Potent Inhibition of Viral Fusion by the Lipophosphoglycan of *Leishmania donovani*[†]

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ABSTRACT: Lipophosphoglycan (LPG) is an amphiphile produced by *Leishmania*. Its chemical structure consists of a hydrophilic flexible polymer of repeating PO_4-6Galβ1−4Manα1 units (on average 16 units) linked via a hexasaccharide core to a lyso-1-*O*-alkyl-PI membrane anchor. In the study of viral fusion we report in this paper, we have introduced LPG into human erythrocyte ghost (HEG) membranes, with the purpose of understanding how the LPG-induced surface-structural changes may modulate the interactions between a viral envelope and the HEG membranes. We have found that LPG, when incorporated at very low concentrations into intact human erythrocyte membranes, strongly inhibits Sendai virus-induced hemolysis. When incorporated into HEGs, it reduces the binding of both Sendai and influenza viruses to HEGs; furthermore, it strongly inhibits the overall viral fusion to HEGs, being among the most potent known inhibitors. We have also shown that LPG stabilizes the bilayer structure of phosphatidylethanolamine against the formation of an inverted-hexagonal structure. We suggest that LPG may give rise to an effective "steric repulsion" between the viral and HEG membranes, thereby modulating some specific modes of interaction between viral-target membranes in the overall fusion process; LPG may also modulate the bending rigidity and the spontaneous curvature of the HEG membrane in the direction of making the destabilization and rearrangement of the underlying lipid bilayer more difficult.

An enveloped virus has a lipid-bilayer membrane which provides a protective barrier for its nucleocapsid. The infectious entry of the virus into its host cell requires the fusion of the viral membrane with target membranes. In this paper, we focus on two representatives of enveloped viruses, Sendai virus and influenza virus. Sendai virus fuses with the plasma membrane of its host cell at neutral pH, while influenza virus fuses with the endosomal membrane upon the acidification of the endosomal lumen after endocytosis of the virion by its host cell (Marsh & Helenius, 1989).

It is widely accepted that specific molecules, such as viral membrane glycoproteins and viral receptors in host-cell membranes, play critical roles in initiating and facilitating viral fusion. However, the collective, structural, and physical properties of the viral and target membranes must influence the fusion process as well, for the following reasons. First, the functioning of specific molecules depends on the physical environment they are in; secondly, interactions between the viral and target membranes, such as electrostatic interactions and hydration forces (Parsegian & Rand, 1991), are involved in the process; and finally, the ultimate coalescence of the two membranes necessarily involves the cooperative structural rearrangement of a large number of membrane-lipid molecules.

Studies have been carried out to seek the relevance of the collective structural and physical properties of lipid-bilayer membranes to viral fusion. A number of amphiphiles which raise the bilayer to inverted hexagonal phase transition temperature (T_H)[1] of phosphatidylethanolamine (PE) have been demonstrated to inhibit viral fusion with human erythrocytes (and ghosts) as well as the fusion with model lipid-bilayer vesicles when introduced into these viral target membranes (Cheetham et al., 1990; Kelsey et al., 1990; Epand, 1992; Chernomordik et al., 1993; Cheetham et al., 1994, Yeagle et al., 1994).

In the study of viral fusion that we report in this paper, we have again emphasized modulation of the surface structural and physical properties of the viral target membrane. We have introduced a novel and natural membrane amphiphile, lipophosphoglycan (LPG), to human erythrocyte ghost (HEG) membranes. The purpose is to understand how the LPG-induced surface-structural changes may modulate the interactions between the viral and HEG membranes, the structural rearrangement of the lipid bilayers, and in turn, the overall viral fusion process.

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[1] Abbreviations: L_α, (bilayer) lamellar, liquid crystallaine phase; H_{II}, inverted hexagonal phase; T_H, L_α to H_{II} phase transition temperature; LPG, lipophosphoglycan; R18, octadecyl Rhodamine B chloride; PE, phosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; DEPE, dielaidoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; HEG, human erythrocyte ghost; MES, 2-(*N*-morpholino)-ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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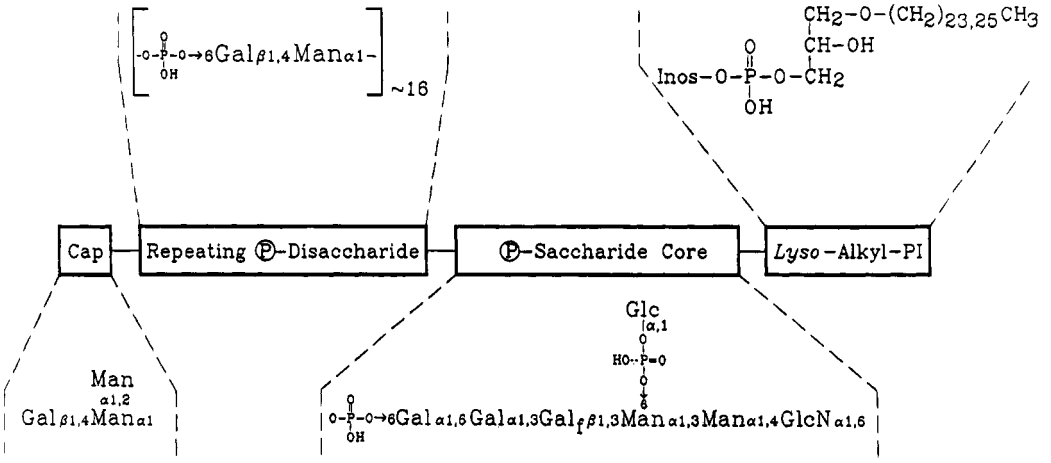

FIGURE 1: Structure of LPG from *L. donovani*.

Lipophosphoglycan is a major cell-surface component of the trypanosomaid *Leishmania donovani*, the etiologic agent of kala azar, which proliferates in hydrolytic environments throughout its digenetic life cycle (Turco & Descoteaux, 1992). Structurally, *L. donovani* LPG is unique among membrane glycoconjugates: It is a polymer of repeating phosphorylated disaccharide units (on average 16 repeats) of PO_4-6Galβ1−4Manα1 linked via a hexasaccharide carbohydrate core to a lyso-1-*O*-alkylphosphatidylinositol lipid anchor (Orlandi & Turco, 1987; Turco et al., 1987, 1989). Even though LPG is only a single chain amphiphile, this chain is composed of a C_{24} or C_{26} saturated, unbranched hydrocarbon, making LPG firmly embedded in the membrane (see Figure 1). LPG plays an important role in the survival of the *Leishmania* parasite at many stages of its life cycle. For example LPG renders *Leishmania major* resistant to complement-induced lysis, even though the binding of complement is not inhibited (Puentes et al., 1988). LPG also protects the *Leishmania* parasite from destruction by macrophages (Handman et al., 1986). It has also been found that LPG inhibits protein kinase C (PKC) activity (McNeely et al., 1989). This is also a property of neutral and zwitterionic amphiphiles which stabilize the bilayer structure of lipid systems by raising T_H[2] (Senisterra & Epand, 1993). Although LPG is neither neutral or zwitterionic, it may still inhibit PKC as a consequence of the fact that it is among the most potent substances in raising T_H of PE (see Results). All of the effects of LPG suggest that the substance has stabilizing effects on membrane bilayers. One might therefore anticipate that LPG would inhibit membrane fusion.

Viral fusion involves the binding of the virus to its target membrane (via the specific molecular bonding between viral glycoproteins and their receptors on the target membrane) and the subsequent, more direct interactions (localized destabilization of membrane bilayer structures and topological merger) between the viral and target membranes, leading to the final fusion. It is the theme of this paper to observe what effects the LPG has on these distinct steps. Such a study may help to elucidate details about the mechanism(s) by which enveloped viruses gain entry to cells, and inhibition of the fusion event may also prove to be a viable strategy for preventing viral infection of cells.

[2] In general, an additive which is able to raise the T_H of PE is defined to be a bilayer stabilizer in the sense that it stabilizes the L_α phase relative to the H_{II} phase. This is a term we will use throughout the text.

EXPERIMENTAL PROCEDURES

LPG. LPG was isolated and purified from *L. donovani* as previously described (Orlandi & Turco, 1987). The M_r of this product was taken as 9500 for calculating mol %.

Virus Preparations. The Cantell strain of Sendai virus was propagated in the allantoic sac of 10-day-old embryonated chicken eggs by incubation at 33 °C for 72 h. Virus was isolated by discontinuous sucrose gradient centrifugation. The virus was washed and the final preparation resuspended in HEPES-buffered saline, pH 7.4, at a viral protein concentration of 1 mg/mL. The virus was stored in the frozen state at −80 °C. Initial studies on influenza virus-induced hemolysis and on influenza viral fusion were done with strain A/Mississippi X-87, recombinant strain H3N2 which was inactivated with β-propiolactone. Subsequent studies and experiments to analyze the kinetics of influenza viral fusion and the effects of LPG were done with active influenza virus, strain A/PR/8/34 (H1N1) purchased from Spafas (Stoors, CT). Qualitatively similar results and effects of LPG were obtained with both strains of influenza virus.

Preparation of Liposomes. Routinely, DOPC and DOPE (Avanti Polar Lipids, Alabaster, AL) were dissolved in chloroform/methanol (2:1, v/v) at desired concentrations, and the solution was dried under a stream of nitrogen to deposit a thin lipid film on the inside of a Pyrex test tube. The film was further subjected to vacuum evaporation for 2−3 h to remove any trace of the solvent. Large unilamellar vesicles (liposomes) were prepared by hydrating the dried lipid film with the proper fusion buffer, then repeatedly freezing and thawing the suspension five times, and finally extruding it 10 times through two polycarbonate filters of pore size 0.1 μm (Nuclepore Filtration Products, Pleasanton, CA) using an Extruder (Lipex Biomembranes) at room temperature (Olson et al., 1979; Mayer et al., 1986). The prepared liposomes were stored at 4 °C until use (typically during the same day as the preparation).

Preparation of Human Erythrocyte Ghosts. The procedure followed that described by Steck and Kant (1974). Five milliliters of packed human erythrocytes was washed three times, each time with 20 mL of 10 mM HEPES and 150 mM NaCl, pH 7.4 (HBS), by centrifugation in an SS34

Sorval Rotor at 5000 rpm, at 4 °C for 5−10 min. The cells were then lysed in approximately 35 mL of 5 mM HEPES, pH 8.0 (lysis buffer), and the membrane fragments were pelleted at 10 000 rpm for at least 10 min at 4 °C and washed with the lysis buffer five or six times. The ghosts were resealed in 5 mM HEPES, 150 mM NaCl, and 1 mM $MgCl_2$, pH 7.4, for 45 min at 37 °C with gentle shaking. The sealed ghosts were then washed and resuspended in 5 mM HEPES, 150 mM NaCl, and 1 mM EDTA, pH 7.4 (HEPES buffer), and protein concentration was determined using the micro BCA assay (Pierce, Rockford, IL). The ghosts were diluted with the HEPES buffer to yield a final (ghost-protein) concentration of 1 mg/mL and stored in the refrigerator until further use.

Incorporation of the LPG into HEG Membranes, Erythrocytes, and Liposomes. In order to achieve an effective incorporation of the LPG into HEG membranes or erythrocytes, the ghosts or erythrocytes prepared according to the above procedure were further treated with papain (Eilam et al., 1985). The papain treatment greatly increased LPG incorporation, but the enzyme treatment itself had no effect on viral fusion. For ghosts not treated with papain, typically only less than 20% of the added LPG became bound to the ghost membrane. Papain treatment increased the extent of LPG incorporation to 80%. We also have done control fusion experiments where LPG was added directly to the system containing virus and untreated ghosts. The results showed that LPG in this case had no effect on viral fusion. The treatment was performed in the procedure described as follows: The ghost suspension (7.5 mL of 1 mg of ghost protein/mL) was mixed well with 625 μL of papain (in HBS buffer at 18 mg/mL) and 790 μL of cysteine (in HBS buffer at 1 mg/mL), and the mixture was then incubated at 37 °C for 30 min. The treated ghosts were pelleted and washed twice with the HEPES buffer by centrifugation in an SS34 rotor at 7000 rpm, at 4 °C for 10 min. The ghost pellet was resuspended in the HEPES buffer to a final concentration of approximately 1 mg/mL (ghost protein). The incorporation of the LPG into the ghost membrane was achieved by adding small aliquots of the LPG stock solution (LPG in double-distilled water) to the suspension of the papain-treated ghosts. The concentration of the LPG was either 10 or 20 μM, and the ghost (protein) concentration was 0.8 mg/mL. The mixture was incubated at 37 °C for 15 min to allow the partition of the LPG molecules into the ghost membrane. Unincorporated LPG molecules were then removed by centrifugation of the ghost membranes in an Eppendorf centrifuge and discarding the supernatant. Such a procedure typically yields a final LPG concentration in the ghost membrane of 1 or 2 mol % (with respect to the ghost phospholipids), as determined by a phosphate assay (Ames, 1966). These ghosts were then used as the target membrane for virus binding and fusion. Similarly prepared erythrocytes were used for the hemolysis study. The incorporation of LPG into liposomes was achieved through direct incubation of LPG with liposomes at a desired concentration.

Sendai Virus-Induced Hemolysis. For this purpose 10^8 erythrocytes were treated with 180 μg of papain and 100 μg of cysteine in 1 mL of HBS, followed by incorporation of LPG as described above. In the hemolysis assay, a small aliquot of Sendai virus stock solution, containing 5 μg of viral protein, was added to and mixed well with the prepared erythrocyte suspension (approximately 10^8 cells) to make a final volume of 500 μL in a microcentrifuge tube. The mixture was then placed on ice for 45 min. After this step the sample was incubated for 3 h in a gently shaking 37 °C waterbath. Following the incubation, the sample was microcentrifuged for 1 min. The supernatant was then removed, and its light absorbance at 540 nm was measured. The extent of hemolysis was determined by normalizing this absorbance with the absorbance of the supernatant from a sample containing cells which were completely lysed by sonication.

Binding of Virus to Human Erythrocyte Ghosts. Most of the binding experiments were performed at 4 °C to reduce fusion and neuraminidase activities of the Sendai viral HN protein. R18-labeled virus was incubated at neutral pH with the target ghosts for 20 min to allow for equilibration of binding, at the same concentrations as those used in the viral fusion assay (see below). Unbound virions were removed by centrifugation in an Eppendorf centrifuge at 4 °C. R18 fluorescence intensity from the pellet and supernatant fractions was measured to determine the fraction of virions bound to the ghosts.

Fusion of Sendai and Influenza Viruses with HEGs. Viral fusion was continuously monitored at 37 °C with the R18 fluorescence assay as described elsewhere (Hoekstra et al., 1984). Fusion of Sendai virus was initiated by rapid injection of R18-labelled virus ($\sim 7 \times 10^9$ virions) into a magnetically stirred cuvette containing 2 mL of the prepared ghost suspension at the desired number of ghosts (typically in the range of $(1-5) \times 10^8$ ghost particles) in 5 mM HEPES, 5 mM MES, 5 mM sodium citrate, 150 mM NaCl, and 1 mM EDTA, pH 7.4 (HEPES/MES/citrate buffer). For the influenza virus, a 10 or 20 min preincubation was done at pH 7.4 after the injection of R18-labeled influenza virus (10^{10} virions typically) into the 2 mL ghost suspension to allow for viral binding to the ghosts at neutral pH. Fusion was then initiated by rapid injection of a small aliquot of citric acid, which lowered the sample pH to 5.

Fluorescence was recorded using an SLM AMINCO Bowman Series 2 Luminescence Spectrometer interfaced with a 386/20 IBM compatible computer. The instrument used a xenon arc light source with a 560 nm band-pass filter (Oriel Corp., Stratford, CT) between the excitation slit and sample, and a 590 nm low-wavelength cutoff filter (Oriel Corp., Stratford, CT) between the sample and the photomultiplier tube to minimize any contribution of light scattering to the observed signal. The excitation and emission monochromators were set at 565 and 600 nm, respectively. Temperature was regulated using a circulating water bath and monitored with a thermocouple (Precision Digital, Needham, MA).

To test whether the papain treatment of the human erythrocyte ghosts alters the overall course of viral fusion in any significant way, we also performed control experiments using various virus-ghost systems. We employed Sendai virus and two strains of influenza virus, A/PR/8 and X31. The results of these control experiments are shown in Figure 2. The quantitative extent of the effect on viral fusion of this enzymatic treatment of the erythrocyte-ghost membrane depends on specifics of the viruses, but the effect on the initial rates is small in all cases. Thus the capacity of the erythrocyte ghost membrane for fusion with viruses largely survived the papain treatment.

Analysis of the Fusion Kinetics Data. With Sendai virus, the initial kinetics of the fusion reaction was monitored over

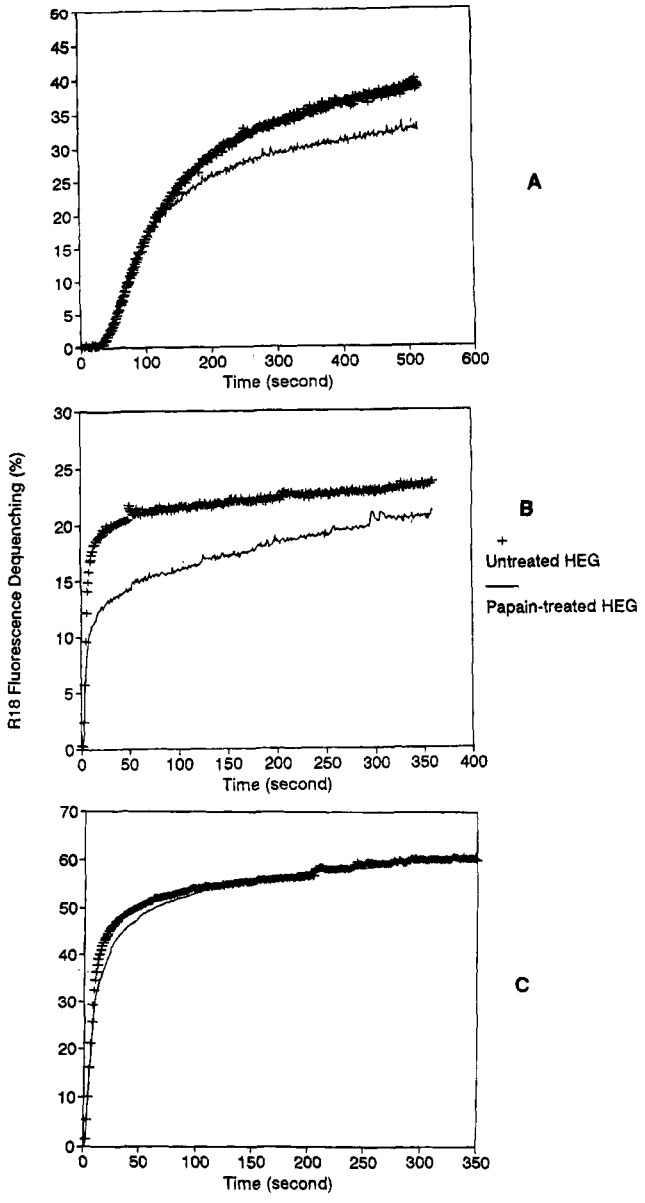

FIGURE 2: Effect of the papain treatment of HEGs on viral fusion with the HEGs. (A) Sendai virus, pH 7.4; (B) A/PR/8 strain influenza virus, pH 5.1; (C) A/X-31 strain influenza virus, pH 5.1.

a period of 10 min at several concentration ratios of virus to ghost. With influenza virus, the initial-kinetics data were taken for a period of 5 min after the acidification. The fraction of bound virions at the end of the prefusion binding period at neutral pH was measured. The final extent of fusion in each assay was measured at 1 or 2 h after the initiation of fusion. Duplicate measurements were made for the same preparations of R18-labeled virus and ghosts. Measurements were also repeated with different preparations of R18-labeled virus and ghosts. Representative runs of the initial-kinetics data were analyzed on the basis of the mass-action models developed for Sendai virus-ghost (Nir et al., 1986) and influenza virus-ghost fusion (Nir et al., 1990; Ramalho-Santos et al., 1993), respectively. Briefly, the Sendai virus-ghost fusion model views the initial kinetics as the result of three distinct processes: a second-order virus adhesion (binding) to ghosts, characterized by the rate constant C (M^{-1} s^{-1}); a first-order virus dissociation from ghosts, described by the rate constant D (s^{-1}); and a first-order fusion of an adhered virion (measured as R18 dilution from the viral to the target membrane), represented by the fusion rate constant f (s^{-1}). The influenza virus-ghost fusion model includes the effect of the loss of viral fusion activity upon the exposure to low pH (inactivation) (Nir et al., 1988; Stegmann et al., 1989). This model employs three kinetic rate constants for the prebound virus: f, γ_1, and γ_2, of which the latter two represent forward and reverse rate constants of the inactivation, respectively (Nir et al., 1990). The kinetics during the first few seconds after the initiation of fusion is dominated by the intrinsic rate constant f; the effect of the inactivation process becomes appreciable at later times, when the fusion kinetics becomes much slower, and is eventually governed by the rate constant $f = f(0)\gamma_2/(\gamma_1 + \gamma_2)$. The optimal values of these parameters are determined from a best fit to the data. Model calculations can yield predictions for additional cases (Nir et al., 1988, 1990; Düzgünes et al., 1992; Ramalho-Santos et al., 1993).

Differential Scanning Calorimetry (DSC). Lipid films were made from DEPE dissolved in chloroform/methanol (2:1, v/v). After solvent evaporation with nitrogen, final traces of solvent were removed in a vacuum chamber for 90 min. The lipid films were suspended in 20 mM PIPES, 1 mM EDTA, and 150 mM NaCl with 0.002% NaN_3, pH 7.40, and added LPG by vortexing at 45 °C for 30 s. The final lipid concentration was 10 mg/mL. The lipid suspension was degassed under vacuum before being loaded into an MC-2 high sensitivity scanning calorimeter (Microcal Co., Amherst, MA). A heating scan rate of 39 °C/h was generally employed. The observed phase transitions were independent of scan rates between 10 and 60 °C/h. The bilayer to hexagonal phase transition was fitted using parameters to describe an equilibrium with a single van't Hoff enthalpy (Sturtevant, 1987) and the transition temperature reported as that for the fitted curve.

RESULTS

Effect of LPG in Raising T_H. The transition temperature T_H of aqueous suspensions of dielaidoylphosphotidylethanolamine (DEPE) is very sensitive to the presence of certain additives in the suspensions (Epand, 1985). Cholesterol phosphorylcholine (CPC) has been shown to be a bilayer stabilizer that inhibits viral fusion (Cheetham et al., 1994). This amphiphile raises the T_H by 286 °C/mole fraction of CPC. By comparison LPG was about 10-fold more effective on a molar basis in raising T_H (Figure 3), but it was only slightly more effective on a weight basis. The results demonstrate that on a molar basis LPG is extraordinarily effective in raising T_H at very small mole fractions. This property has been found to be associated with the inhibition of membrane fusion. However, one cannot quantitatively compare the shift in T_H of DEPE caused by LPG with its effect on inhibiting membrane fusion to liposomes or erythrocyte ghosts. The bilayer-stabilizing effects of LPG were dependent on the presence of its large (polymeric) polar and anionic headgroup. If the LPG is depolymerized to remove the repeating units by the treatment with 0.02 N HCl for 5 min at 100 °C, its remaining fragment (hexasaccharide core linked to lyso-1-*O*-alkyl-PI) lowered T_H (Figure 3).

Inhibition of Sendai Virus-Induced Hemolysis by LPG. Virus-induced hemolysis is related to viral fusion with erythrocytes (Bodian et al., 1993) although with Sendai virus

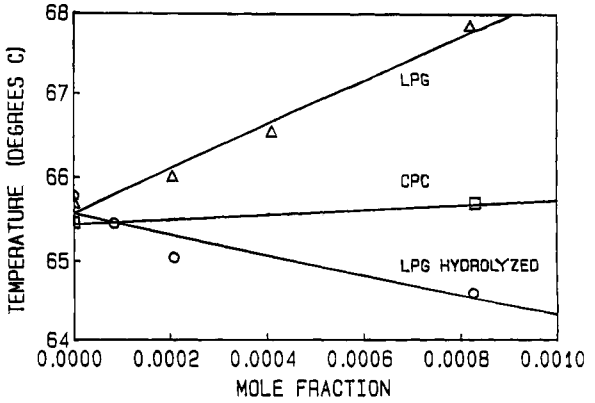

FIGURE 3: Effect of LPG (△), LPG hydrolyzed for 5 min at 100 °C with 0.02 N HCl to hydrolyze the repeating disaccharide units (○), and CPC which is cholesteryl phosphorylcholine (□) on T_H of DEPE at pH 7.4. The T_H is determined from DSC heating scans and is plotted against the mole fraction of amphiphile added to the membrane.

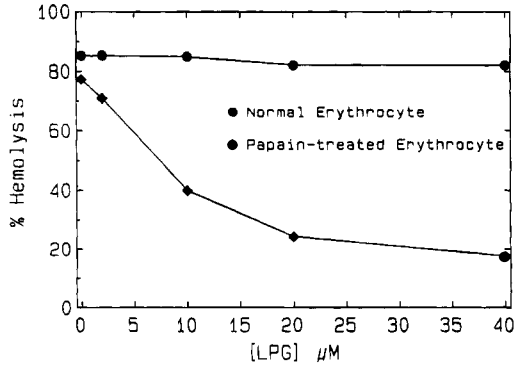

FIGURE 4: Effect of LPG on Sendai virus-induced hemolysis. The virus concentration is 10 μg of viral protein/mL; the erythrocyte concentration is 10^8 cells/mL. The effective incorporation of LPG into the erythrocyte membrane, achieved through the papain treatment of the erythrocytes leads to the inhibition of the hemolysis (◆); LPG does not partition into the membrane of untreated erythrocytes, having no effect on the virus-induced hemolysis (●).

it has been shown that only virus preparations that have been frozen and thawed are hemolytic (Shimizu et al., 1976). The extent of Sendai virus-induced hemolysis was greatly reduced when a low amount of LPG was incorporated into the erythrocyte membrane (Figure 4). If the erythrocytes are not subject to the papain treatment, no LPG was incorporated into the membrane and there was no inhibition of hemolysis even though LPG was present in the system throughout the hemolysis process (Figure 4). The amount of erythrocyte membrane protein in the hemolysis assay is estimated to be about one-tenth that used in fusion assays with ghosts. Thus, the effects of LPG on hemolysis are not directly comparable to its inhibitory effect on fusion which is often greater (Bodian et al., 1993).

Inhibition of Binding of Sendai and Influenza Viruses to Human Erythrocyte Ghosts by LPG. The interactions governing the process of the binding of a Sendai virion to its receptor-bearing target membrane at low temperature, e.g., 4 °C, involve primarily the specific binding of the HN glycoprotein to sialic acid residues on the receptors, but nonspecific physical forces may also play a role (see Discussion). The binding of influenza virus to its receptor-containing target membranes at neutral pH is a similar process, only that the molecular binding is between the viral HA protein (which is also directly involved in the actual

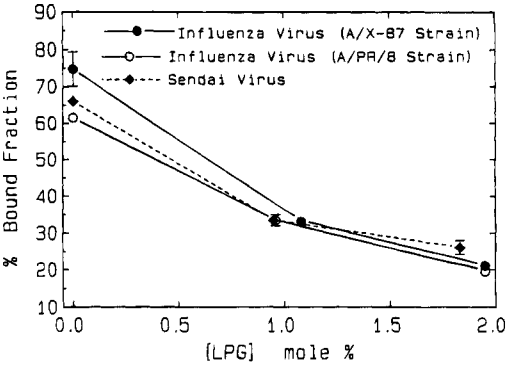

FIGURE 5: Effect of LPG on viral binding to papain-treated HEGs at pH 7.4. The virus concentration is 5 μg (viral protein) per 2 mL, and the ghost concentration is 200 μg (ghost protein) per 2 mL. The concentration of LPG incorporated into the HEG membrane is given in units of mol % with respect to the mole number of ghost-membrane phospholipids. Binding of influenza virus to HEGs was studied at 37 °C, and that of Sendai virus to HEGs was measured at 4 °C.

membrane fusion event, i.e., the bilayer destabilization and the topological merger) and sialoglycoconjugate receptors. We directly measured the relative number of virions in the bound state.

LPG, when incorporated into ghost membranes at very low mole fractions, significantly reduced virus binding in a concentration-dependent manner (Figure 5). At only 2 mol % of LPG, the reduction was approximately 60% (compared to the control case). The extent of this reduction in binding was similar for both Sendai virus and influenza virus. Such a significant extent of inhibition of viral binding has not been observed in previous cases where other fusion-inhibiting amphiphiles or anionic polymers interacting with lipid bilayers through adsorption were used to study viral binding (Cheetham et al., 1994; Ohki *et. al.*, 1992).

Inhibitory Effect of LPG on Viral Fusion with Human Erythrocyte Ghosts and Virus Receptor-Free Liposomes. The presence of LPG in HEG membranes had a dramatic inhibitory effect on viral fusion with human erythrocyte ghosts. This inhibition occurred for both viruses studied, despite differences in assay conditions (e.g., difference in pH).

Figures 6 and 7 show the dose-dependent inhibition of viral fusion by LPG incorporated in the HEG membrane, as measured through R18 fluorescence dequenching, for two different systems: the Sendai virus-ghost system and the A/PR/8-strain influenza virus-ghost system. As unequivocally demonstrated by these experimental observations, LPG is potent in arresting the initial fusion reactions in all these systems at very low concentrations, typically 2 mol % (with respect to the ghost phospholipids). We have also found a similar inhibition by LPG of the fusion of the X87-strain influenza virus with HEGs. The observed fusion rates for Sendai virus are determined by virus binding to target membranes, the fusion rate constant, and the final extent of fusion. In the case of influenza where the virus is prebound, the principle factors determining the rate are the fusion rate constant, the final extent of fusion, and the rate of viral inactivation, the latter parameter being particularly important at longer times. The rate constants for these various processes are quantitatively analyzed (see next section).

Influenza virus also fuses effectively with liposomes composed of certain glycerophospholipids, in the absence

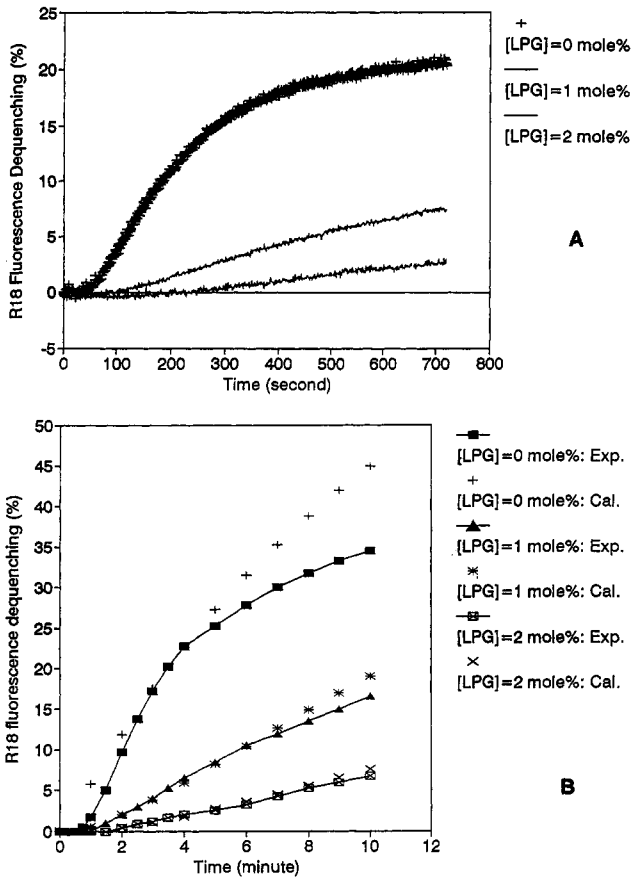

FIGURE 6: LPG dose-dependent inhibition of Sendai viral fusion with papain-treated HEGs at pH 7.4 and 37 °C. The virus concentration is 5 μg (viral protein) per 2 mL, and the ghost concentration is 50 (A, top) or 200 (B, bottom) μg of ghost protein per 2 mL: (A) Set of typical curves of initial kinetics of Sendai viral fusion with HEGs, as monitored by R18 fluorescence dequenching assay. Fusion with HEG not containing LPG is the upper curve marked with (+). The middle curve is for 1 mol % LPG and the lower curve for 2 mol % LPG. A horizontal line is shown at 0% dequenching. (B) Experimental data (Exp) and their numerical fit (Cal). The various rate constants employed in the simulations to generate the numerical fit are given in Table 1.

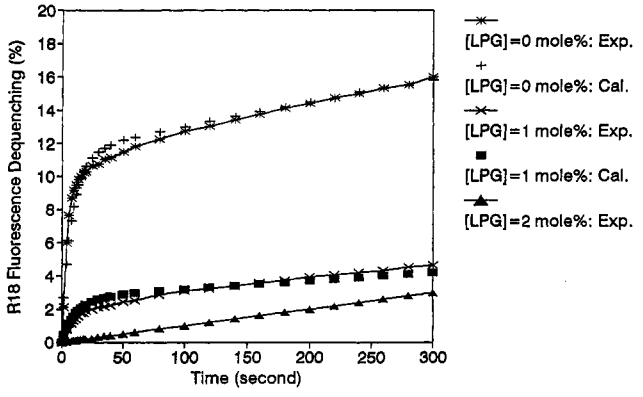

FIGURE 7: LPG dose-dependent inhibition of the low-pH-induced influenza viral (A/PR/8 strain) fusion with papain-treated HEGs at 37 °C. The virus concentration is 5 μg (viral protein) per 2 mL, and the ghost concentration is 200 μg (ghost protein) per 2 mL. Fusion was initiated by lowering the pH of the buffer to 5.1. The experimental data (Exp) of the initial fusion kinetics were fitted numerically (Cal), using those rate constants given in Table 1.

of viral receptors (Nir et al., 1988, 1990; Stegmann et al., 1989). We have also studied the effect of LPG on the fusion reaction between influenza virus and liposomes composed of DOPE/DOPC (3:1). Again, the low pH-induced fast fusion was completely abolished when LPG was present in the membrane of the liposomes at a very low concentration (data not shown). Thus the inhibitory effect of LPG also operates in the absence of viral receptors in the target membrane.

Analysis of the Kinetics of Viral Fusion. A brief description of the mass-action models developed to describe kinetic processes of viral fusion with biological membranes is given in the Experimental Procedures. The results of the analysis are summarized in Table 1. It is apparent from the analysis that the first-order fusion step *per se* is the one affected most dramatically by the presence of LPG in the HEG membrane: 2 mol % LPG in the HEG membrane leads to one to two orders-of-magnitude reduction in the fusion rate constant characterizing this first-order kinetic step. Other kinetic steps involved are also affected. For example, the rate constant C, which characterizes the second-order adhesion process of Sendai virus to human erythrocyte ghosts, was reduced by a factor of 2, a result which is consistent with the observations from our studies of viral binding (adhesion) to human erythrocyte ghosts.

DISCUSSION

This study was aimed at investigating the effect of the *Leishmania* LPG as an inhibitor of viral fusion. The principal finding is that LPG, when incorporated into HEG membranes, leads to an unprecedented inhibition of overall fusion of both Sendai and influenza viruses with human erythrocyte ghosts. The glycoconjugate also strongly modulates those cooperative physical properties which determine the relative stability of lamellar and hexagonal phases and which also may be relevant to the viral fusion process.

A number of steps in viral fusion are affected by LPG. The most dramatic effect is on the fusion rate constant, f, which is lowered 100-fold for the fusion of Sendai virus with HEG by the incorporation of only 2 mol % LPG into the HEG membrane (Table 1). It may be noted that, in the cases studied here of Sendai virus fusing with HEG, the outcome of fusion is most sensitive to f values during the first 2−5 min. After 10 min the effect of addition of LPG on the ratio between the values of fluorescence intensity increase is reduced. On the other hand at 3 min the extents of virions fusing with HEG are reduced by about 20-fold by the inclusion of 2% LPG in the HEG membranes. At shorter times the ratios between the corresponding extents of fusion are larger, but the relative experimental uncertainty is also larger. The kinetics of fusion is also affected by the kinetics of virus adhesion to the HEG, which is reduced by about 2-fold in the presence of 2% LPG. The LPG content is expressed as a percent of erythrocyte phospholipid, and it should be noted that LPG is incorporated only into the outer monolayer of the membrane. Weightwise, 1 or 2 mol % of LPG corresponds to a fraction of approximately 5 or 10%, respectively, of the total weight of the ghost membrane, as erythrocyte phospholipid is only about half of the total membrane weight and LPG has a molecular weight (9500), roughly 10 times that of the average weight of a membrane phospholipid. Clearly LPG is an effective inhibitor of viral fusion when present as only a minor component of the membrane. In the case of influenza virus, the corresponding reduction in f is 15-fold. For both Sendai and influenza virus, there is also a reduction in q, the percent of virions capable

Table 1: Effects of LPG on the Kinetics of Viral Fusion with Human Erythrocyte Ghosts

Sendai virus-ghost fusion[a]					influenza virus-ghost fusion[b]				
[LPG] (mol %)	f (s^{-1})	C (M^{-1} s^{-1})	D (s^{-1})	q (% of virus fusing)	[LPG] (mol %)	f (s^{-1})	γ_1 (s^{-1})	γ_2 (s^{-1})	q (% of virus fusing)
0	0.100	6×10^9	0.001	85	0 (exp. 1)	0.06	0.1	0.002	43
					0 (exp. 2)	0.08	0.06	0.002	30
					0 (average)	0.07	0.08	0.002	37
1	0.005	3.5×10^9	0.001	73	1 (exp. 1)	0.02	0.075	0.002	38
					1 (exp. 2)	0.022	0.015	0.003	15
					1 (average)	0.02	0.045	0.0025	27
2	0.001	3.5×10^9	0.001	73	2	0.004	$\leq 10^{-5}$		18

[a] See Nir et al. (1986) for a detailed description of calculations. The estimated uncertainties are 10%, 20%, 30%, and 50% in q, C, f and D, respectively. [b] See Nir et al. (1990) for details of computational procedure. The estimated uncertainties are 20%, 20%, 70% and 70%, for q, f, γ_1, and γ_2. For the last case (2% LPG) γ_2 could not be determined.

of fusing. It has been found in several cases that a lower extent of fusion occurs if the virions which are bound in a nonfusogenic manner are also not rapidly released from this binding site, or else they are likely to fuse at another site (Nir et al., 1990; Ramalho-Santos, Pedroso de Lima, and Nir, unpublished results). In addition, in the case of influenza virus there is also a marked decrease in the rate constant of low pH inactivation, γ_1 (Table 1). These effects of LPG are another example of the fact that the rate constants of fusion and inactivation of influenza virus fusing with target membranes respond in a similar way to changes in conditions (Ramalho-Santos et al., 1993). In the latter study it was suggested that the observed concomitant changes in f and in γ_1 with pH and temperature were a result of a common rate-limiting step involving a rearrangement of the viral hemagglutinin protein at the contact points with the target membrane.

Several properties of LPG may contribute to its inhibitory activity. The presence of small mole fractions of LPG, but not of its hydrolysis products, in the DEPE dispersion in excess water markedly raised the bilayer to H_{II} phase transition temperature of this lipid system (Figure 2). Several other compounds which raise T_H also inhibit viral fusion (Cheetham et al., 1990, 1994; Kelsey et al., 1990; Epand, 1992; Chernomordik et al., 1993; Vogel et al., 1993; Yeagle et al., 1994). Membrane fusion has been modeled as the formation of a stalk between two juxtaposed membranes which then expands radially. This expansion results in contact between the two monolayers which were oriented trans to the two monolayers that formed the initial contact (Siegel, 1993). Calculations of the energetics of fusion intermediates have suggested that the transmonolayer contact is inhibited by agents which reduce negative curvature strain (Siegel, 1993). This may also be a result of placing a polymer at the surface of the membrane. It has been shown (de Gennes, 1990) that a layer of polymer molecules adsorbed onto a lipid monolayer has two principal effects on the mechanical properties of the lipid monolayer: (1) It leads to the rigidification of the original monolayer, i.e., the modified surface has a larger bending rigidity; (2) it tends to bend the monolayer away from the side of polymer adsorption. Bending the monolayer away from the polymer induces positive curvature, opposite to the monolayer curvature required for membrane fusion. The fact that LPG effectively raises T_H suggests that it induced positive monolayer curvature.

In addition to the effects of LPG in inhibiting lipid rearrangements required to form fusion intermediates, the ability of LPG to relieve negative curvature strain may also inhibit structural rearrangements of the viral fusion proteins. Functional activities of a number of other proteins such as alamethicin (Keller et al., 1993), rhodopsin (Gibson & Brown, 1993), and protein kinase C (Senisterra & Epand, 1993) are also inhibited by membrane components that raise T_H, perhaps also by making the bilayer more stable and thereby inhibiting rearrangements of proteins within the membrane. This would explain why both the fusion and inactivation rate constants are strongly inhibited by LPG (Table 1) in analogy with the inhibition of both of these rates by lower temperature and higher pH (Ramalho-Santos et al., 1993). It is known that an acid-induced conformation change of the HA protein leads to both membrane fusion and virus inactivation. Since in the presence of target membrane part of the HA protein interacts with this membrane, this conformational change will be affected by the nature of this membrane. In addition it is possible that LPG provides a steric barrier to fusion. Studies of the three-dimensional structure of LPG reveal that while the molecule exhibits limited mobility about the Galβ1−4Man linkages, a variety of stable rotamers exist about the Manα1−PO_4−6Gal linkages (Homans et al., 1992). These torsional rotations confer upon the LPG molecule a large number of conformational degrees of freedom and the structural flexibility to contract or expand, resulting in a molecule whose length is potentially 9 nm when fully contracted and 16 nm when fully expanded, if an average of 16 repeats is assumed. The large configurational entropy resulting from this molecular structure is decreased when some forms of the conformation of the incorporated LPG molecules are disfavored by the proximity of another surface, e.g., a viral membrane surface, opposing the LPG-anchoring surface. This decrease in entropy can lead to an effective "steric" repulsion between the two interacting membranes. However, the observation that LPG lowers the adhesion rate constant, C, by 2-fold does not contradict this inhibitory mode of action of LPG, since this steric effect may not only influence the energetics but also alter the structural details of the binding of virus to the target membrane. For example, the virus may be bound to the target membrane at a location far away from the lipid bilayer. This lipophosphoglycan also lowers the percent of viruses capable of fusing, q. This may be a result of LPG interacting with the virus causing it to bind in a state that does not lead to fusion at the point of contact or that LPG reduces the probability of other regions of the virus forming contacts with the target membrane at other points. We have also tested the effect of truncated forms of LPG made by partial acid hydrolysis of the phosphoglycan portion of the molecule. These fragments were less effective in inhibiting

viral fusion. They would be expected to offer less steric hindrance, but they also are less potent in raising T_H (unpublished observations).

The molecular mechanism by which LPG inhibits viral fusion remains to be firmly established. It is clear, however, that LPG is a very effective inhibitor of viral fusion and is particularly effective in lowering the fusion rate constant even when present at very low mole fractions in the membrane. Since LPG adversely affects the fusogenic capacities of membranes, this may be a natural role of LPG in protecting *Leishmania* parasites from digestion in host lysosomes through inhibition of the fusion of parasite membranes with the host phagosomal membranes. Furthermore, in addition to inhibiting membrane fusion, LPG has been suggested to preclude macrophage activation through inhibition of protein kinase C (Descoteaux & Turco, 1993). This action of LPG may also be a consequence of this lipophosphoglycan altering the membrane physical properties which modulate the activity of this enzyme (Epand & Lester, 1990; Zidovetzki & Lester, 1992).

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