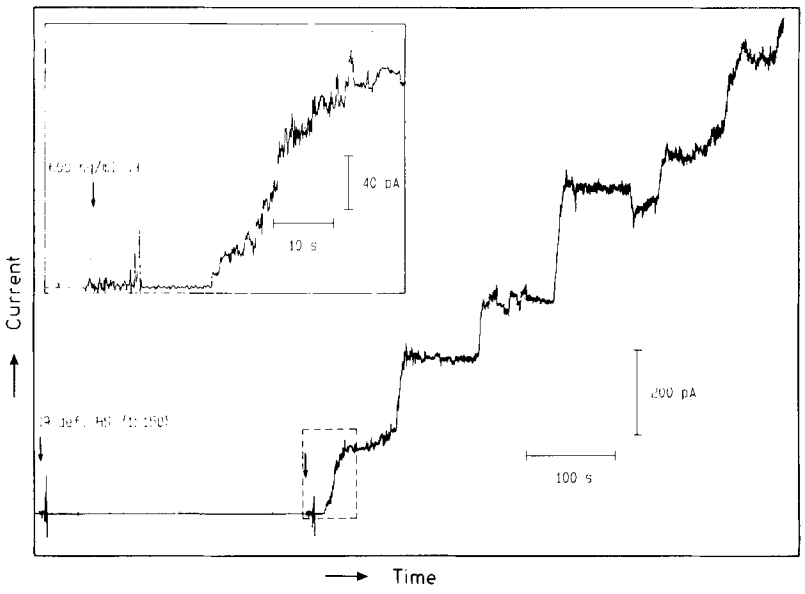

Fig. 6. Macroscopic current response of asymmetric planar LPS/PL bilayer upon addition of C9defHS (final dilution 1 : 150) and, after a period of 5 min, subsequent addition of human C9 to a final concentration of 600 ng/ml. The membrane was disrupted at the end of the trace. Both components were applied to the aqueous solution at the LPS side. Inset shows enlargement of marked area. Conditions with respect to bathing solution and temperature as in Fig. 3

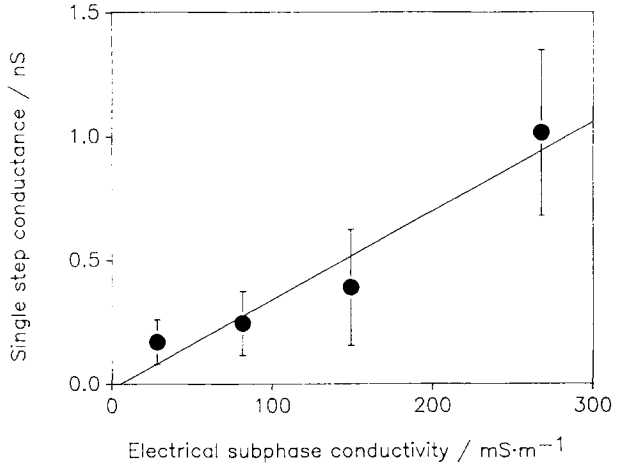

Fig. 7. Plot of mean single step conductance *versus* specific conductivity of the membrane bathing solution. For all points the number of events exceeds 30 and error bars refer to standard deviation

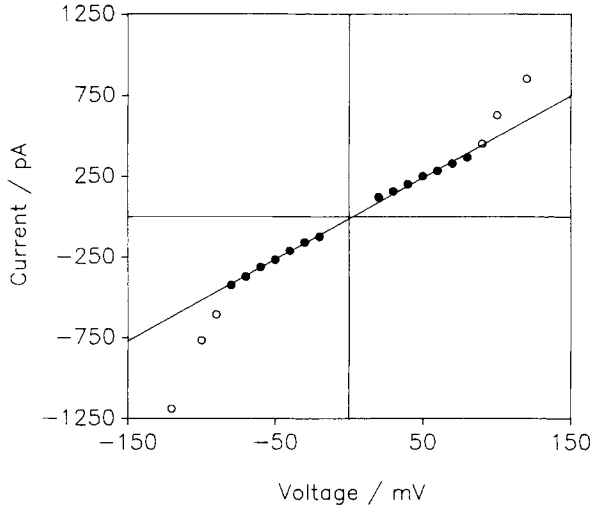

Fig. 8. Current-voltage relationship of asymmetric membranes obtained for experimental conditions as in Fig. 3. Regression curve was calculated only for the measuring points represented by filled circles

brane of Gram-negative bacteria. The presented results confirm earlier data (del Castillo et al., 1966; Barfort et al., 1968; Wobschall & McKeon, 1975) that no reactivity of serum can be observed against bilayers composed of various phospholipids (PE, PG and CL as well as mixtures from PC and PS). We observed, however, marked effects when WHS was applied to the LPS side of the asymmetric LPS/PL membranes.

Previous studies on the action of complement on planar bilayers employed either the principle of reactive lysis (Michaels et al., 1976; Benz et al., 1986) with isolated C5b6 or the activation of complement was provoked by sensitization or modification of phospholipid membranes with certain antigens, e.g., crystallized albumins (del Castillo et al., 1966; Wobschall & McKeon, 1975; Jackson et al., 1981).

In our study we were able to utilize whole human serum as the complement source, since the LPS membrane system itself acted as an activator surface independent of specific antibodies against the LPS. This fact provides the advantage that purification or isolation of complement components is not neces-

sary, thus avoiding possible alterations of the natural system caused by such procedures.

The experiments with C9defHS clearly demonstrated that the conductance changes were complement dependent since there was an absolute requirement for C9. Our results are in agreement with the observation of Benz et al. (1986) who detected no conductance changes in the absence of C9.

Initiation of the conductance changes required no antibodies directed against the respective LPS. However, activation appeared to involve the classical pathway because it was blocked by EGTA. These findings are in agreement with the statement of Vukajlovich, Sinoway and Morrison (1986) that Re LPS activates exclusively the classical pathway of complement. We consider it likely that complement activation is mediated via a direct interaction of C1 with LPS (Morrison & Cline, 1977; Clas, Schmidt & Loos, 1988).

The detection of discrete conductance steps provided direct evidence for the generation of transmembrane pores induced by the interaction of serum with the asymmetric LPS/PL membrane system. However, the conductance changes observed in our experimental setup differ in many features from those described for pure PL membrane systems (Benz et al., 1986). These differences are probably due mainly to differences in the physico-chemical properties of the lipid components.

On the basis of the data obtained, we advance the following models for possible modes of pore formation by C5b-9 in the asymmetric LPS/PL bilayers (Fig. 9*a* and *b*):

The formation of the complement pore proceeds within the membrane via insertion of C9 monomers and their subsequent aggregation to water-filled channels. The discrete conductance changes which are detected within the bursts, result from a stepwise increase of the channel size due to an uptake of monomers into the aggregate. When the number of monomers in the aggregate reaches a certain limit (which might be variable) the aggregation is terminated. The burst itself, accordingly, corresponds to the formation of a whole pore. Events of type A (Fig. 4a), would be most compatible with the occurrence of noncylindrical geometries which are not entirely lined by protein but on one side by phospholipids (Amiguet, Brunner & Tschopp, 1985). The schematic in Fig. 9*a* illustrates, that for this mode of C9 assembly no strict correlation between the number of C9 monomers and pore size or conductance, respectively, but that, in any case, an increase in pore conductance with increasing number of monomers is to be expected. Patterns of conductance changes resembling type B (Fig. 4*b*) may result from

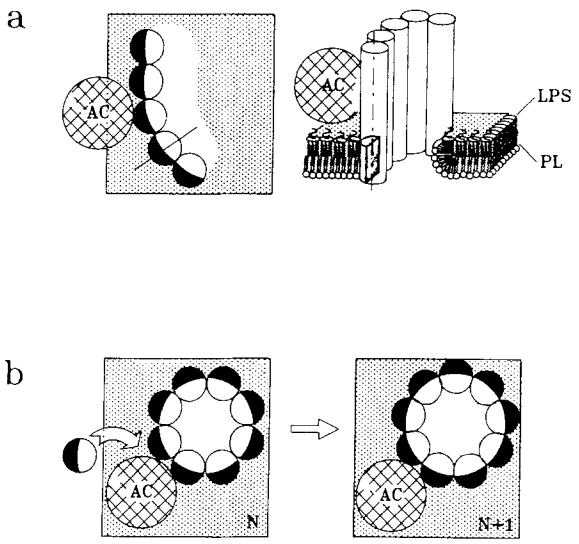

Fig. 9. Schematic models for the assembly of the complement pore via aggregation of C9 monomers. Transitions between two neighboring conductance levels N to $N + 1$ result from irreversible uptake of one C9 monomer. The hatched parts of the pore-forming units represent hydrophobic regions. *AC*: Active (C5b-8) complex which mediates the insertion and aggregation process. (*a*) Formation of noncircular, not fully C9-lined pores. (*b*) Formation of circular pores with a development of pore conductance according to Eq. (5). The pore is seen in axial projection

a circular assembly of C9 monomers in which the uptake of additional monomers would lead to a stepwise increase of the pore diameter and with that to increasing spacings between subsequent conductance levels. The following considerations collectively led to this model:

(*i*) The occurrence of the discrete conductance steps in bursts is difficult to interpret as formation of single statistically occurring and spatially separated pores, because a physical coupling between individual pore-forming complexes, which would lead to a cooperative opening of a certain number of pores, is unlikely to occur.

(*ii*) For the amplitude of the conductance increase within one burst, a mean of (8.3 ± 4.0) nS was determined. Since the conductance induced by serum seems to follow basically the specific conductivity σ of the bathing solution and exhibits, at least for low voltages, an ohmic current-voltage relation, it is justified to assume that the ionic mobility within the complement lesion is similar to that in the bathing solution. This is in agreement with previous investigations of Wobschal and McKeon (1975) and of Benz et al. (1986). Thus, a water-filled pore seems to be an adequate model of the complement-induced lesion. Under the assumption of a cylindrical geometry for

the pore (length l and radius r), the geometry of the tubule and its conductivity G are related by

$$G = \frac{I_P}{U_M} = \frac{\pi \cdot \sigma \cdot r^2}{l}. \quad (1)$$

For a length of the pore of 15 nm, indicated by electron microscopic analysis, a diameter of (7.2 ± 5) nm is derived. This is in good agreement with the diameter of 10 nm of the complement lesion approximated by electron microscopic studies (Tranum-Jensen & Bhakdi, 1983) and the value of 8.3 nm obtained from the single-channel analysis of Benz et al. (1986). In contrast, if one assumes that the mean conductance of 0.39 nS of the microscopic steps within the bursts corresponds to the conductance of an individual pore a diameter of only 1.6 nm is derived. This value is considerably lower as compared with the cited results.

(*iii*) A mean number of 9 ± 3 events per burst was detected, a finding that we interpret in the sense that the conductance increase during each burst of events results from an aggregation of C9 monomers to form a pore. One single step may correspond to the uptake of one monomer into the aggregate, which is accompanied by an increase of the cross-section of the pore and thus by a stepwise increase of the pore conductance. Previous studies have indicated a marked heterogeneity in C9 content of individual lesions and there is evidence correlating increased C9 content with larger pore diameters in phospholipid bilayers (Bhakdi & Tranum-Jensen, 1984, 1986; Ramm, Whitlow & Mayer, 1985; Silversmith & Nelsestuen, 1986; Malinski & Nelsestuen, 1989). The mean ratio of C9 : C8 in C5b-9 complexes generated on serum-sensitive *E. coli* is approximately 6–8 : 1 (Bhakdi, et al., 1987). Summarized, it seems therefore attractive to postulate that the variations in amplitude of individual conductance changes observed in the present study directly reflect the C9-dependent size heterogeneity of the pores.

(*iv*) From the aggregation model (Fig. 9*b*) it is expected that the spacing between neighboring conductance levels of the pore (denoted by M and M + 1, M = 1, 2, 3 . . .) shows a tendency to increase with increasing number N of monomers in the aggregate. This is derived from the following consideration: For a rough approximation it is assumed that the circumference U of the inner circular cross-section of the pore, which contributes to the conductance, is a linear function of the number N of segments (C9 monomers) forming the rim of the water-filled cylinder:

$$U(N) = N \cdot \delta l = 2 \cdot \pi \cdot r \quad \text{for } N = N_0 + M \quad (2)$$

where δl is the contribution of each segment to the circumference. The lower limit for N was set to N_0 + 1 (N_0 natural number) since it is expected from the model that structures with less than N_0 monomers do not form conducting pores. A reasonable number for N_0 might be 2. Thus, the radius r of the cylinder and its cross-section A can be expressed as function of N:

$$r(N) = \frac{N \cdot \delta l}{2 \cdot \pi} \quad (3)$$

and

$$A(N) = \pi \cdot r^2 = \frac{\delta l^2}{4 \cdot \pi} \cdot N^2. \quad (4)$$

If it is further assumed that the conductance $g(N)$ of the pore in state N depends linearly on the cross-section, one finds

$$g(N) = g_0 \cdot N^2 \quad (5)$$

for the pore conductance and

$$i(N) = i_0 \cdot N^2 \quad (6)$$

for the current through the pore, where g_0 and i_0 are constants, and for the spacing between the conductance levels $g(N)$ and $g(N + 1)$:

$$g(N + 1) - g(N) = g_0 \cdot (2 \cdot N + 1). \quad (7)$$

For comparing the experimentally found data with the theoretical model it is useful to rewrite Eq. (6) in terms of M:

$$i(M) = i_0 \cdot (N_0 + M)^2. \quad (8)$$

In the experiments we observe within the bursts jumps from a base current level i_0, which is set for convenience to 0, to higher levels i_M. The experimentally found sequence i_1, i_2, i_3 . . . was tested against Eq. (8) by fitting the two parameters i_0 and N_0 using a least-squares routine and calculating theoretical values for the currents of the consecutive levels with these parameters. From this fit the following parameters were found: N_0 = 1, i_0 = 1.32 pA. As can be seen from Fig. 4*b* a reasonable fit of the model to the experimental data is achieved with deviations between experimental and theoretical values in the range of a few percent.

However, it should be emphasized once more that a marked increase of the amplitude of consecutive steps according to Eq. (8) occurs only at a low frequency, suggesting that the model outlined in Fig.

9*b* is an idealization and that the more realistic case should be that of noncircular, not entirely protein-lined complement lesions for which a relationship between channel size and number of C9 molecules in the C5b-9 complex is expected to occur with an exponent <2 (*see* Eq. (5)) as discussed by Malinski and Nelsestuen (1989) and Ramm et al. (1985).

The concept that pore formation requires binding of more than one C9 molecule per complex is in accordance with previous work demonstrating a requirement for binding of multiple C9 molecules to C5b-8 for expression of bactericidal activity (Joiner et al., 1985; Bhakdi et al., 1987).

The low frequency of occurrence of downward current steps indicates that the pore is stable as a consequence of an irreversible aggregation. This is in contrast to pore formation by, e.g., alamethicin, in which both uptake and release of monomers from the aggregate take place (Boheim, 1974).

The intensive current noise after formation or insertion of a small number of pores has previously been described (Benz et al., 1986). Since this noise is far too large as to result simply from transport noise it should originate from rapid transitions between various conductance levels of the pore. Such fluctuations may, according to Malinski and Nelsestuen (1989), occur especially in pores which are not entirely protein lined.

The experiments with different LPS show that the activation of complement at this membrane is widely independent of the specific composition of the polysaccharide moiety, differing largely for LPS R595, Rz and R5, e.g., having different terminal sugars as Kdo, heptose and glucose, respectively.

It has been discussed that the differences in serum sensitivity of rough-mutant and wild-type Gram-negative strains may result from phospholipid domains in the outer leaflet in the case of the former (Joiner et al., 1982*a*,*b*). The existence of such phospholipid domains, proposed by Nikaido and Vaara (1985), would facilitate the insertion of terminal complement complexes due to the higher fluidity of these domains as compared to the surrounding LPS. Our results, however, that transmembrane pores were generated by complement in a membrane which was composed on one side exclusively from deep rough-mutant LPS shows that the presence of phospholipid is not necessary for the generation of pores by complement.

To accumulate data on the role of O-antigen in the activation of complement, in particular on the importance of the primary carbohydrate structure of the O-polysaccharide (Liang-Takasaki, Mäkelä & Leive, 1982) we will attempt to form asymmetric membranes from LPS with more complete O-antigen structures.

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