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Factors Affecting Surface Expression of Glycolipids: Influence of Lipid Environment and Ceramide Composition on Antibody Recognition of Cerebroside Sulfate in Liposomes†

Simon J. Crook,‡ Joan M. Boggs,*,‡ Arnt Inge Vistnes,§ and Kalavelil M. Koshy‡

Department of Clinical Biochemistry, University of Toronto, and Department of Biochemistry, Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8, and Institute of Physics, University of Oslo, Blindern, Oslo 3, Norway

Received June 2, 1986; Revised Manuscript Received August 1, 1986

ABSTRACT: The reactivity of the acidic glycolipid cerebroside sulfate (CBS) with antibody was studied as a function of its lipid environment in vesicles and of its ceramide composition. The lipid environment was varied by using phosphatidylcholine of varying chain length with cholesterol in a phosphatidylcholine:cholesterol:cerebroside sulfate molar ratio to glycolipid of 1:0.75:0.1. The ceramide structure of CBS was varied by using synthetic forms containing palmitic acid, lignoceric acid, or the corresponding α-hydroxy fatty acids. Reactivity with antibody was determined by measuring complement-mediated lysis of the vesicles containing a spin-label marker, tempocholine chloride. The data were analyzed by a theoretical model which gives relative values for the dissociation constant and concentration of antibodies within the antiserum which are able to bind to the glycolipid. If the phosphatidylcholine chain length was increased, increasing the bilayer thickness, only a small population of high-affinity antibodies were able to bind to cerebroside sulfate, suggesting decreased surface exposure of the glycosyl head group. A larger population of lower affinity antibodies were able to bind to it in a shorter chain length phosphatidylcholine environment. However, if the chain length of the cerebroside sulfate was increased, it could be recognized by more antibodies of lower affinity than the short chain length form, suggesting that an increase in chain length of the glycolipid increased surface exposure. Hydroxylation of the fatty acid inhibited antibody binding; only a smaller population of higher affinity antibodies was able to bind to the hydroxy fatty acid forms. This suggests that hydroxylation may decrease the surface exposure or alter the head-group conformation so that most of the antibodies in this polyclonal antiserum are unable to bind. Thus, changes in the lipid composition of plasma membranes may affect the surface exposure of glycolipids or the ability of receptors to bind to them and thus alter cell recognition.

Glycolipids are present in the plasma membrane of a great number of different types of cells. Their location and the existence of a multiplicity of different carbohydrate structures suggest that they play more than a structural role in the membrane. Indeed, glycolipids have been implicated in cell development, differentiation, and carcinogenic transformation (Hakomori, 1981). The ability of glycolipids to act as haptens and elicit immune responses suggests the possibility that they may also play a role in autoimmune diseases such as multiple sclerosis (Webb & Fazakerley, 1984; Offner et al., 1981). The expression or exposure of glycolipids on the cell surface may be altered in malignant tissue and different cells in cases where there is no change in glycolipid content (Young et al., 1981; Kannagi et al., 1982; Nudelman et al., 1982). It is therefore

† This investigation was supported by the Multiple Sclerosis Society and the Medical Research Council of Canada, by a Scientist Award from the Medical Research Council to J.M.B., and by a studentship from the Multiple Sclerosis Society to S.J.C.

* Correspondence should be addressed to this author at the Department of Biochemistry, Hospital for Sick Children, Toronto.
‡ University of Toronto and Hospital for Sick Children, Toronto.
§ University of Oslo.

0006-2960/86/0425-7488$01.50/0

important to understand how the carbohydrate moiety of glycolipids is exposed on the surface of membranes and what factors can influence this expression.

Studies of the reactivity with antibody of glycolipids and other lipid haptens in liposomes have shown that the membrane surface expression of the lipid head group is influenced by the surrounding lipid environment. The effect of fluidity and lateral mobility, varied by using different forms of phosphatidylcholine (PC)[1] with different transition temperatures in the presence or absence of cholesterol, on the reactivity of lipid haptens has been studied extensively (McConnell, 1978; Petrossian & Owicki, 1984; Stanton et al., 1984). The effect of bilayer thickness, varied by using synthetic forms of PC with fatty acid chains of different length or sphingomyelin (SM), in vesicles containing high cholesterol content (so that all the bilayers are in a similar state of fluidity) has also been studied (Alving & Richards, 1977; Alving et al., 1974; Shin et al., 1978; Utsumi et al., 1984; Inoue et al., 1971).

Although the glycosyl portion of a glycolipid defines its unique specificity to antibody, enzymes, or other receptors, its hydrophobic portion also may influence the availability of its carbohydrate structure at the membrane surface. Variations in the reactivity of different cells with antibody specific for certain glycolipids was associated with changes in the ceramide composition of the glycolipid and not its amount in the membrane (Kannagi et al., 1982). Cells in which the glycolipid had long chain length (Nudelman et al., 1982) or hydroxy fatty acids (Young et al., 1981) bound monoclonal antibody better than cells in which it had shorter chain length non-hydroxy fatty acids.

In intact cells, other changes in composition may occur simultaneously with changes in ceramide composition, making it difficult to determine the predominant factor affecting antibody binding. Study of antibody reactivity with model membranes of well-defined auxiliary lipid composition and a homogeneous form of the lipid hapten is a better way to determine the factors which affect lipid carbohydrate surface exposure. Such a study of model membranes containing synthetic forms of galactosyl cerebroside (Alving & Richards, 1977) also indicated that an increase in chain length of the glycolipid increases antibody binding. It was concluded that only high-affinity antibodies were able to bind to a shorter chain length form. Study of antibody reactivity with natural forms of α-L-fucopyranosylceramide (Yoshino et al., 1982) and lactosylceramide (Symington et al., 1984) also suggested that an increase in chain length of the glycolipid increases antibody binding. However, the ceramide structure of the glycolipids used in the latter two studies was more heterogeneous. The effect of hydroxylation of the ceramide on glycosyl surface expression in model membranes has not yet been reported although Hansson et al. (1983) found that a number of strains of bacteria adhered better to lactosylceramide if the ceramide portion was hydroxylated.

In the present study, we have determined the influence of both the lipid environment and of the ceramide composition of the acidic glycolipid, cerebroside sulfate on anti-cerebroside sulfate antibody reactivity with natural and synthetic forms of cerebroside sulfate of varying chain length and degree of hydroxylation. Antibody-dependent, complement-mediated lysis of liposomes formed of synthetic PC of varying chain length or SM, cholesterol, and cerebroside sulfate was measured from the release of a water-soluble spin-label, tempocholine chloride, as a marker.

Most of the previous studies of this problem, with the exception of that of Alving and Richards (1977), compared only the degree of the effect on different types of vesicles caused by antisera at a particular concentration or the antiserum concentrations required to cause the same degree of effect. Variations in either could be a result of differences in the affinity or concentration or both of the specific antibodies in the serum which are able to bind to different types of vesicles. In the present study, we have used a theoretical model developed to compare the relative concentrations and affinities of antibodies causing lysis of liposomes (Vistnes, 1984) in order to quantitatively assess the influence of lipid composition on glycosyl surface exposure and reactivity with antibody.

Materials and Methods

Lipids. Bovine brain cerebroside sulfate was prepared from an acetone-precipitated, ether-insoluble fraction rich in cerebroside and sulfatide, purchased from Avanti Polar Lipids Inc. (Birmingham, AL), by an adaptation of the method of Svennerholm and Thorin (1962). Synthetic forms of cerebroside sulfate (CBS) containing palmitic acid (NFA-P-CBS), α-hydroxypalmitic acid (HFA-P-CBS), lignoceric acid (NFA-L-CBS), and α-hydroxylignoceric acid (HFA-L-CBS) were prepared by deacylation of bovine brain CBS and reacylation as described (Koshy & Boggs, 1982, 1983). Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and distearoylphosphatidylcholine (DSPC) were purchased from Sigma Chemical Co., St. Louis, MO. Bovine brain sphingomyelin (SM) was from Avanti, and cholesterol was from Fluka (Buchs, Switzerland). Synthetic sulfated galactosyl diglyceride (3-*O*-[β-D-galactopyranosyl 3-sulfate]-2-*O*-hexadecanoyl-1-*O*-hexadecyl-L-glycerol) synthesized by Dr. R. Gigg, National Institute for Medical Research, London, England (Gigg, 1979), was provided by Dr. C. Lingwood, Hospital for Sick Children, Toronto.

Anti-Cerebroside Sulfate Antiserum. Male 5-kg rabbits were immunized by the procedure of Zalc et al. (1977) with minor modifications. Cholesterol-rich particles were prepared by adding ethanolic solutions containing 40.7 mg/1 mL cholesterol, 16.6 mg/0.3 mL DSPC, and 4.3 mg/0.2 mL bovine brain CBS, in that order and in a dropwise manner with strong stirring to 50 mL of distilled water. The ethanol was removed by evaporation under reduced pressure at 50 °C for 20 min. Methylated bovine serum albumin (Sigma) in distilled water (40 mg/2 mL) was added, and the solution was left overnight at room temperature. The suspension was centrifuged at 27200*g* for 1 h at 4 °C, the supernatant was removed and discarded, and the pellet was resuspended in 20 mL of distilled water, using a sonicating bath to aid in resuspension. The emulsion was divided into 1.0-mL aliquots and stored at −20 °C. Rabbits received twice-weekly injections of 1.0 mL of the suspension into the ear veins. They were bled twice a week, and the serum was tested for anti-sulfatide activity. Serum was aliquoted and stored at −20 °C. The antiserum used for this study was obtained on the 21st day after immunization from a rabbit which had received 6 immunizing doses of cholesterol-rich particles. It was complement inactivated by heating at 56 °C for 30 min.

Preparation of Liposomes for Immune Lysis. Multilamellar vesicles containing PC/Chol/CBS (DMPC, DPPC, or DSPC)

[1] Abbreviations: PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; SM, sphingomyelin; CBS, cerebroside sulfate; SGG, sulfogalactoglycerolipid; NFA, non-hydroxy fatty acid form; HFA, hydroxy fatty acid form; P-CBS, palmitoyl form of CBS; L-CBS, lignoceroyl form of CBS; VBS, veronal-buffered saline; Chol, cholesterol; Tris, tris(hydroxymethyl)aminomethane; EPR, electron spin resonance; GG, galactosyl diglyceride.

or SM/Chol/CBS in the molar ratio 1.0:0.75:0.1 were prepared by dissolving the lipids together in 2:1 chloroform/methanol and evaporating the solvent under nitrogen. The dried lipid was stored under vacuum for 1 h. An aqueous solution of 0.1 M tempocholine chloride, synthesized by the method of Kornberg and McConnell (1971), in 0.01 M Tris buffer and 0.04 M NaCl at pH 7.4, was added so that the lipid concentration was 1 μmol of PC (or SM)/0.1 mL. The suspension was dispersed at a temperature 5–10 °C above the lipid transition temperature for a period of 10 min with intermittent vortex shaking in the presence of small glass beads. The liposome suspension was diluted in 0.5 mL of veronal-buffered saline (VBS) containing 0.145 M NaCl, 1.8 mM sodium diethylbarbiturate, 3.1 mM diethylbarbituric acid, 0.5 mM $MgCl_2$, and 0.15 mM $CaCl_2$ at pH 7.5 and dialyzed against a further 2 L of VBS overnight at 4 °C.

Spin Immunoassay for Complement-Mediated Lysis of Liposomes. The spin immunoassay was carried out as described earlier with minor modifications (Boggs et al., 1983; Rosenqvist & Vistnes, 1977). Guinea pig serum from female Hartley strain guinea pigs was used as a source of complement. The serum was aliquoted at 4 °C and stored at −70 °C. Appropriate dilutions of liposomes, antiserum, and guinea pig serum were made in VBS. The dilution of guinea pig serum to be used was optimized on the highest concentration of vesicles to be used for each serum and was generally 1:16 to 1:4. Liposomes were used at dilutions of 1:240 to 1:30 (7–55 μM PC or SM); 25 μL of the antiserum, 50 μL of the liposomes, and 25 μL of the guinea pig serum were added to a 6 × 25 mm borosilicate culture tube, shaken gently, and incubated in a water bath at 37 °C for 20 min. Preparation and incubation of the tubes were staggered so that each sample was measured within 2 min of completion of its incubation at 37 °C. The solution was withdrawn into a 100-μL microcapillary pipet and the end sealed with Critoseal (Fisher Scientific) for measurement of the amount of spin-label released due to lysis of the vesicles, from the height of the electron spin resonance spectrum. The low-field peak of the electron spin resonance spectrum of the sample was measured in a Varian E 104B EPR spectrometer at room temperature.

At the concentration of tempocholine chloride trapped inside the vesicles, 0.1 M, the electron spin resonance spectrum is strongly broadened and reduced in amplitude as a result of spin exchange. After lysis of the vesicles, the spin-label becomes diluted in the external aqueous medium, exchange broadening is abolished, and the height of the spectrum is greatly increased. The height of the spectrum is proportional to the concentration of spin-label outside the vesicles, and thus, the degree of lysis can be quantitated from the height of the peak. The total amount of spin-label trapped inside is determined from the height after complete lysis of the vesicles with an equal volume of 2-chloroethanol. The maximum lysis obtained in the presence of optimal amounts of antibody and complement was 50–60% for all types of vesicles, indicating that antibody and complement were present in excess and that complement was able to act on all types of vesicles. The maximum lysis was redefined as 100% for the purpose of finding the $[Ab]_{50}$ values. The antiserum did not cause lysis of vesicles lacking the antigen or in the presence of inactive complement. Lysis in the absence of antibody was less than 10%.

Data Analysis. The theoretical model used to analyze the data (Vistnes, 1984) was adapted from equilibrium binding theory where the lipid vesicle is assumed to behave as a macromolecule with n identical binding sites (the lipid antigen) for the ligand (antibody) with a microscopic dissociation constant k. At equilibrium, the number of moles of antibody bound to the vesicle is v. The degree of lysis of the vesicles is related to the binding of antibody to the CBS antigen in the liposomes by assuming that for a given degree of lysis, the relative number of antibodies required to be bound per vesicle is constant, regardless of vesicle concentration. Then, for 50% lysis of the vesicles, the number of moles of antibody bound per vesicle is v_{50}. The antiserum concentration causing 50% lysis, $[Ab]_{50}$, depends on the vesicle concentration, [V], and on the dissociation constant, k_i, and concentration, c_i, of the ith population of specific antibodies in the serum which binds to the antigen, according to the equation:

$$c_i[Ab]_{50} = v_{50}[V] + v_{50}k_i/(n - v_{50}) \quad (1)$$

Since v_{50} and n are unknown but constant, let $c_i' = c_i/v_{50}$ and $k_i' = k_i/(n - v_{50})$ be relative values for the concentration and dissociation constant of the antibodies. Substituting and inverting eq 1 give

$$[V] = c_i'[Ab]_{50} - k_i' \quad (2)$$

By plotting experimental values of [V] vs. $[Ab]_{50}$ (2) a straight line with slope c_i' and intercept $-k_i'$ results.

This method is a quantitative way to compare the effect of changes in lipid composition and of the ceramide structure of the antigen on reactivity with antibody. By titrating the same antiserum against vesicles of different composition, the relative concentrations and dissociation constants, c_i' and k_i', of the population of antibodies which binds to the vesicles can be compared. Since the antiserum is polyclonal, these values reflect those of the majority of the antibodies from the antiserum which are able to bind to the vesicles.

It is necessary to assume that n and v_{50} are not affected by changes in composition of the vesicles. The molar ratio of the antigen, CBS, to the auxiliary lipids was kept constant for different types of vesicles and different forms of CBS. The lipid concentration of the vesicles was determined by phosphorus analysis by the method of Bartlett (1959). Differences in the size of liposomes of different auxiliary lipid composition would affect the number of liposomes and the total concentration of surface antigen available and thus would affect the amount of antiserum required to cause lysis and the c_i' value, although not k_i'. However, Schwartz and McConnell (1978) have shown that the chain length of PC or the presence of cholesterol has no effect on the total surface area of the liposomes. Care was taken to prepare the liposomes in the same way each time. Vesicles were used only 1 day after preparation. The assay was repeated using several different preparations of vesicles of the same composition to ensure reproducibility. Plots of [V] vs. $[Ab]_{50}$ for any one experiment, obtained by using four concentrations of vesicles, were fit to a straight line by computer. Values for k_i' and c_i' from a number of different experiments, performed on different days and using different preparations of vesicles of the same composition, were averaged.

It is also necessary to assume that complement is equally effective on all types of vesicles used. Complement was always used in excess, and the same maximal degree of lysis was obtained for all types of vesicles, at all dilutions used, at sufficiently high concentrations of antiserum and guinea pig serum.

Results

Effect of Lipid Environment. The lipid environment of the CBS antigen was varied by using synthetic forms of PC of different chain length, DMPC, DPPC, and DSPC, or by using

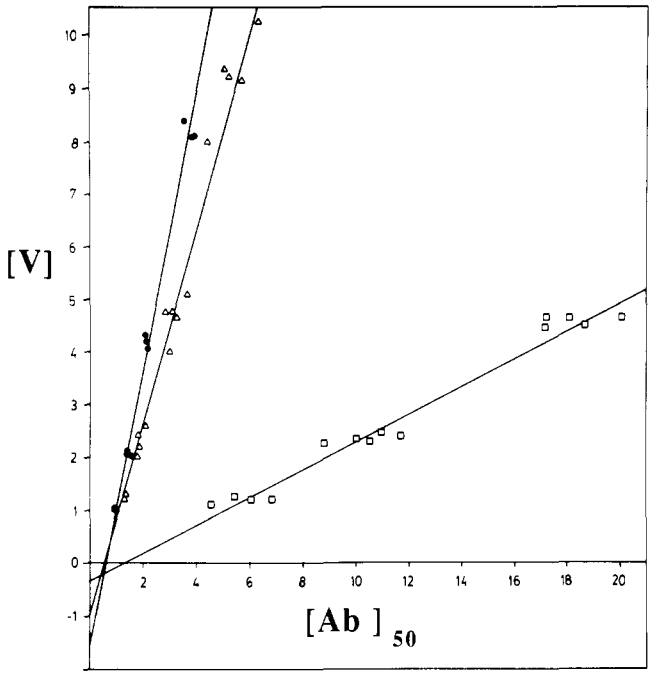

FIGURE 1: Effect of changes in the lipid environment on reactivity of natural CBS with anti-CBS antiserum. The concentration of lipid vesicles, [V], in relative units, is plotted against the concentration of antiserum, in relative units, required to cause 50% complement-mediated lysis of the vesicles. Lipid vesicles contain synthetic PC of varying chain length and cholesterol in the molar ratio PC: cholesterol:CBS 1:0.75:0.1. DMPC (●), DPPC (△), and DSPC (□). Each point represents a complete titration curve of a particular concentration of vesicles with at least 10 different concentrations of antiserum. Data from three to four separate experiments for each type of vesicle are shown.

Table I: Effect of Lipid Environment on Antibody Reactivity with Natural Cerebroside Sulfate[a]

lipid[b]	k_i'	c_i'	n^c
DMPC	1.60 ± 0.20	2.69 ± 0.26	4
DPPC	1.45 ± 0.55	2.13 ± 0.37	8
DSPC	0.30 ± 0.22[e]	0.27 ± 0.04[d]	7
SM	1.39 ± 0.22	1.59 ± 0.17[e]	8

[a] Parameters from immune lysis of lipid vesicles. [b] In mixtures with cholesterol of PC(SM):cholesterol:CBS mole ratio =1:0.75:0.1. [c] Number of separate experiments using different preparations of liposomes. [d] Significantly less than values for DMPC and DPPC; $P < 0.001$. [e] Significantly less than values for DMPC and DPPC; $P < 0.005$.

SM instead of PC. Natural CBS from bovine brain was used for these studies. Cholesterol was always present at a concentration of 40 mol % of the total lipid so that the lipids are all in a state intermediate between the gel and liquid-crystalline phases and of similar fluidity. Figure 1 shows plots of [V] vs. $[Ab]_{50}$ for several different experiments on PC vesicles of varying chain length. The slope decreased, and the intercept became less negative with increase in chain length. The average values of c_i' and k_i' from a number of different experiments are given in Table I. The dissociation constant decreased as the chain length increased, indicating that the affinity of the antibodies binding to CBS increased. The difference was highly significant for the increase in chain length from 16 to 18 carbons, but that from 14 to 16 carbons was not. However, it required a much higher concentration of antiserum to lyse DSPC vesicles, and the relative concentration of antibodies binding with high affinity to CBS in DSPC vesicles was considerably less than those binding with lower affinity to DMPC and DPPC vesicles. Thus, only a smaller population of higher affinity antibodies is able to bind to CBS in DSPC while a larger population of lower affinity antibodies is also able to bind to the glycolipid in a shorter chain length PC environment.

Table II: Effect of Variations in Hydrophobic Portion of Sulfogalactosyl Lipids on Their Reactivity with Anti-CBS Antiserum[a]

sulfogalactosyl lipid antigen[b]	k_i'	c_i'	n^c
NFA-P-CBS	1.18 ± 0.56	2.71 ± 0.87	8
HFA-P-CBS	0.47 ± 0.35[d]	1.59 ± 0.68[e]	8
NFA-L-CBS	2.06 ± 0.54[g]	5.34 ± 1.47[g]	8
HFA-L-CBS	1.32 ± 0.12[d,g]	3.39 ± 1.78[d,f]	4
SGG	1.12 ± 0.38	0.72 ± 0.05[h]	6

[a] Parameters from immune lysis of lipid vesicles. [b] In a constant lipid environment of DPPC and cholesterol in a DPPC:cholesterol:lipid antigen mole ratio of 1:0.75:0.1. [c] Number of separate experiments using different preparations of liposomes. [d] Values for the HFA form are significantly less than values for the NFA form of the same fatty acid chain length; $P < 0.025$. [e] Values for the HFA form are significantly less than values for the NFA form of the same fatty acid chain length; $P < 0.005$. [f] Values for the longer chain length form are significantly greater than values for the shorter chain length form of the same type (NFA or HFA); $P < 0.025$. [g] Values for the longer chain length form are significantly greater than values for the shorter chain length form of the same type (NFA or HFA); $P < 0.005$. [h] Significantly less than for NFA-P-CBS; $P < 0.005$.

The dissociation constant of the antibodies binding to CBS in the SM environment was similar to that in DPPC, but the concentration of antibodies able to bind was less, and it required a higher concentration of antiserum to cause a comparable degree of lysis.

Effect of Variation in the Hydrophobic Portion of the Glycolipid. The effect of hydroxylation of the fatty acid of CBS and of an increase in fatty acid chain length of CBS from 16 to 24 carbons on the reactivity with antibody was determined by using synthetic forms of CBS. The lipid environment was kept constant by using DPPC/cholesterol, 1:0.75. Values of k_i' and c_i' for antibody binding to these different lipids are given in Table II. The dissociation constant was significantly less for binding to HFA-P-CBS than for NFA-P-CBS, indicating higher affinity antibodies bind to the HFA form. However, the concentration of antibodies reacting with HFA-P-CBS was also less, indicating that this lipid selects out a population of higher affinity antibodies from the antiserum. A smaller but still significant difference was found between the HFA and NFA forms of L-CBS. Thus, only a smaller population of higher affinity antibodies is able to bind to the HFA forms while a larger population of lower affinity antibodies can also bind to the NFA forms. An increase in chain length of the fatty acid of CBS also allows binding of a larger population of lower affinity antibodies, while higher affinity antibodies are required to bind to the shorter chain length forms. The effect of an increase in chain length is particularly dramatic for the hydroxylated forms.

The reactivity of anti-CBS antibody with CBS was also compared to that with another sulfogalactosylated lipid based on glycerol instead of sphingosine, sulfogalactodiglyceride (SGG). The dissociation constant for antibody binding to SGG was similar to that for NFA-P-CBS (Table II). The SGG is a synthetic form with two chains of 16 carbons in length and thus resembles P-CBS more than the other forms. However, the concentration of antibodies able to bind to SGG was much less than that for P-CBS.

Discussion

The theoretical model used to analyze the data obtained from antibody-dependent, complement-mediated lysis of li-

posomes provides a relative value for the concentration and dissociation constant of the population of antibodies present in the serum which reacts with the antigen and is thus a more quantitative method of comparing the effect of changes in lipid composition on the reactivity of lipid haptens with antibody than has generally been applied to this question. Further, although the effect of lipid composition on the reactivity of antibodies with some neutral lipid haptens and glycolipids has been studied, that with an acidic glycolipid has not. The acidic glycolipid cerebroside sulfate has the advantage that both hydroxy and non-hydroxy fatty acid synthetic forms of varying chain length are available (Koshy & Boggs, 1983; Boggs et al., 1984). It can be considered to be a model for other charged glycolipids and is also of intrinsic interest because it is one of the two major glycolipids of myelin, being 3.8 mol % of the total lipid and 11% of the total sphingolipid (Norton, 1977). Myelin has only low concentrations of more complex glycolipids. CBS occurs at lower concentrations in a number of other membranes as well (Karlsson, 1982), and the related sulfogalactoglycerolipid, SGG, is the major glycolipid of spermatocytes (Kornblatt et al., 1974).

The results indicate that as the chain length of the PC environment is increased, antibodies of higher affinity are required to recognize and bind to the natural CBS head group. This suggests that the head group is less exposed in bilayers of greater thickness. It is also possible that the specificity of these higher affinity antibodies differs from that of the lower affinity antibodies present. They may recognize a different portion of the head group. Decreased exposure of the head group in thicker bilayers is consistent with results obtained by others where reactivity with antibody was generally assessed from the degree of antibody-dependent agglutination or complement-mediated lysis of liposomes or from the amount of antiserum required to cause the observed effect (Alving et al., 1974; Shin et al., 1978; Utsumi et al., 1984). Alving and Richards (1977) showed, in addition, that the effect of anticerebroside antibodies eluted from liposomes containing DSPC, DPPC, or DMPC on target liposomes increased with increase in PC chain length of the absorbing liposomes even though unabsorbed serum had less effect on longer chain length liposomes. This suggested that the affinity of the antibodies bound to the longer chain length liposomes was greater than that bound to the shorter chain length liposomes, in agreement with our results.

However, Shin et al. (1978) found that the degree of lysis caused by activated components of complement (reactive lysis system), in the absence of antibody, also decreased as the chain length or bilayer thickness of the liposomes increased. This finding suggested that the effect of lipid environment on the degree of lysis of liposomes found in many studies might be a result of an effect on complement activity, rather than antibody binding. However, where complement is present in excess and differences in antibody concentration are found to be required to cause the same degree of maximum lysis, as in our study, these differences must be a result of differences in antibody binding, and not complement fixation or channel formation.

The relative affinity of the antiserum for CBS in a SM environment was similar to that of DPPC. However, the concentration of antiserum required to cause lysis and the concentration of antibodies reacting were less, suggesting that the specificity of the population of antibodies which reacts with CBS in SM may be different from that which reacts with CBS in DPPC. Others also have found that a higher concentration of antiserum was required to cause lysis of SM vesicles compared to PC (Inoue et al., 1971) and that less antibody bound to a glycolipid in SM liposomes compared to PC (Alving & Richards, 1977).

Comparison of antibody reactivity with different synthetic forms of CBS with varying chain length indicated that higher affinity antibodies also were required to recognize and bind to the shorter chain length form, P-CBS, in a DPPC/cholesterol environment, indicating that the glycosyl head group may be less exposed on the surface than for the longer chain length form, L-CBS. This is also consistent with conclusions reached by others from results on whole cells (Nudelman et al., 1982), isolated glycolipids (Symington et al., 1984), or liposome lysis (Alving & Richards, 1977; Yoshino et al., 1982). Of these studies, only that by Alving and Richards (1977) used well-defined synthetic forms of a glycolipid of varying fatty acid chain length in a model membrane system. They showed that antibody eluted from liposomes containing palmitoylcerebroside was more reactive on target liposomes than antibody eluted from liposomes containing lignoceroylcerebroside, even though the unabsorbed serum caused less lysis of liposomes containing the shorter chain length form than the longer. This suggested that the affinity of the antibodies bound to the shorter chain length form was greater, although their concentration was lower, in agreement with our results on different chain length forms of CBS.

The effect of hydroxylation of the fatty acid of a glycolipid on its reactivity with antibody has not previously been studied in model membranes nor with well-defined synthetic lipids. Our results on the effect of hydroxylation of the fatty acid of CBS are not consistent with the results of studies of whole cells. The latter found that high-expressor cell lines, reactive with a monoclonal antibody to ganglio-*N*-triaosylceramide (Gg_3Cer), contained three species of this lipid with NFA C-16, NFA C-20 to C-24, and HFA C-16 fatty acids while low-expressor cell lines contained the first two species of Gg_3Cer but lacked that with HFA C-16 (Young et al., 1981; Kannagi et al., 1982; Nudelman et al., 1982; Hakomori & Kannagi, 1983). This led to the suggestion that the presence of the HFA C-16 species was responsible for the reactivity of the high-expressor cell lines with antibody. However, our results indicate that hydroxylation of the fatty acid of CBS decreases the reactivity with antibody. Higher affinity antibodies are required to bind to it, suggesting that its head group may be less exposed or altered in conformation. The effect of hydroxylation was diminished but not abolished by an increase in the chain length. This inconsistency with the results from studies of whole cells may be a consequence of the different carbohydrate structures of Gg_3Cer and CBS, of the different lipid environments in the cell and liposome membranes, or of other differences in composition between the high- and low-expressor cell lines, which may be responsible for the low reactivity rather than the absence of the HFA C-16 form. For example, the low-expressor cell lines also have increased content of more complex glycolipids, which may cause crypticity of the Gg_3Cer through steric interference (Kannagi et al., 1983). It may also be due to the specificity of the monoclonal antibody used in those studies.

The effect of modifications to the ceramide portion on the recognition of the glycolipid head group may depend on the type of receptor and its specificity. These modifications could alter the head-group conformation, either in a direct way or as a consequence of its altered location in the bilayer. Some receptors may have a specificity for this altered conformation. For example, Hansson et al. (1983) found that one strain of bacteria bound better to NFA forms of lactosylceramide al-

though the other strains tested bound better to HFA forms, indicating that the effect of hydroxylation of the ceramide depended on the specificity of the bacterial lectin receptor. Some monoclonal antibodies might also have a specificity which reacts better with the hydroxylated form. Therefore, hydroxylation may not always decrease cell surface glycolipid recognition.

Differences in the hydrophobic structure between SGG and CBS also seem to affect reactivity of the sulfated galactosyl head group with anti-CBS antibody. The affinity of antibodies binding to the synthetic SGG with two C-16 hydrocarbon chains was similar to those binding to NFA-P-CBS, but a higher concentration of antiserum was required to cause lysis of liposomes containing SGG, and the concentration of antibodies reacting with it was less than of those reacting with P-CBS, suggesting that populations of antibodies of differing specificities react with these two glycolipids. It is possible that the glycosyl head group has two different conformations in CBS and SGG. There is evidence that this may be the case for the nonsulfated form of these lipids, galactosyl cerebroside and galactosyl diglyceride, since galactose oxidase can react with cerebroside but not with galactosyl diglyceride (GG) (Lingwood, 1979). A monoclonal antibody specific for sulfomonogalactosylglycolipids, which could not distinguish between CBS and SGG in serological tests, bound only to tissues containing SGG and not tissues containing CBS without SGG, suggesting that in the membrane environment the head groups of these two lipids acquire different spatial configurations which can be distinguished by the antibody (Goujet-Zalc et al., 1986; Guerci et al., 1986). An X-ray crystallography study of cerebroside (Pascher & Sundell, 1977) has shown that the amide nitrogen is involved in an intramolecular hydrogen bond with the oxygen of the glycosidic linkage, fixing the galactose ring in a shovel conformation. It is not known whether this also occurs in CBS, but it could not occur in GG or SGG without the amide moiety.

Calorimetry and spin-label studies (Boggs et al., 1984) of synthetic forms of cerebroside sulfate have shown that the length and hydroxylation of the fatty acid chain have significant effects on its phase behavior and organization in lipid vesicles containing only this lipid. The influence on antibody reactivity reported in this study shows that these structural variations also affect the behavior of CBS in mixtures with PC or SM and cholesterol. Further studies of the structure and organization of different forms of CBS in lipid mixtures using physical techniques may help to understand the mechanism of the effects of chain length and hydroxylation on reactivity with antibody.

These results suggest that changes in membrane lipid composition or the ceramide structure of glycolipids could lead to changes in glycosyl surface expression and to altered recognition of cell surfaces. Decreased expression could lead to loss of contact inhibition of cell growth or prevent destruction of malignant cells by the immune system. Increased expression might lead to increased susceptibility to bacterial infection or to autoimmunity. Altered ceramide structure of certain glycolipids has been found in transformed malignant tissues (Kannagi et al., 1982, 1983). Altered lipid composition may also occur in myelin in patients with multiple sclerosis (MS) [see Boggs & Moscarello (1980) and references cited therein], a disease in which demyelination is mediated by the immune system. Multiple sclerosis patients have been reported to have immune response to brain gangliosides, and this response was greater to gangliosides isolated from MS brains than from normal human brains (Offner et al., 1981).

ACKNOWLEDGMENTS

We thank Drs. C. Lingwood and R. Gigg for providing a sample of synthetic SGG.

Registry No. DMPC, 18194-24-6; DPPC, 2644-64-6; DSPC, 816-94-4; cholesterol, 57-88-5; 3-*O*-(β-D-galactopyranosyl 3-sulfate)-2-*O*-hexadecanoyl-1-*O*-hexadecyl-L-glycerol, 79645-30-0.

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Motion and Surface Accessibility of Spin-Labeled Lipids in a Model Lipoprotein Containing Cholesteryl Oleate, Dimyristoylphosphatidylcholine, and Apolipoprotein E†

Martha P. Mims,‡ Mohan V. Chari,‡ and Joel D. Morrisett*,‡,§
Departments of Medicine and Biochemistry, Baylor College of Medicine and The Methodist Hospital, Houston, Texas 77030
Received June 16, 1986; Revised Manuscript Received August 21, 1986

ABSTRACT: A series of spin-labeled phosphatidylcholines (PCs) and cholesteryl esters (CEs) bearing the paramagnetic 2,2-dimethyloxazolidinyl-1-oxy (doxyl) group at fatty acyl carbon C5′, C12′, or C16′ were used to study acyl chain motions in the polar surface shell and hydrophobic core domains of microemulsion (ME) particles containing cholesteryl oleate and dimyristoylphosphatidylcholine (DMPC), and of particles with apolipoprotein E (apoE) bound to their surfaces. Electron paramagnetic resonance data obtained with the doxyl-labeled PCs indicated a gradient of motion in the ME surface monolayer similar to that observed with the same probes in a bilayer. The 5- and 12-doxyl-CEs clearly demonstrated a higher degree of order for the cholesteryl ester rich core than the corresponding doxyl-PCs showed for the phospholipid-rich surface over the entire range 10–60 °C. The temperature dependencies of spectra of the 16-doxyl-CE in the core and PC in the surface of the ME were almost identical, suggesting that there was no sharp boundary between core and surface domains. None of the probes detected either the surface phospholipid transition (31 °C) or the cholesteryl ester core transition (46 °C) measured previously by differential scanning calorimetry and ^{13}C nuclear magnetic resonance. Binding of apoE to spin-labeled DMPC vesicles increased the order of the 5′-position of the *sn*-2 acyl chain over the range 15–33 °C; the thermal transition was broadened and its midpoint elevated. The effect of protein binding was not as striking for the ME particles. In separate studies, the rates of ascorbate-induced reduction of the nitroxyl moiety in ME labeled with either 5-doxyl-PC or 5-doxyl-CE were measured to determine the accessibility of each lipid type to the aqueous phase and the core → surface mobility of the nonpolar lipids. Reduction of 5-doxyl-PC in the ME was monophasic; the rates were comparable to those of 5-doxyl-CE in vesicles, but much lower than those of 5-doxyl-PC in vesicles. This result indicated that the C5′-position of the *sn*-2 acyl chain of PC in the microemulsion was less accessible (by bulk water molecules) than the corresponding position in the vesicle. Reduction of 5-doxyl-CE in the ME was also monophasic and dependent on ascorbate concentration at every temperature studied. Thus, CE movement from the core to the surface was more rapid than the rate of doxyl group reduction. Calculations based on these results suggested that a significantly larger fraction of CE may be present in the ME surface monolayer than in the vesicle bilayer.

The capacity of spin-labeled lipids to detect phase transitions in phospholipid vesicles (Barrett et al., 1969; Hubbell & McConnell, 1971) and neat lipids (Morrisett et al., 1984) is well documented. Binding of peptides or proteins to spin-labeled vesicles has been shown to shift and broaden the electron paramagnetic resonance (EPR)[1] phase transition (Yu et al.,

† This work has been supported by Specialized Center of Research in Atherosclerosis Grant HL-27341 and by Grant Q-837 from the Robert A. Welch Foundation. M.P.M. is the recipient of National Research Service Award HL-07341 from the National Heart, Lung, and Blood Institute.
* Address correspondence to this author at the Department of Medicine, The Methodist Hospital.
‡ Department of Medicine.
§ Department of Biochemistry.

[1] Abbreviations: TLC, thin-layer chromatography; CO, cholesteryl oleate; DMPC, dimyristoylphosphatidylcholine; doxyl, 2,2-dimethyloxazolidinyl-1-oxy; CE, cholesteryl ester; PC, phosphatidylcholine; CER-VLDL, cholesteryl ester rich very low density lipoprotein, a lipoprotein isolated at $d < 1.006$ g/mL from the plasma of hypercholesterolemic rabbits (Morrisett et al., 1984); EPR, electron paramagnetic resonance; DSC, differential scanning calorimetry; SUV, small unilamellar vesicles of 220-Å diameter (Laggner et al., 1979); ME, microemulsion particles containing DMPC and CO, with diameters of ~750 Å (Mims et al., 1986); apoE, apolipoprotein E; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

0006-2960/86/0425-7494$01.50/0