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Complement Activation by Bacterial Surface Glycolipids: A Study with Planar Bilayer Membranes

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Abstract. Planar asymmetric glycolipid/phospholipid bilayer membranes were used as a reconstitution model of the lipid matrix of the outer membrane of Gram-negative bacteria to study complement (C) activation by various bacterial surface glycolipids with the aim of defining the C activation pathway. As glycolipids the lipopolysaccharides of *Salmonella enterica* serovar Minnesota R mutant strains R595 (Re LPS) and R4 (Rd_2 LPS), pentaacyl lipid A from the LPS of the *Escherichia coli* Re mutant F515, and glycosphingolipid GSL-1 of *Sphingomonas paucimobilis* IAM 12576 were used. Methylester and carboxyl-reduced derivatives of GSL-1 were used to elucidate the role of the carboxyl group as common functional group of LPS and GSL-1 for C activation. The formation of lytic pores was monitored *via* the measurement of changes in membrane current. For all glycolipids we observed a considerable increase in membrane current soon after addition of whole human serum due to the formation of lytic pores in the membranes. Pore formation was dependent on the presence of C9, indicating that the observed current changes were due to C activation.

We found that in our reconstitution system of the outer membrane lipid A, Re LPS, and Rd_2 LPS activated the classical pathway, the activation being independent of specific anti-LPS antibodies. In contrast, GSL-1 and the methylester derivative of GSL-1 activated the alternative pathway even at the low serum concentrations used in this study (about 0.2% v/v). Interestingly, the carboxyl reduced GSL-1 activated the classical pathway.

Key words: Complement — Lipopolysaccharide — Glycosphingolipid — Planar lipid bilayer — Outer membrane — Gram-negative bacteria

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Introduction

The complement (C) system consists of at least 20 serum proteins and plays an important role in the early host defense against invading bacteria (Morgan, 1995). Its activation can lead to the direct lysis of bacteria *via* the formation of transmembrane pores built up by the alignment of C9 monomers (Bloch et al., 1987; Tschopp, Engel & Podack 1984; Bhakdi & Tranum-Jensen, 1983). The C system can be activated along at least two distinct, the classical and the alternative pathways. Both pathways can be initiated with or without the participation of antibodies (Lachmann & Hughes-Jones, 1984). For C activation by carbohydrates, a Ca^{2+}-dependent lectin pathway is discussed, which is initiated after binding of collectins such as the mannan-binding protein (MBP) (for review *see* Holmskov et al., 1994; Matsushita, 1996). Thus, this protein would act like C1q in the classical pathway. Gram-negative bacteria activate C mainly by the surface glycolipids anchored to the outer leaflet of the outer membrane. Thus, the C cascade is activated by surface components (Cooper, 1993) and directs its lytic activity against the surfaces of invading bacteria (Bhakdi et al., 1987). In particular, the formation of the membrane attack complex, which finally leads to cell lysis, is catalyzed by the bacterial surface.

The lipid matrix of the outer membrane is extremely asymmetric with respect to the distribution of the lipids and glycolipids forming the two opposing leaflets. Whereas the inner leaflet is composed of a mixture of phospholipids, the outer leaflet is assumed to be built up exclusively of glycolipids, usually lipopolysaccharides (LPS) (Nikaido & Vaara, 1985; Rietschel et al., 1988; Rietschel et al., 1994). LPS consists of a poly- or oligosaccharide portion of varying length, depending on the bacterial mutant strain, which is covalently linked to lipid A which anchors the molecule to the membrane.

Enterobacterial lipid A consists of a β-(1′→6)-interlinked D-GlcN disaccharide backbone which is phosphorylated in positions 1 and 4′ and carries in ester and amide linkage up to seven hydroxylated and nonhydroxylated saturated fatty acid residues (Rietschel et al., 1996; Zähringer, Lindner & Rietschel, 1994). LPS and lipid A are membrane bound toxins and display a variety of pathophysiological activities in mammals and are, therefore, termed endotoxins (Westphal, Westphal & Sommer, 1977).

Recently, it was found that in the outer membrane of the Gram-negative species *Sphingomonas paucimobilis* IAM 12576 (formerly *Pseudomonas paucimobilis*) LPS is completely substituted by glycosphingolipids (GSL) (Kawahara et al., 1991). In GSL, the hydrophobic moiety is heterogeneous with respect to the dihydrosphingosine residue, but is in each case quantitatively substituted by an amide-bound (S)-2-hydroxymyristic acid at the dihydrosphingosine. The sugar moieties of the two main fractions found in *Sphingomonas paucimobilis*, GSL-4A and GSL-1, consist of Man-Gal-GlcN-DlcA and GlcA, respectively, which are α-linked to the lipid portion (Kawahara et al., 1991).

The activation of the classical pathway by dispersed endotoxin aggregates was found to depend on the physicochemical properties of the applied preparations such as the aggregate size of the lipids (Wilson & Morrison, 1982; Fulop, Webber & Manchee, 1993; Galanos & Lüderitz, 1976; Oshima, Soma & Mizuno, 1993). Therefore, the study of C activation requires comparability of the physicochemical properties of the various activating surfaces. This requirement is fulfilled when planar membrane reconstitution models are used. However, only few investigations into C action on planar bilayer systems have been performed, and in all these studies symmetric phospholipid bilayers have been used (del Castillo et al., 1966; Barfort, Arquilla & Vogelhut, 1968; Wobschall & McKeon, 1975; Michaels et al., 1976; Benz et al., 1986; Young & Young, 1990; Shiver, Dankert & Esser, 1991). C activation was either achieved by reactive lysis (Michaels et al., 1976; Benz et al., 1986; Young & Young, 1990; Shiver et al., 1991) with isolated C5b6 or by sensitization or modification of the phospholipid membranes with certain antigens such as crystallized albumins and addition of antibodies (del Castillo et al., 1966; Wobschall & McKeon, 1975; Jackson, Stephens & Lecar, 1981).

In previous publications (Schröder et al., 1990; Wiese et al., 1996), we have introduced a reconstitution model of the lipid matrix of the outer membrane of Gram-negative bacteria as an asymmetric planar bilayer membrane to study the activation pathway of the C system by membrane glycolipids and details of the formation of lytic pores *via* electrical measurement of changes of the transmembrane current (membrane conductance). Limited or even contradictory information is available on the role of polar headgroups in glycolipids responsible for the initiation of the antibody-independent C activation along the two pathways. Our model, now, resembles in composition and geometry the outer membrane to a far extent, because the membrane-forming lipid molecules are arranged in a lamellar array with the polar glycolipid headgroups responsible for activation facing the aqueous phase containing serum or serum components.

In earlier studies utilizing this asymmetric planar membrane system, we found that C is activated by the LPS of *Salmonella enterica* sv. Minnesota strains R595 (Re mutant), Rz (Rd_1 mutant), and R5 (Rc mutant) (Schröder et al., 1990) as well as by GSL-1 of *Sphingomonas paucimobilis* IAM 12576 (Wiese et al., 1996). We could, furthermore, show that Re LPS activated C along the classical pathway and that not only circular pores (these pores are completely surrounded by C9 monomers), but also leaky patches (these lesions are aligned only on one side by C9 monomers and the other side is constituted by the lipid matrix of the membrane) were formed.

The present study aims at defining the activation pathway for GSL-1 but also for LPS with longer core structures and the functional groups responsible for C activation. To this end, we have investigated C activation by surfaces made from Re LPS and Rd_2 LPS as well as pentaacyl lipid A from LPS of *Escherichia coli* strain F515 and GSL-1.

Since one common feature of the chemical structures of Re LPS and GSL-1 is the presence of a negatively charged carboxyl group, it seemed reasonable to study its influence on C activation. Therefore, we have synthesized carboxyl-reduced and methylester derivatives of GSL-1 and investigated C activation by membrane surfaces composed of these derivatives.

Materials and Methods

Lipids and Other Chemicals

Re LPS and Rd_2 LPS were extracted from *Salmonella enterica* sv. Minnesota (*S. minnesota*) strains R595 and R4, respectively (chemical structures *see* Fig. 1), by the phenol/chloroform/petroleum ether method (Galanos, Lüderitz & Westphal, 1969), purified, lyophilized and used in the natural salt form (Re LPS) or the triethylamine (TEN) salt form (Rd_2 LPS). In the latter case, the TEN salt form was a prerequisite for solubility in chloroform. Pentaacyl lipid A was derived from *Escherichia coli* Re mutant strain F515 (Zähringer et al., 1996) and used in the TEN salt form. In this lipid A, the secondary fatty acids from acyl-oxyacyl residues in position 2 of the reducing (16:0) and position 3′ (14:0) of the non-reducing glucosamine, respectively, found in *S. minnesota* lipid A (Fig. 1) are lacking.

GSL-1 was extracted from *Sphingomonas paucimobilis* IAM 12576 with chloroform/methanol (Kawahara et al., 1991) and eluted from a silica gel column by stepwise increase in methanol content.

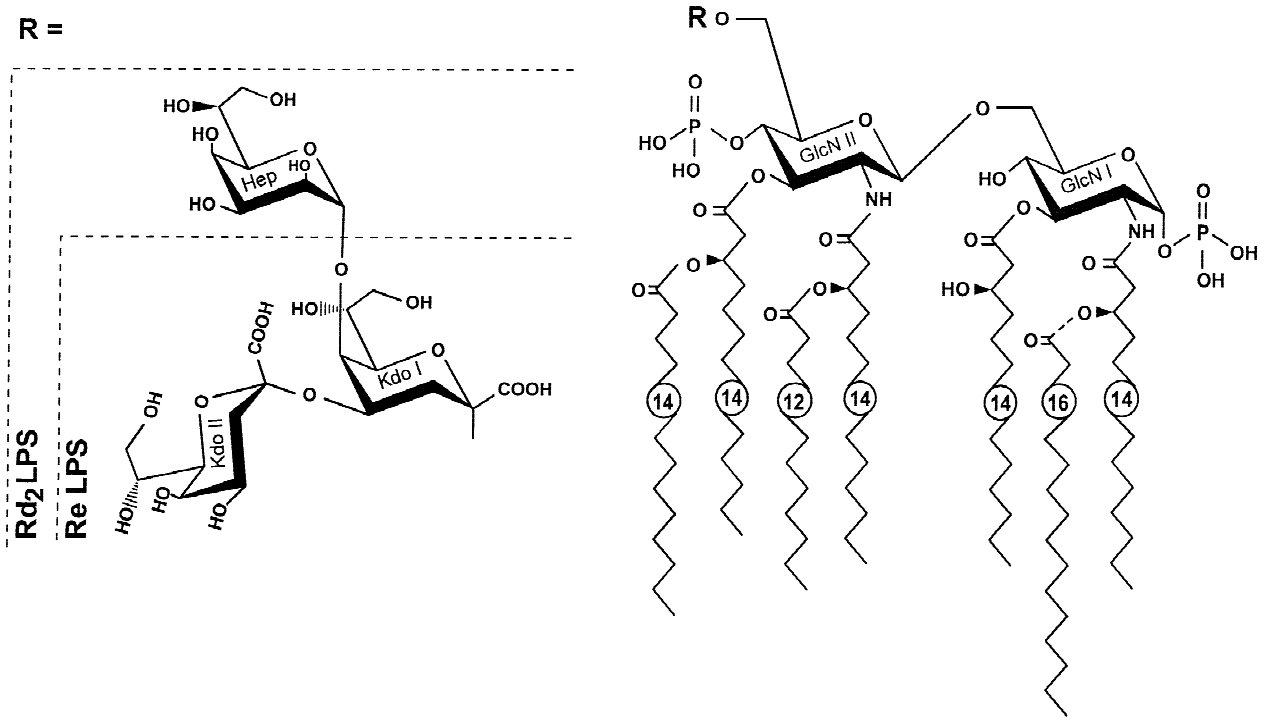

Fig. 1. Chemical structures of Re and Rd_2 LPS from *Salmonella enterica* serovar Minnesota strains R595 and R4, respectively. The right part shows the lipid A structure (R = H). The dashed line indicates a non-stoichiometric substitution by the 16:0 fatty acid residue. Hep: L-*glycero*-D-*manno*-hepto-pyranose; Kdo: 3-deoxy-D-*manno*-oct-2-ulosonic acid.

LPS was not present in these preparations as was confirmed chemically by the lack of β-hydroxylated fatty acids and Kdo. The hydrophobic moiety of GSL-1 is heterogeneous with respect to the occurrence of different sphingosine derivatives, the two main of which are *erythro*-1,3-dihydroxy-2-amino-octadecane (R_1) and *erythro*-1,3-dihydroxy-2-amino-*cis*-13,14-methylene-eicosane (R_2), which are present in a ratio of approximately 1:4 (*see* Fig. 2, left).

GSL-1 was derivatized with respect to the carboxyl group of the glucuronic acid. In one derivative (GSL-1 Me, Fig. 2, center), the carboxyl group was methylated, in the other (GSL-1 red, Fig. 2, right) it was reduced. The modification was performed according to chemical standard derivatization procedures. Briefly, GSL-1 of *S. paucimobilis* (40 mg, free acid) was transformed to the methyl ester with etheral diazomethane. The resulting methylester derivative of GSL-1 was purified on a small silica gel column (1 × 12 cm) by stepwise elution with chloroform-methanol with increasing polarity whereby 26.7 mg pure GSL-1 Me was obtained. One aliquot of GSL-1 Me (17.5 mg) was dissolved in 22 ml of methanol-water (10:1, by vol.) to which 35 mg $NaBH_4$ were added and reduction was allowed to proceed for 24 hr at 4°C. The reduced GSL-1 derivative containing a terminal Glc (GSL-1 red) was purified on a silica gel column as described above whereby 16.7 mg GSL-1 red were obtained in pure form. The structural analysis by NMR spectroscopy and MALDI- and GC-MS of both derivatives revealed that during derivatization of the glycosyl moiety neither the sphinganine nor the fatty acid part in the GSL-1 molecule had been affected.

Phosphatidylethanolamine (PE) from bovine brain (type I), phosphatidylglycerol (PG) from egg yolk lecithin (sodium salt), and diphosphatidylglycerol (DPG) from bovine heart (sodium salt) were purchased from Sigma (Deisenhofen, Germany). All lipids were used without further purification.

For membrane formation, the phospholipids (0.74 mg/ml) were dissolved in chloroform, glycosphingolipids (0.73 mg/ml) in chloroform/methanol (10:1 v/v) at room temperature, and LPS (2.6 mg/ml) in chloroform/methanol (10:1 v/v) and heated to 95°C for 5 min.

Membranes were prepared (*see below*) in bathing solutions containing 0.1 M KCl, 5 mM $MgCl_2$, and 5 mM HEPES, adjusted with KOH to pH 7.4 at a temperature of 37°C. The specific electrical conductivity was 16.3 mS cm^{-1} at 37°C. In some cases, a bathing solution containing 0.1 M KCl, 2 mM $MgCl_2$, 8 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), and 5 mM HEPES (EGTA-bathing solution) adjusted to pH 7.4 was used.

Sera and Complement Components

Whole human serum (WHS) was prepared from the blood of healthy volunteers. Calcium-depleted WHS (EGTA-WHS) was obtained by dilution (1:10) of WHS in EGTA-bathing solution. Heat-inactivated serum (HI-WHS) was prepared by incubation of WHS at 56°C for 30 min. Human sera depleted of components C1q or C9 (C1qdepHS or C9depHS, respectively) were purchased from Sigma (Diesenhofen, Germany), factor B depleted human serum (BdepHS) was from Calbiochem (San Diego, CA). Purified complement factor B was purchased from Sigma and used without further purification. All sera were diluted 1:10 before addition to the bathing solution. In the case of BdepHS, 3 mM $CaCl_2$ were added.

As antiserum the rabbit antiserum K236d58 (Swierzko et al., 1993) against Rd_2 LPS was used. The antiserum was obtained from blood drawn 8 days after the last boost. Before use, the C system of the antiserum was inactivated by incubation at 56°C for 30 min. Antibody titers were determined in the passive hemolysis test (Brade et al., 1987).

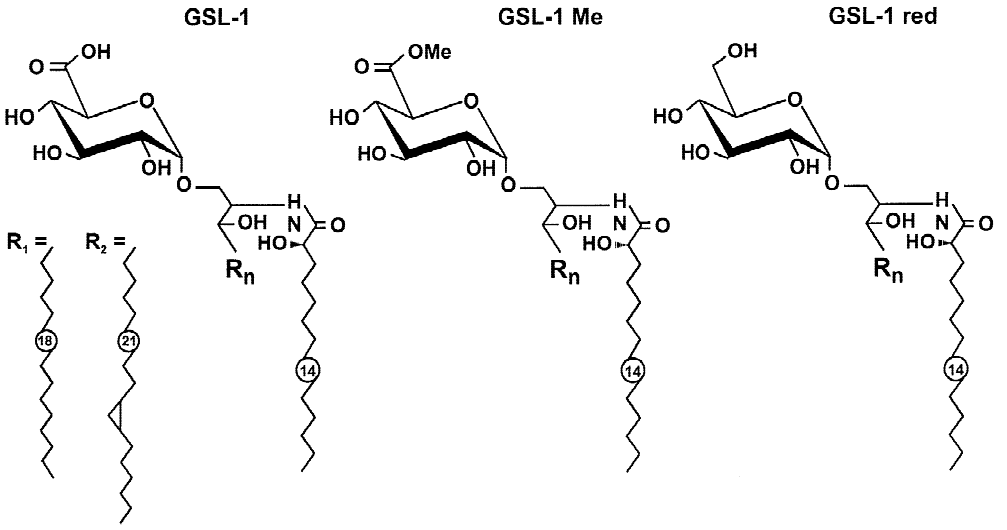

Fig. 2. Chemical structures of the glycosphingolipid GSL-1 from *Sphingomonas paucimobilis* IAM 12576 (left) and its carboxyl-methylated (GSL-1 Me, center) and carboxyl-reduced (GSL-1 red, right) derivatives.

Preparation of Planar Bilayers and Electrical Measurements

The preparation of virtually solvent-free asymmetric planar bilayers according to the Montal-Mueller technique (Montal & Mueller, 1972) has been described previously (Seydel, Schröder & Brandenburg, 1989). Briefly, asymmetric bilayers were formed by apposing two lipid monolayers prepared on aqueous bathing solutions from chloroformic solutions of the lipids at a small aperture (diameter (125 ± 5) μm) in a thin Teflon septum (12.5 μm thickness). The apparatus for membrane formation consisted of two Teflon compartments of 1.5 ml volume each. Prior to membrane formation, the septum was pretreated with a hexane/hexadecane mixture (20:1 v/v).

For electrical measurements, planar membranes were voltage-clamped *via* a pair of Ag/AgCl-electrodes (type IVM E255, Advanced Laboratory Research, Franklin, MA) which were connected with the headstage of a BLM 120 bilayer membrane amplifier (Biologic, Claix, France) with a feedback resistor of 1 GΩ. For the conductance measurements, a voltage of 20 mV was applied to one electrode, and the other was grounded. Membrane current and clamp voltage were stored using a DAT-tape recorder (DTR 1200, Biologic). The stored signals were sent to the microcomputer system filtered by a 4-pole low-pass Bessel filter (Ithaco Scientific Instruments, Ithaca, NY), the corner of which was adjusted to 10 Hz (−3 dB) and digitized at a sampling rate of 10 Hz with a PCI 20428W-2 multipurpose card (Intelligent Instrumentation, Leinfelden, Germany). Current was positive, when cation flux was directed towards the grounded compartment. For C activation measurements, sera were added to the grounded compartment, giving the indicated final dilutions followed by 40 seconds of stirring with magnetic bars.

For the reconstitution of the outer membrane we used the glycolipids on one side and a phospholipid mixture (PL) composed of PE (phosphatidylethanolamine), PG (phosphatidylglycerol), and DPG (diphosphatidylglycerol) in a molar ratio of 81:17:2 on the other resembling the phospholipid composition of the inner leaflet of the outer membrane of *S. enterica* sv. Typhimurium (Osborn et al., 1972).

For the formation of lipid A/PL bilayers, instead of hexaacyl lipid A, which is the main lipid A fraction of most enterobacterial LPS, pentaacyl lipid A was used, because this preparation guaranteed a sufficient degree of acyl chain mobility (fluidity) at 37°C (Brandenburg, Kusumoto & Seydel, 1997) which is a prerequisite for membrane stability.

Results

In our studies we utilize an experimental membrane model to determine the lytic C activity induced by different glycolipid surfaces as an increase of membrane current/conductivity. To obtain comparable and reproducible results for the C activation, in all experiments WHS from only one batch has been used. Thus, the activity should depend only on the composition of the membrane surface facing the side to which complement was added. To compare the lytic activity triggered by the different glycolipid surfaces, the ‘activation time’ and the ‘activation rate’ were introduced. Their meaning is explained in Fig. 3 for the example of activation of WHS by an asymmetric Re LPS/PL bilayer. Some minutes after addition of WHS to the LPS side, a rapid increase in membrane current is observed. The activation rate is given by the slope of the current increase and is taken as a measure of C activity. In many cases, however, in particular when membranes were formed from GSL-1 and its derivatives, no macroscopic current response could be observed prior to spontaneous membrane rupture after C activation. Furthermore, in particular bilayers made from the derivatives showed an overall lower stability and were thus very sensitive to structural rearrangements during pore formation. In these cases, the ‘activation time’, which is defined as difference between the time of serum addition ($t = 0$ sec) and the time at which the current at a clamp voltage of 20 mV exceeds 100 pA, provides information on C activity (Fig. 3, in-

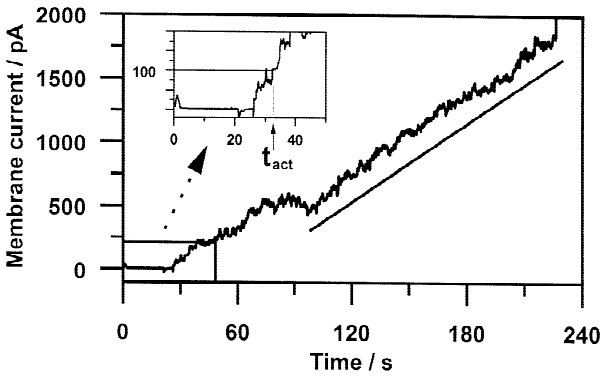

Fig. 3. Macroscopic current increase in dependence on time after addition of 0.1% whole human serum to the lipopolysaccharide side of an asymmetric Re LPS/PL membrane. Bathing solution: 0.1 M KCl, 5 mM $MgCl_2$, 5 mM HEPES, pH 7.4, T = 37°C. Serum was added at time t = 0 sec. The activation time (t_{act}) is defined as the time at which the current exceeds 100 pA (*see* insert: detail of the initial phase).

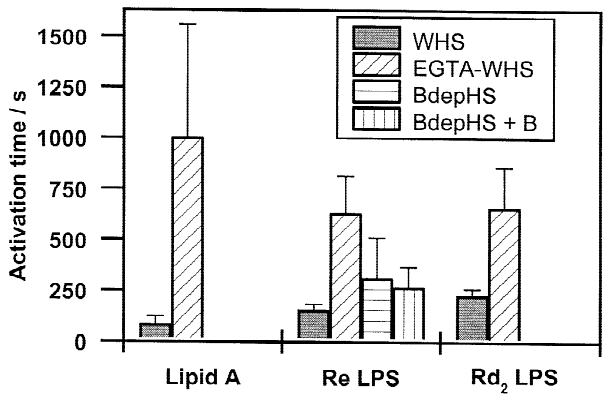

Fig. 4. Activation times for pentaacyl lipid A/PL, Re LPS/PL, and Rd_2 LPS/PL membranes after the addition of WHS or EGTA-WHS to the endotoxin side of the bilayer. For Re LPS, also the activation times after the addition of BdepHS and in the absence and presence of factor B are depicted. Serum concentrations were 0.1% (pentaacyl lipid A), 0.3% (Re LPS), and 0.4% (Rd_2 LPS).

sert). In the absence of serum or when no C activation occurred, this value represents the 'natural' lifetime of the membranes. In those cases, however, in which C activation is observed, the activation time is the total time required for diffusion of the C proteins to the lipid bilayer surface, initiation of the activation cascade, and formation of the first C pore (leading to a current increase of at least 100 pA).

Negative Controls

To test whether the observed current changes and the activation time are indeed C-dependent, we added C9-depleted serum (C9depHS) to each membrane system as negative control. In these experiments, no increase in transmembrane current was observed. Thus, the activation time represented in each case the natural lifetime of the membranes. Similar observations were made with heat-inactivated WHS (HI-WHS).

Complement Activation by Endotoxin Surfaces and Determination of Activation Pathway

In an earlier study on C activation by asymmetric Re LPS/PL bilayers, we found that Re LPS activates C in a Ca^{2+}-dependent manner, i.e., along the classical pathway. Here, we extended the studies to asymmetric membranes, where the activating surface was built up from lipid A or Rd_2 LPS. Activation pathways were determined by selective blocking of one of the pathways, the classical by chelation of Ca^{2+} ions with EGTA from the serum and the alternative pathway by depletion of factor B.

In Figure 4, the results for the activation times for the various endotoxin/PL bilayers exposed to different sera are summarized. The data obtained for Re LPS (concentration of sera 0.3%) clearly show that removal of Ca^{2+} (EGTA-WHS) leads to inactivation. Serum depleted of factor B (BdepHS) showed a slightly lower activity than WHS which could not be enhanced by addition of purified factor B to reach near physiological concentration of 250 μg/ml in the serum (BdepHS+B).

To test the influence of the composition of the sugar moiety of endotoxin, we also studied C activation by lipid A and Rd_2 LPS (Fig. 4). After addition of 0.1% WHS to the bathing solution on the lipid A side of a lipid A/PL bilayer, a rapid current increase was observed leading to a mean activation time of 90 sec. The addition of EGTA-WHS did not result in current changes, and the activation time was approximately 1000 sec. Also in the case of Rd_2 LPS/PL bilayers, the addition of WHS to the LPS side resulted in membrane current increase. As in the case of lipid A, C activity could be inhibited significantly by chelation of Ca^{2+}. C1qdepHS had no effect on lipid A, Re LPS, or Rd_2 LPS. The additional positive control using BdepHS was performed only with Re LPS (Fig. 4) as representative for all endotoxins tested, which could all be shown to activate the classical pathway. It should be noted that WHS concentrations required for the induction of comparable activation times differed for the three different bilayer surfaces. They were 0.1% for lipid A, 0.3% WHS for Re LPS, and 0.4% for Rd_2 LPS. At serum concentrations $\lesssim$0.5%, the Re LPS/PL bilayers were usually stable enough to allow the observation of a macroscopic current increasing about linearly over time. In these cases, a linear relation between the activation rate and the serum concentration was found (Fig. 5, solid line). The activation time decreased with increasing serum concentration (data not shown). At the highest serum concentrations, in a few experiments a superlinear

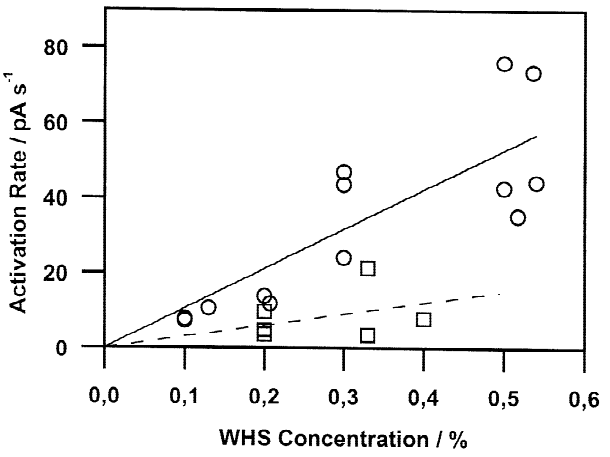

Fig. 5. Activation rates with linear fits in dependence on serum concentration on the glycolipid side for Re LPS/PL (circles, solid line, R = 0.85) and GSL-1/PL bilayers (squares, dashed line, R = 0.3). Bathing solution: 0.1 M KCl, 5 mM $MgCl_2$, 5 mM HEPES, pH 7.4, T = 37°C.

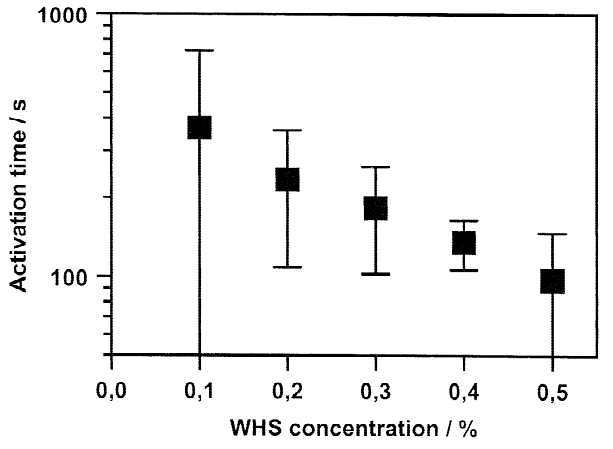

Fig. 6. Activation time in dependence on serum concentration on the GSL side for GSL-1/PL bilayers. Bathing solution: 0.1 M KCl, 5 mM $MgCl_2$, 5 mM HEPES, pH 7.4, T = 37°C.

increase of membrane current over time was observed. For Rd_2 LPS/PL bilayers, at a WHS concentration of 0.4% an activation rate of (6.1 ± 3.7) pA sec^{-1} was found (*data not shown*).

To exclude that the different C activities induced by Re LPS and Rd_2 LPS were caused by different antibody titers of the sera, we studied the influence of antibodies on C activation. The antibody titers were determined to 64 for Re LPS antibodies and to below the detection limit for those directed against Rd_2 LPS. Therefore, we added 3 μl of rabbit antiserum K236d58 directed against Rd_2 LPS 3 min after membrane formation and 2 min prior to WHS addition (3 μl) to the LPS side of Rd_2 LPS/PL bilayers. However, neither the activation rate nor the activation time were influenced by the presence of the antibodies (*data not shown*).

Complement Activation by GSL-1 and GSL-1 Derivatives

As pointed out before, asymmetric GSL-1/PL bilayers tended to break in an early phase after serum addition. Thus, in most experiments it could be observed only from a first increase of membrane current that activation took place, and only a few membranes were stable enough to allow the observation of a linear current increase with time. In those cases, the dependence of the activation rate on WHS concentration was not as pronounced as observed for Re LPS/PL membranes, and, furthermore, the activation rates for GSL-1 were overall lower (Fig. 5, squares). The activation time showed a significant decrease over serum concentration (Fig. 6), and the activation time at comparable concentrations was as high as for Re LPS/PL membranes (Fig. 4). For very low serum concentrations ([WHS] <0.1%), the activation time was determined by the natural lifetime of the membranes, for higher concentrations it decreased exponentially with serum concentration. For very high serum concentrations ([WHS] >0.5%), the activation time approached the minimal time required for serum addition and stirring. To define the pathway along which GSL-1 activates C, we used EGTA-WHS (depleted of Ca^{2+}) and C1qdepHS serum instead of WHS. In these experiments, serum concentration in the bathing solution was 0.3%. For the depleted sera, no increase in activation time was observed as compared to that for WHS (Fig. 7). C1qdepHS, on the contrary, led to a decrease in activation time. This might be explained by *a priori* not necessarily equal activities of C1qdepHS and WHS. It should, furthermore, be mentioned that even the chelation of calcium ions from C1qdepHS did not result in a higher activation time (*data not shown*). These results are indicative of C activation by GSL-1 along the alternative pathway.

To further confirm these results, we performed additional experiments with serum depleted of factor B (BdepHS), because factor B is an essential component of the alternative pathway. Addition of BdepHS (0.3%) to the GSL-1 side of GSL-1/PL bilayers did not induce any lytic activity. Activity of BdepHS could, however, be restored by the addition of purified factor B in a concentration of 250 μg per ml of added serum corresponding to the physiological concentration of factor B (Fig. 7). To study the influence of the negative charge represented by the carboxyl group of GSL-1 on C activation, we used derivatives of GSL-1, in which the carboxyl group was either methylated (GSL-1 Me) or reduced (GSL-1 red).

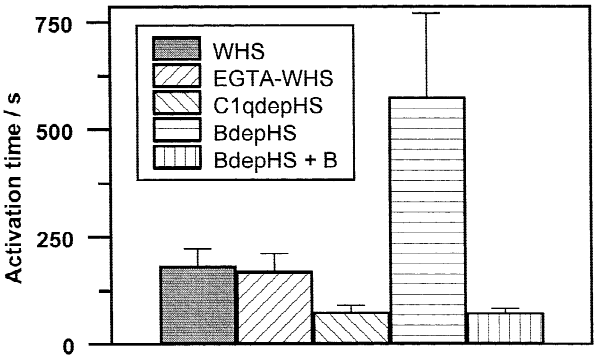

Fig. 7. Activation time for different sera on the GSL side of GSL-1/PL bilayers. Concentration of sera: 0.3%. Bathing solution: 0.1 M KCl, 5 mM $MgCl_2$, 5 mM HEPES, pH 7.4, T = 37°C.

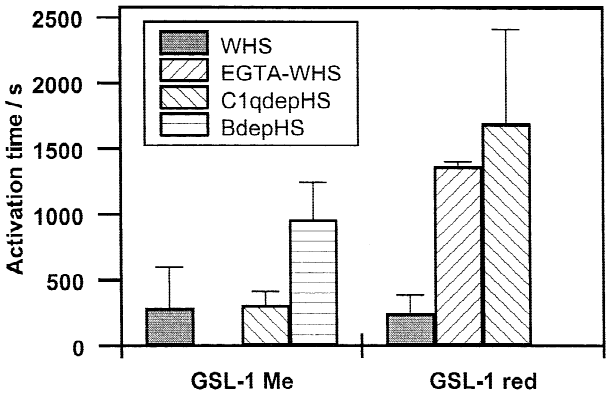

Fig. 8. Activation time for different sera on the GSL side of GSL-1 Me/PL (left) and GSL-1 red/PL bilayers (right). Concentration of sera: 0.2%. Bathing solution: 0.1 M KCl, 5 mM $MgCl_2$, 5 mM HEPES, pH 7.4, T = 37°C.

Both derivatives activated WHS, and the activation times were of the same order as in the case of native GSL-1 (Figs. 6 and 8).

In subsequent experiments, we have determined the activation pathways for the two GSL-1 derivatives. In the case of GSL-1 Me (Fig. 8, left panel), C1qdepHS had the same activity as WHS, whereas BdepHS led to a significantly higher activation time. These results are clearly indicative of an activation along the alternative pathway. For GSL1-red, the activation time was significantly prolonged, when C1qdepHS or EGTA-WHS were used and was comparable to the natural lifetime of the membranes (Fig. 8, right panel), thus indicating an activation along the classical pathway. Additional positive controls as, for instance, experiments with BdepHS reconstituted with purified factor B in the case of GSL-1 ME or with BdepHS in the case of GSL-1 red were not performed, because the experiments described above already allowed an unequivocal identification of the pathway.

Discussion

The addition of WHS to the glycolipid side of asymmetric glycolipid/phospholipid bilayers resulted in a considerable increase in membrane current (Fig. 3) and a decrease in the activation time as compared to the natural lifetime of the membranes (Figs. 4, 7 and 8) for all glycolipids tested. In each case, increases in membrane current occurred only in the presence of C9 which has previously been described to insert into the target membrane and form lytic pores (Bhakdi & Tranum-Jensen, 1983; Zalman & Müller-Eberhard, 1990; Schröder et al., 1990). The pore-forming activity of the serum could be completely abolished by incubating the serum at 56°C for 30 min. This treatment is known to lead to a complete inactivation of the C system and gives further evidence that the observed current changes are C-dependent.

For a comparison of the C-activating capabilities of the different glycolipids, we determined the activation rates and the activation time. For Re LPS/PL bilayers, the activation rates were found to be proportional to serum concentration (Fig. 5, solid line). For different endotoxin preparations, we found that the activation decreased from lipid A — which showed the highest C activation — with increasing core sugar chain length (lipid A > Re LPS > Rd_2 LPS), taken from the increasing serum concentration required to obtain comparable activation times (Fig. 4). Thus, at a given WHS concentration (0.4%), the activation rate observed for Rd_2 LPS/PL bilayers (6.1 ± 3.6 pA/s) was significantly lower than that obtained from the fit in Fig. 5 for Re LPS/PL bilayers (42 pA/s).

First experiments with LPS from *Salmonella enterica* sv. Minnesota strain R5 (Rc chemotype) (Rc LPS) on the glycolipid side of the asymmetric membranes provided evidence for a still lower C activating capacity of this LPS which would thus fit in the above sequence.

For the glycosphingolipid GSL-1/PL bilayers, the activation rates were lower than that observed for Re LPS/PL bilayers at all WHS concentrations (Fig. 5). Obviously, the activation rate for GSL-1/PL bilayers does not show a pronounced dependence on serum concentration. However, the activation time, i.e., the difference between the time of serum addition and the time at which the current becomes larger than 100 pA, showed an exponential dependence on WHS-concentration (Fig. 6). At a WHS concentration of 0.3%, the activation time was comparable for GSL-1/PL and LPS Re/PL bilayers (Figs. 4 and 7). The different behavior of the two bilayer systems with respect to the activation rate and the activation time may be indicative of different kinetics of C activation by the two glycolipids.

The derivatization of the carboxyl group of GSL-1 by either methylation (GSL-1 Me) or reduction (GSL-1 red) did not influence the C activating capacity of the glycolipid (Figs. 7 and 8). From this observation it may be concluded that the presence of a negative charge as represented by the carboxyl group is not a prerequisite for C activation. To further confirm this assumption further experiments with neutral glycolipids of various chemical structures (their synthesis is on the way) will be conducted.

For Re LPS, C activation was independent of the presence of factor B, but dependent on Ca^{2+} (Fig. 4). The reduced activity of BdepHS with or without addition of factor B is probably due to a reduction of classical pathway activity by the depletion procedure or to an *a priori* different activity of the original sera. Also for (penytaacyl) lipid A and Rd_2 LPS, activation was dependent on the presence of Ca^{2+} (Fig. 4). These results indicate that activation for all three endotoxin structures occurs along the classical pathway. As C1qdepHS was not activated by the endotoxin surfaces, an activation of the lectin pathway can be ruled out. These findings and the observation that with increasing length of the sugar moiety the C activating capability decreases, suggest that the recognition site for the classical pathway is located in the lipid A region, i.e., in the bisphosphorylated diglucosamine backbone as was proposed by Morrison and Kline (1977). This region, however, is progressively hidden with increasing number of core sugar components. To further elucidate this hypothesis, experiments with lipid A derivatives, in which the phosphate groups are either substituted by other negatively charged groups such as carboxylate or partly or totally removed, will have to be done. Our finding that Rd_2 LPS activates the classical pathway is in contradiction to previous work of Vukajlovich, Hoffman & Morrison (1987) who found that all LPS structures with a sugar moiety longer than that of Re chemotype LPS activate the alternative pathway. The additional L-*glycero*-D-*manno*-heptose in Rd_2 chemotype LPS was interpreted by these authors to have a critical recognition role for the activation of the alternative pathway and a downregulation of the classical pathway activation. For an understanding of this discrepancy, it has again to be considered that in the studies of Vukajlovich and coworkers (1987) LPS was applied in aggregated form in aqueous buffer. Therefore, the spatial arrangement of the target structure for the C proteins is poorly defined. Bogard et al. (1987), using monoclonal antibodies, showed that structural determinants located in the lipid A region are exposed to a higher degree on bacteria than on the surface of LPS aggregates. Planar bilayers as used in this study, however, resemble the geometry of the outer membrane to a high degree. Also Brade et al. (1987) found that the physicochemical state and the environment of lipid A modulated its antigenicity. Using liposome-incorporated instead of aggregated antigens as inhibitors in the passive-hemolysis inhibition assay, they could show that nonspecific reactions were avoided and specific ones were enhanced. Furthermore, Tenner, Ziccardi & Cooper (1984) could show that whole *E. coli* J5 bacteria which express an Rc chemotype LPS on their surface activate C1 and with that the classical pathway.

In earlier experiments with Re LPS/PL membranes, we could show that the removal of antibodies against Re LPS did not influence the C activity (Schröder et al., 1990). In the present study, we did respective experiments with Rd_2 LPS/PL membranes. However, since anti-Rd_2 LPS antibodies were not detectable in WHS, we incubated the membranes with anti-Rd_2 LPS antiserum (antibody titer 6400) prior to WHS addition. This procedure did not enhance C activity. This observation shows that in our test system the C activation by antibodies can be neglected in comparison to the antibody-independent C activation. These results confirm the findings of Schröder et al. (1990). The obvious irrelevance of antibodies in physiological concentrations in our system is most likely due to the low serum concentration used in these experiments or to the short time needed until current changes are observed. This time is probably not sufficiently long for an effective binding of antibodies to the membrane surface.

In the case of GSL-1/PL bilayers, neither the depletion of C1q nor the chelation of Ca^{2+} ions had an influence on the activation. Furthermore, serum from which factor B was depleted (BdepHS) showed no activity on GSL-1/PL bilayers (Fig. 7). Activity could be restored by addition of physiological concentrations of purified factor B. From these results it is concluded, that GSL-1 activates the alternative C pathway. Obviously, alternative pathway activation and the first steps of membrane attack occur even at low serum concentrations as used in this study (dilution 1:500), whereas the lysis of *E. coli* K12 W1485 was found to be completely inhibited by serum dilution by a factor of 16 or more (Schreiber et al., 1979). Alternative pathway activation by GSL-1 is surprising in the light of the situation observed for endotoxin preparations. The activation of the alternative pathway was found for LPS with longer sugar chains, whereas GSL-1 contains only one sugar. Also gangliosides with 5 or more monosaccharides were shown to activate the alternative C pathway (Oshima et al., 1993). This deviating behavior suggests, in agreement with Grossman & Leive (1984), that the chemical character of the sugar rather than the length of the sugar moiety determines the activation pathway.

The observation of C activating capability of the GSL-1 derivatives was surprising. It had been expected that the negatively charged carboxyl group is a necessary functional group for C activation. The experiments

aimed at defining the activation pathway, however, clearly showed that the C6 of the glucose plays a critical role: A carboxyl group (GSL-1) and its methyl ester (GSL-1 Me) in 6-position lead to alternative pathway activation, whereas a hydroxyl group ($-CH_2OH$; GSL-1 red) triggers the classical pathway (Fig. 8). These results suggest that not the presence of a negatively charged group but rather the chemical nature of the substituent determines the pathway of C activation.

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