Dependence of the Surface Expression of the Glycolipid Cerebroside Sulfate on Its Lipid Environment: Comparison of Sphingomyelin and Phosphatidylcholine†

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ABSTRACT: The influence of the membrane lipid environment on the reactivity with antibody of the acidic glycolipid cerebroside sulfate (CBS) was examined by using a spin membrane immunoassay. Fewer antibodies in a polyclonal anti-CBS antiserum recognized the antigen in a bovine brain sphingomyelin/cholesterol (SM/CHOL) environment than in dipalmitoylphosphatidylcholine/cholesterol (DPPC/CHOL). Changes in the CBS ceramide group appeared to have less influence on antibody recognition of CBS in SM/CHOL than in DPPC/CHOL [Crook et al. (1986) *Biochemistry 25*, 7488–7494]. Although the fatty acid chain length of phosphatidylcholine strongly influences CBS recognition, the fatty acid chain length of sphingomyelin had only a moderate effect on CBS recognition and did not account for the decreased recognition in SM compared to in DPPC. Inhibition studies revealed that the antibodies which recognize CBS in SM/CHOL (S antibodies) form a population distinct from those which recognize CBS in DPPC/CHOL (P antibodies). The specificity of the P and S antibodies was examined further by comparing the efficacy of various substances, which share chemical features with the components of CBS in a SM/CHOL or DPPC/CHOL environment, to inhibit lysis of liposomes containing CBS. Intact CBS, cholesterol, and a phosphocholine lipid, at certain antigen densities, were required for optimal recognition of the antigen, especially by the P antibodies, suggesting that a complex of all three lipids in a multivalent array may be recognized by these antibodies. The S antibodies may recognize a smaller complex or monomers of CBS. Although increasing the molar ratio of CBS in SM above an optimal ratio 0.05 to 0.1 had little effect on the recognition of CBS in SM, increasing the molar ratio of CBS in DPPC above this optimal ratio reduced the recognition of the antigen in DPPC by both pools of antibodies. This suggested that the organizations of CBS in SM/CHOL and in DPPC/CHOL may differ and that the S and P antibodies recognize this difference.

Glycolipids are an important class of membrane components found in virtually all cells. The carbohydrate head groups of glycolipids have been associated with numerous recognition processes including cell adhesion, interaction with biological ligands, and adhesion of microbial organisms (Hakomori, 1981; Hansson et al., 1983). Changes in glycolipids have also been noted during cell growth, differentiation, and oncogenesis (Hakomori, 1984; Kannagi et al., 1981). In addition, glycolipids are immunogenic and can serve as cell markers. Antibodies to glycolipids have been identified in a number of autoimmune diseases and may be responsible for the pathology in some cases (Marcus, 1984; Quarles et al., 1986).

One of the most striking observations concerning the expression or exposure of glycolipids, found in certain transformed cell lines, is that during oncogenesis the expression can change despite little or no change in the chemical quantity of the glycolipid (Hakomori, 1984; Kannagi et al., 1982, 1983). This suggests that the exposure of the carbohydrate head group of glycolipids can be influenced by various other changes in the membrane composition and organization. It further implies that changes in the membrane composition other than the concentration of a particular glycolipid may result in alterations in cell contact, cell growth and regulation, susceptibility to infection, or immune response, as a result of changes in the exposure of the glycolipid carbohydrate. Thus, an understanding of how glycolipid expression can be affected by changes in the membrane composition is important for a complete understanding of glycolipid function.

The recognition of glycolipids by antibody provides a convenient model to investigate the factors influencing the exposure of glycolipids at the membrane surface and to examine the immune recognition of glycolipids in more detail. The degree of antibody binding can be used as a measure of accessibility at the membrane surface. A useful method for detecting antibody binding to glycolipids in a membranous environment is antibody-dependent, complement-mediated lysis of liposomes containing lipid haptens, as first described by Kinsky and colleagues (Haxby et al., 1968). The glycolipids can be incorporated into liposomes of defined lipid composition, permitting examination of the influence of each component of the membrane environment on recognition of the glycolipid. Studies with lipid haptens incorporated into liposomes have shown that the antigenic recognition of glycolipids is modulated by the membrane environment, the antigen density, and the ceramide composition of the glycolipid itself [cf. Kinsky and Nicolotti (1977), McConnell (1977), and Alving and Richards (1983) for reviews].

In a previous study we examined the effects of changing both the ceramide composition and the phospholipid environment of the acidic glycolipid cerebroside sulfate on its antigenic expression (Crook et al., 1986). By use of a theoretical model of data analysis developed by Vistnes (1984), the effect of these changes on both the concentration and affinity of antibodies

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recognizing cerebroside sulfate (CBS)[1] was determined. Most earlier studies of antibody recognition of lipid haptens determined only the effect of changes in lipid composition on the titer of the antiserum. However, an immunoabsorption study by Alving and Richards (1977) obtained information about the affinity of the antibodies reacting with the lipid hapten. Our previous studies using polyclonal anti-CBS antiserum led to the conclusion that in a PC environment increasing the fatty acid chain length of phosphatidylcholine (PC) restricted recognition of CBS to a low concentration of relatively high affinity antibodies, whereas increasing the chain length of CBS allowed a higher concentration of lower affinity antibodies to bind. Hydroxylation of the CBS fatty acid also restricted the recognition of CBS to a lower concentration of higher affinity antibodies.

The study by Crook et al. (1986) also showed that the recognition of CBS was somewhat reduced in bovine sphingomyelin (bSM) compared to that in DPPC. Moreover, the data suggested that CBS might be recognized in bSM by a subpopulation of antibodies with a different specificity from those recognizing CBS in PC. Thus, the current study was undertaken to compare the recognition of CBS in bSM and PC in more detail and to compare the specificity of the antibodies binding to CBS in these two phospholipids. The effects of changes in ceramide composition of CBS, as well as the chain length of SM, on the antigenic expression of CBS in SM were examined and compared to the effects these changes have on CBS expression in PC. The importance of the lipid environment for recognition of CBS by the antiserum suggests that other liposomal components in addition to CBS could contribute to the antigenic epitope. The fine specificity of the antibodies recognizing CBS in both SM and PC was thus probed through the use of competitive inhibitors that share structural features with CBS, cholesterol, or phosphocholine.

Materials and Methods

Lipids. Bovine brain cerebroside sulfate (galactosylceramide I^3-sulfate), bovine brain sphingomyelin (bSM), phosphatidic acid (PA), and 2,4-dinitrophenyldipalmitoylphosphatidylethanolamine (DNP-PE) were purchased from Avanti Polar Lipids. Bovine brain galactosylceramide (GalC) was purchased from Supelco. Dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC), distearoylphosphatidylcholine (DSPC), egg sphingomyelin (egg SM), bovine erythrocyte sphingomyelin (rbc SM), dipalmitoylphosphatidylethanolamine (DPPE), *N*-methyldipalmitoylphosphatidylethanolamine (*N*-Me-PE), *N*,*N*-dimethyldipalmitoylphosphatidylethanolamine (*N*,*N*-diMe-PE), and cholesterol 3-sulfate (CHOL-SO_4) were purchased from Sigma Chemical Co. Cholesterol (CHOL) was purchased from Fluka. All lipids were chromatographically pure and were used as supplied. 4-(*N*,*N*-Dimethyl-*N*-*n*-hexadecylammonio)-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (H-TEMPO) was purchased from Molecular Probes. 5-Doxylstearate (5-S-SL) and 16-doxylstearate (16-S-SL) were purchased from Syva. Lyso-CBS was obtained by deacylation of bovine brain CBS (Koshy & Boggs, 1982). Semisynthetic species of CBS containing palmitic (PCBS), α-hydroxypalmitic (POHCBS), and lignoceric (LCBS) acids were prepared by reacylation of lyso-CBS and purified as described (Koshy & Boggs, 1983).

Reagents and Inhibitors. All general chemicals were of reagent grade. Galactose 1-phosphate, galactose 6-phosphate, *p*-nitrocatechol sulfate, *p*-acetylphenyl sulfate, choline chloride, phosphocholine, glycerophosphocholine, glycerophosphate, 2,4-dinitrophenol (DNP), and 2,4-dinitrophenyllysine (DNP-lysine) were obtained from Sigma. Veronal-buffered saline (VBS) contained 0.145 M NaCl, 1.8 mM sodium diethyl barbiturate, 3.1 mM diethylbarbituric acid, 0.5 mM $MgCl_2$, and 0.15 mM $CaCl_2$, pH 7.4. The water-soluble spin label tempocholine chloride was prepared according to the method of Kornberg and McConnell (1971).

Antisera. The antisera (serum B and serum C) were raised by immunization of New Zealand White rabbits, purchased from Reimans, with intravenous injection of cholesterol-rich particles containing cholesterol, DSPC, CBS, and methylated bovine serum albumin as described by Crook et al. (1986, 1987). Serum C was used in our previous study (Crook et al., 1986) but was in limited supply, so serum B was used for most of the experiments in this study. They behaved similarly (Crook et al., 1987). The antisera were stored frozen in small aliquots, and a new aliquot was used for each experiment. Anti-DNP-BSA serum was purchased from Miles-Yeda.

Spin Membrane Immunoassay. The spin membrane immunoassay to measure antibody-dependent, complement-mediated lysis of multilamellar liposomes from the release of the spin label tempocholine chloride was performed as described previously (Crook et al., 1986, 1987). The liposomes typically contained bSM (or DPPC)CHOL/CBS in the molar ratio of 1:0.75:0.1 and were prepared as described (Crook et al., 1986). The indicator liposomes contained 0.1 M tempocholine chloride, while those used for competitive inhibition contained VBS only. The inhibitor liposomes were serially diluted 10-fold in VBS.

For quantitative comparison of the reactivity of the antiserum with different types of liposomes, the tempocholine-containing liposomes at a PC or SM concentration of 1 μmol/mL were diluted 1/10 to 1/80 by 2-fold serial dilutions, with final phospholipid concentrations ranging from 5 to 50 μM as determined by phosphorous analysis (Bartlett, 1959). The guinea pig serum, used as a source of complement, was stored in small aliquots at −70 °C and diluted 1/4 to 1/8 in VBS before use. The antiserum was serially diluted 2-fold from 1/10 to 1/10 240 in VBS.

Quantitative Data Analysis. The reactivity of the antiserum with the various synthetic forms of CBS in DPPC/CHOL and SM/CHOL liposomes was analyzed quantitatively by using the theoretical model of Vistnes (1984), allowing determination of relative values for both concentration and affinity of antibodies binding to CBS. The detailed description of the theory and application for this use is given elsewhere (Vistnes, 1984; Crook et al., 1986). Briefly, the theory relates the concentration of antiserum required to achieve a given degree of lysis of the liposomes (in this case it is 50% lysis), [AB_{50}], the relative concentration, *c*, of antibodies binding to the vesicles, the vesicle concentration, [V] (assumed to be proportional to

[1] Abbreviations: BSA, bovine serum albumin; CBS, cerebroside sulfate; nCBS, natural bovine brain CBS; LCBS, lignoceroyl form of cerebroside sulfate; PCBS, palmitoyl form of cerebroside sulfate; POH-CBS, α-hydroxypalmitoyl form of cerebroside sulfate; CHOL, cholesterol; CHOL-SO_4, cholesterol sulfate; DNP, 2,4-dinitrophenol; DNP-lysine, (2,4-dinitrophenyl)lysine; DNP-PE, (2,4-dinitrophenyl)dipalmitoylphosphatidylethanolamine; DPPE, dipalmitoylphosphatidylethanolamine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; ESR, electron spin resonance; GalC, galactosylceramide; H-TEMPO, 4-(*N*,*N*-dimethyl-*N*-*n*-hexadecylammonio)-2,2,6,6-tetramethylpiperidine-1-oxyl iodide; 5-S-SL, 5-doxylstearic acid; PA, phosphatidic acid; VBS, veronal-buffered saline; *N*-Me-PE, *N*-methyldipalmitoylphosphatidylethanolamine; *N*,*N*-diMe-PE, *N*,*N*-dimethyldipalmitoylphosphatidylethanolamine; lyso-CBS, psychosine sulfate; bSM, bovine sphingomyelin; egg SM, egg sphingomyelin; rbc SM, erythrocyte sphingomyelin.

the lipid concentration), and the relative dissociation constant, k, by the expression

$$[V] = c[AB_{50}] - k$$

Experimental values of [V] and $[AB_{50}]$ are obtained, and the resultant straight line is computed by regression analysis to give c as the slope of the line and $-k$ as the ordinate intercept. The value obtained for k represents an average dissociation constant for all the antibodies that bind. Repetition of the assay with the same or different sets of liposomes over a period of a week or two gave reproducible values for k and c, although repetition of the experiments after a longer time period often gave different values, for unknown reasons. However, when different types of lipsomes were compared, the same relative differences were obtained when the experiment was repeated at different times even though the absolute values might differ. Therefore, the differences in the absolute values did not seem to be due to variations in different preparations of the same type of liposomes. Different types of liposomes to be compared were always measured on the same day by using the same aliquot of antiserum, guinea pig serum, buffers, etc. The data shown in Table I are organized into sets. The data for any one set were determined at the same time and can be compared. However, the data obtained for different sets were obtained at different times, and it may not be possible to directly compare the data in one set with those in another set.

Surface Area of Liposomes. The surface area of liposomes of different composition was determined by measuring the extent of reduction of the spin-labeled fatty amine H-TEMPO, incorporated into the lipid bilayer, by ascorbic acid. Liposomes containing either DMPC, DPPC, DSPC, or bSM, together with cholesterol and natural bovine CBS (nCBS), were prepared in the presence of H-TEMPO in the molar ratio choline lipid/cholesterol/CBS/H-TEMPO 1:0.75:0.1:0.005 in VBS at a concentration of 30 μmol of phospholipid/mL. H-TEMPO inserts into the bilayer with the nitroxide group exposed to the bilayer surface and with a small proportion free in solution. The ESR signal due to lipid-bound probe was quantitated by subtracting the height of the high-field line due to probe free in solution from the center line, which is the sum of peaks due to lipid-bound probe and probe in solution. Samples were kept on ice. An aliquot was taken and diluted with an equal volume of cold VBS or 0.5 M sodium ascorbate in VBS and incubated for 5 min at 4 °C, and the signal was measured on the EPR spectrometer at 4 °C. Ascorbate is a relatively nonpermeant reducing agent that reduces the exposed nitroxide spin label resulting in signal loss (Kornberg & McConnell, 1971). Thus, the reduction in the ESR signal upon addition of ascorbate reflects the number of spin-labeled molecules exposed at the surface and, therefore, the overall surface area of the liposomes.

The motion of H-TEMPO and 5-S-SL in different lipid environments was also determined at 9 °C from the value of the maximum hyperfine splitting, T_{max}, of its spectrum, which is inversely proportional to the degree of motion (Esser, 1980).

Purification of CBS Antibodies. Separate populations of high- and low-affinity antibodies were purified by a liposome elution method adapted from the methods of Alving and Richards (1977), Lingwood et al. (1980), and Coulon-Morelec (1972). One milliliter of undiluted antiserum was adsorbed with 1 mL of DPPC/CHOL/POHCBS or DPPC/CHOL/LCBS liposomes containing 1 μmol of DPPC, 0.75 μmol of cholesterol, and 0.1 μmol of CBS by incubating at room temperature for 1 h. These two liposome compositions were chosen because they have previously been shown to result in the largest differences in k and c values for the antiserum (Crook et al., 1986). After the liposomes were washed three times in 10 mL of cold VBS by centrifugation at 30000g at 4 °C in a Beckman L8-55 ultracentrifuge, the bound antibodies were eluted from the liposomes by incubating at room temperature with 1.0 mL of 2.5 M NaCl/0.5 M $BaCl_2$ (Lingwood et al., 1980), followed by extraction with an equal volume of diethyl ether containing 0.1% lecithin (PC) (Coulon-Morelec, 1972). The aqueous phase was then dialyzed extensively against VBS. The reactivity of the purified antibodies with both DPPC/CHOL/LCBS and DPPC/CHOL/POHCBS liposomes was analyzed by the method of Vistnes (1984).

Inhibition Assay. Ten-fold dilutions of various inhibitors were added to a standardized indicator system. The effectiveness of each inhibitor was compared from the concentration at which 50% inhibition was achieved. The sulfate concentration of inhibitors containing sulfate was determined by the method of Rosenberg (1963). The indicator system comprised the following (in order of addition): 25 μL of antiserum diluted such that 80% of the maximum lysis was achieved in the absence of inhibitor; 100 μL of VBS or inhibitor diluted in VBS; 50 μL of indicator liposomes containing tempocholine, composed of either DPPC/CHOL/LCBS or bSM/CHOL/LCBS, and diluted to 2.5 μM CBS; and 25 μL of guinea pig serum. Each tube was incubated at 37 °C for 20 min and the ESR signal measured as described above. A similar study was carried out by using DPPC/CHOL/PA/DNP-PE liposomes in the molar ratio 1:0.75:0.1:0.1 and anti-DNP antiserum.

Results

Comparison of CBS Expression in SM and DPPC. Previous studies have shown that for natural CBS, prepared from bovine brain, fewer antibodies bound to CBS in bSM than in DPPC, although the relatively affinity of the antibodies recognizing the antigen was similar (Crook et al., 1986). (In all liposomes containing CBS referred to, 43% cholesterol was also present, unless otherwise noted.) Since in a PC environment the fatty acid chain composition of CBS had been shown to greatly affect antibody binding to CBS, the recognition of synthetic species of CBS with varying fatty acid composition was compared in bSM and DPPC. Table I, sets 1 and 2, shows that the recognition of CBS in bSM was reduced compared to that in DPPC regardless of the CBS fatty acid chain length. A lower concentration of antibodies bound to CBS in bSM regardless of whether the CBS species had 16-carbon (PCBS) or 24-carbon (LCBS) fatty acid chains. However, the affinity (inverse of the dissociation constant k) of the antibodies recognizing either species in bSM and DPPC was relatively similar. Note that two different antisera, B and C, were used in sets 1 and 2 so that the data on LCBS could not be compared directly with those on PCBS.

The effect of ceramide composition of CBS on its recognition in a bSM environment was examined more directly by comparing the reactivity of antiserum B with different synthetic forms of CBS in bSM/CHOL liposomes. Table I, set 3, shows that neither the fatty acid chain length nor the presence of an α-hydroxy group on the fatty acid had much effect on the k and c values for antibody recognition of CBS in bSM; the antigen seems to be recognized by the same population of antibodies with the same affinity in each case. In contrast, earlier studies using antiserum C showed that the concentration and affinity of antibodies binding to CBS in DPPC were significantly dependent on the ceramide composition of CBS. This was confirmed for POHCBS and LCBS by using antiserum C in Table I (set 4). In addition, Table I shows that antiserum B behaved similarly in discriminating

Table I: Comparison of the Relative Affinity and Concentration of Antibodies to Synthetic CBS Species with Different Fatty Acid Substituents in SM and DPPC

liposomes[a]	serum	k^b	c^b
	Set 1		
DPPC/CHOL/LCBS	C	0.38 ± 0.09	1.08 ± 0.14
bSM/CHOL/LCBS	C	0.53 ± 0.17	0.44 ± 0.04
	Set 2		
DPPC/CHOL/PCBS	B	0.26 ± 0.05	0.52 ± 0.06
bSM/CHOL/PCBS	B	0.28 ± 0.05	0.27 ± 0.02
	Set 3		
bSM/CHOL/POHCBS	B	0.26 ± 0.03	0.32 ± 0.02
bSM/CHOL/PCBS	B	0.27 ± 0.04	0.37 ± 0.07
bSM/CHOL/LCBS	B	0.26 ± 0.10	0.39 ± 0.04
	Set 4		
DPPC/CHOL/POHCBS	C	0.06 ± 0.02	0.31 ± 0.06
DPPC/CHOL/LCBS	C	0.73 ± 0.11	1.08 ± 0.21
	Set 5		
DPPC/CHOL/POHCBS	B	0.32 ± 0.08	0.40 ± 0.02
DPPC/CHOL/LCBS	B	0.63 ± 0.07	1.37 ± 0.25
	Set 6[c]		
egg SM/CHOL/LCBS	B	0.17	0.47
egg SM/CHOL/POHCBS	B	0.16	0.29
rbc SM/CHOL/LCBS	B	0.33	0.40
rbc SM/CHOL/POHCBS	B	0.19	0.18

[a] The molar ratio of DPPC (or bSM)/CHOL/LCBS was 1.0:0.75:0.1. [b] The data shown for each set of lipid vesicles to be compared were obtained over a short period of time (1–2 weeks). Data shown for different sets were obtained at different times or with different sera. Only data from a single experiment can be compared quantitatively. Data are expressed as the average of five to seven determinations ± standard deviation. [c] Data are from a representative experiment.

between POHCBS and LCBS in DPPC (set 5), in apparent contrast to bSM (set 3). It was concluded previously that antibody binding to POHCBS in DPPC was restricted to a low concentration of high-affinity antibodies, while PCBS was recognized by a somewhat higher concentration of antibodies with slightly lower average affinity, and LCBS was recognized by a higher concentration of antibodies with a much lower average affinity (Crook et al., 1986).

To test this conclusion, the antibodies bound to POHCBS and LCBS in DPPC liposomes were eluted, and each antibody population was tested on DPPC/CHOL liposomes containing either POHCBS or LCBS. The antibodies purified from POHCBS liposomes bound to the indicator liposomes with higher affinity than those purified from LCBS, regardless of whether the indicator liposomes contained POHCBS or LCBS (Table II). This confirmed that the population of antibodies in the whole antiserum which bound to POHCBS had a higher average intrinsic affinity than the population which bound to LCBS. In addition, these results show that both populations of antibodies bound to LCBS with a higher affinity than to POHCBS. The difference is especially striking for the antibodies eluted from POHCBS and indicates that these antibodies did not have greater specificity for POHCBS than for LCBS.

Bovine brain sphingomyelin has a heterogeneous fatty acid composition with predominantly 18:0 and 24:1 fatty acids (Calhoun & Shipley, 1979). Recognition of CBS in PC was found to decrease with increasing chain length and bilayer thickness of the PC (Crook et al., 1986). To determine if the reduced expression of CBS and apparent lack of dependence on ceramide composition in bSM compared to DPPC results from the relatively long bSM chain lengths, the recognition of CBS was compared in egg SM, which contains predominantly 16:0 fatty acid chains, and bovine erythrocyte sphingomyelin (rbc SM), which contains predominantly 24:0 fatty acids (Calhoun & Shipley, 1979). The sphingosine species of all three types of SM is predominantly 18-carbon chains (Calhoun & Shipley, 1979). The data, shown in Table I, set 6, indicate that egg SM behaved similarly to bSM in that the k values were similar for POHCBS and LCBS. However, the c value was higher for LCBS. The rbc SM behaved more like PC in that POHCBS was recognized only by a lower concentration of higher affinity antibodies, while LCBS was recognized by a larger population of lower affinity antibodies, although the difference was not as great as that found for DPPC.

Table II: Binding of CBS Antibodies Purified from Liposomes Containing Different Forms of CBS: Comparison of the Exposure of POHCBS and LCBS in DPPC

liposome source of eluted antibodies	indicator liposomes[a]			
	POHCBS[b]		LCBS	
	k	c	k	c
POHCBS	0.64	0.60	0.04	0.89
LCBS	2.4	0.48	1.07	0.95

[a] The molar ratio of DPPC/CHOL/LCBS (or POHCBS) in the indicator liposomes was 1:0.75:0.1. [b] Data are from a representative experiment.

The apparent lack of dependence on ceramide structure of antibody binding to CBS in egg SM and bovine brain SM may be due to opposing effects on the dissociation constant. An increase in fatty acid chain length of CBS may allow antibodies to bind with a higher affinity, as well as allow additional antibody populations with lower intrinsic affinity to bind. Attempts to elute the bound antibodies from CBS in bSM were unsuccessful, so this could not be investigated. The fatty acid chain length of SM seems to have some influence on the recognition of CBS, although less than for PC. The affinity of antibody binding to LCBS in egg SM was greater than in rbc SM. Since the concentrations of antibodies recognizing CBS in egg SM and in rbc SM were similar, the same population of antibodies may be involved. However, it may bind to LCBS in egg SM with a higher affinity than in rbc SM, because of the smaller bilayer thickness of egg SM allowing greater exposure of LCBS. In any case, CBS was not recognized by as high a concentration of antibody in any of the three types of SM as it was in DPPC.

Surface Area of Liposomes. Determination of the relative k and c values using the Vistnes model requires the assumption that the relative surface area and hence amount of available CBS is independent of the lipid composition of the liposomes. To test the validity of this assumption, the relative surface area of liposomes of different composition was determined by incorporating the spin-labeled fatty amine H-TEMPO into the liposomes. Since this spin label remains largely membrane embedded, addition of a water-soluble, nonpenetrating reducing agent such as sodium ascorbate will reduce only the H-TEMPO exposed on the outermost surface of the liposomes. Thus, the proportion reduced by the addition of ascorbate reflects the relative surface area of each liposome preparation. Table III shows that the amount of H-TEMPO reduced by ascorbate was relatively similar regardless of the membrane composition. Increasing the PC chain length or substituting bSM for PC did not alter the surface area of the liposomes enough, or in the right direction, to account for the differences in concentration of antibodies reacting with CBS in bSM and DPPC found in this study and in different chain length PCs found previously (Crook et al., 1986).

Motional Restriction of H-TEMPO at the Membrane Surface. One interpretation of the results indicating that

Table III: Effect of the Lipid Environment on the Relative Amount of the Fatty Amine H-TEMPO Exposed at the Membrane Surface and the Motional Freedom of H-TEMPO and 5-S-SL As Measured by the Value of the Hyperfine Splitting T_{max}

lipid environment[a]	% reduced[b]	T_{max}[c] H-TEMPO	T_{max}[c] 5-S-SL
DMPC/CHOL/CBS	16	29.4	29.7
DPPC/CHOL/CBS	20	30.3	30.1
DSPC/CHOL/CBS	22	31.1	30.4
bSM/CHOL/CBS	17	31.8	30.9
egg SM/CHOL/CBS	nd[d]	31.8	31.0
rbc SM/CHOL/CBS	nd	31.5	30.6

[a] The molar ratio of PC (or SM)/CHOL/CBS is 1:0.75:0.1. [b] Percent reduced = (signal with buffer − signal with ascorbate)/signal with buffer. [c] The measurement of T_{max} is significant to ±0.2 G. [d] nd, not determined.

antibody recognition of CBS depends on the lipid environment is that CBS becomes more deeply embedded within the bilayer as its thickness (fatty acid chain length) increases. X-ray diffraction studies show that increasing fatty acid chain length of PC and SM increases the bilayer thickness, although the effect of cholesterol on this is not known for SM (McIntosh, 1978; Maulik et al., 1986). The effect of different lipid environments on the maximum hyperfine splitting, T_{max}, of H-TEMPO supported this conclusion for PC but not for SM. Although this probe is located in the polar head group region of the bilayer, the large T_{max} value of its spectrum at 9 °C (Table III) indicates that its motion in these lipids was restricted. The motional restriction of H-TEMPO increased with increase in the fatty acid chain length of PC, suggesting that the nitroxide group on the probe penetrated further into the bilayer as its thickness increased. The motion of a spin label with the nitroxide group in the hydrocarbon region of the bilayer, 5-S-SL, was less affected by the increase in PC chain length (Table III), while that of a probe with the nitroxide group located near the center of the bilayer, 16-S-SL, had virtually identical motion in all the lipids. Thus, the fluidity of the PC-CHOL bilayers was relatively independent of chain length. The mobility of H-TEMPO was lowest when in SM, but the motion of 5-S-SL was also reduced more relative to the PC's, indicating that SM/CHOL was more ordered than PC/CHOL. Furthermore, the mobility was similar in all three types of SM regardless of the fatty acid chain length.

Inhibition of Lysis by CBS in PC and bSM. The fact that the concentration of antibodies which bound CBS in SM was less than those which bound to it in DPPC, even though they bound with a similar affinity in both cases, suggests that two different populations of antibodies may be involved and that the similarity in affinity may be coincidental. The population of antibodies recognizing CBS in SM may have a different specificity from those which recognized CBS in PC. An alternative explanation is that the relative c values determined in this way cannot be compared for vesicles of such different lipid composition due to differences in the relationship between number of bound antibodies and degree of lysis.

Since antibodies could not be eluted from CBS in SM liposomes, the specificity of the antibodies for CBS in PC and SM was compared directly by determining the ability of liposomes containing either bSM/CHOL/LCBS or DPPC/CHOL/LCBS to inhibit the lysis of both DPPC/CHOL/LCBS and bSM/CHOL/LCBS indicator liposomes. As shown in Table IV, the concentration of SM/CHOL/CBS liposomes required to achieve 50% inhibition of lysis of DPPC/CHOL/LCBS liposomes was 44-fold higher than for DPPC/CHOL/CBS liposomes. Thus, the population of antibodies that recognized CBS in a PC environment recognized

Table IV: Competitive Inhibition of the Lysis of Liposomes Containing DPPC/CHOL/CBS[a] or bSM/CHOL/CBS by Different Membrane-Bound and Soluble Substances

inhibitor[c]	IC_{50}[b] indicator liposomes: DPPC/CHOL/LCBS[a] (μM)	IC_{50}[b] indicator liposomes: bSM/CHOL/CBS[a] (μM)
DPPC/CHOL/LCBS	0.13	8.9
bSM/CHOL/LCBS	5.7	0.46
DPPE/CHOL/LCBS	1.6	nd[e]
N-Me-PE/CHOL/LCBS	0.35	nd
N,*N*-diMe-PE/CHOL/LCBS	1.6	nd
egg PC/CBS (1:0.1)	9	nd
DPPC/CBS (1:0.1)	14.5	nd
DPPC/CHOL	*d*	*d*
LCBS	7	8.75
PCBS	25	nd
nCBS	50	nd
LCBS/CHOL (0.5:0.4)	17.5	15
PCBS/CHOL (0.5:0.4)	20	nd
nCBS/CHOL (0.5:0.4)	14.5	nd
DPPC/CHOL/GalC	50	nd
lyso-CBS	85	22
DPPC/CHOL/lyso CBS	500	500
DPPC/CHOL-SO_4 (1:0.75)	150	nd
DPPC/CHOL/CHOL-SO_4 (1:0.65:0.1)	*d*	nd
bSM/LCBS	nd	29
bSM/CHOL	nd	*d*
Na_2SO_4	5000	5000
Na_2HPO_4	5000	5000
p-acetylphenyl sulfate	5000	5000
p-nitrocatechol sulfate	5000	5000
NaCl	150000	>150000
phosphocholine	5000	5000
glycerol phosphate	5000	5000
glycerophosphocholine	5000	5000
choline	*d*	>150000
heparin	100	nd
dextran-sulfate	100	nd
dextran	*d*	*d*
galactose 1-phosphate	5000	5000
galactose 6-phosphate	5000	5000
galactose	*d*	*d*

[a] The molar ratio of DPPC/CHOL/CBS or bSM/CHOL/LCBS in the indicator liposomes was 1:0.75:0.1. [b] IC_{50} is the concentration of CBS, sulfate, or molecule indicated that inhibits lysis of DPPC/CHOL/LCBS or bSM/CHOL/LCBS indicator liposomes by 50%. [c] The inhibitors were diluted in VBS. Liposome inhibitors did not contain trapped tempocholine. The CBS/phospholipid molar ratio was 0.1, except as indicated. Phospholipid/CHOL molar ratio was 1.0:0.75. [d] No inhibition occurred up to a concentration of 300 000 μM. [e] nd, not determined.

CBS in a SM environment only poorly. However, SM/CHOL/CBS liposomes can be lysed by a concentration of antiserum only 2–3 times higher than required for DPPC/CHOL/CBS liposomes. This paradox is resolved by the fact that when the indicator liposomes were composed of SM/CHOL/LCBS, a much higher (19-fold) concentration of DPPC/CHOL/CBS liposomes was also required to achieve 50% inhibition than for SM/CHOL/CBS liposomes (Table IV). These data indicate that the CBS is recognized in PC and SM by separate subpopulations of antibody. The antibodies that bind preferentially to CBS in DPPC are referred to as P antibodies, and those that are specific for CBS in bSM are termed S antibodies. Thus, the difference in antiserum recognition of CBS in SM and DPPC vesicles as indicated by the k and c values is not an artifact due to differences in the ability of antibody and complement to cause lysis of the vesicles. Indeed, the difference in recognition of CBS in the two types of lipid environment by a specific population of antibodies is even greater than indicated by these values, which

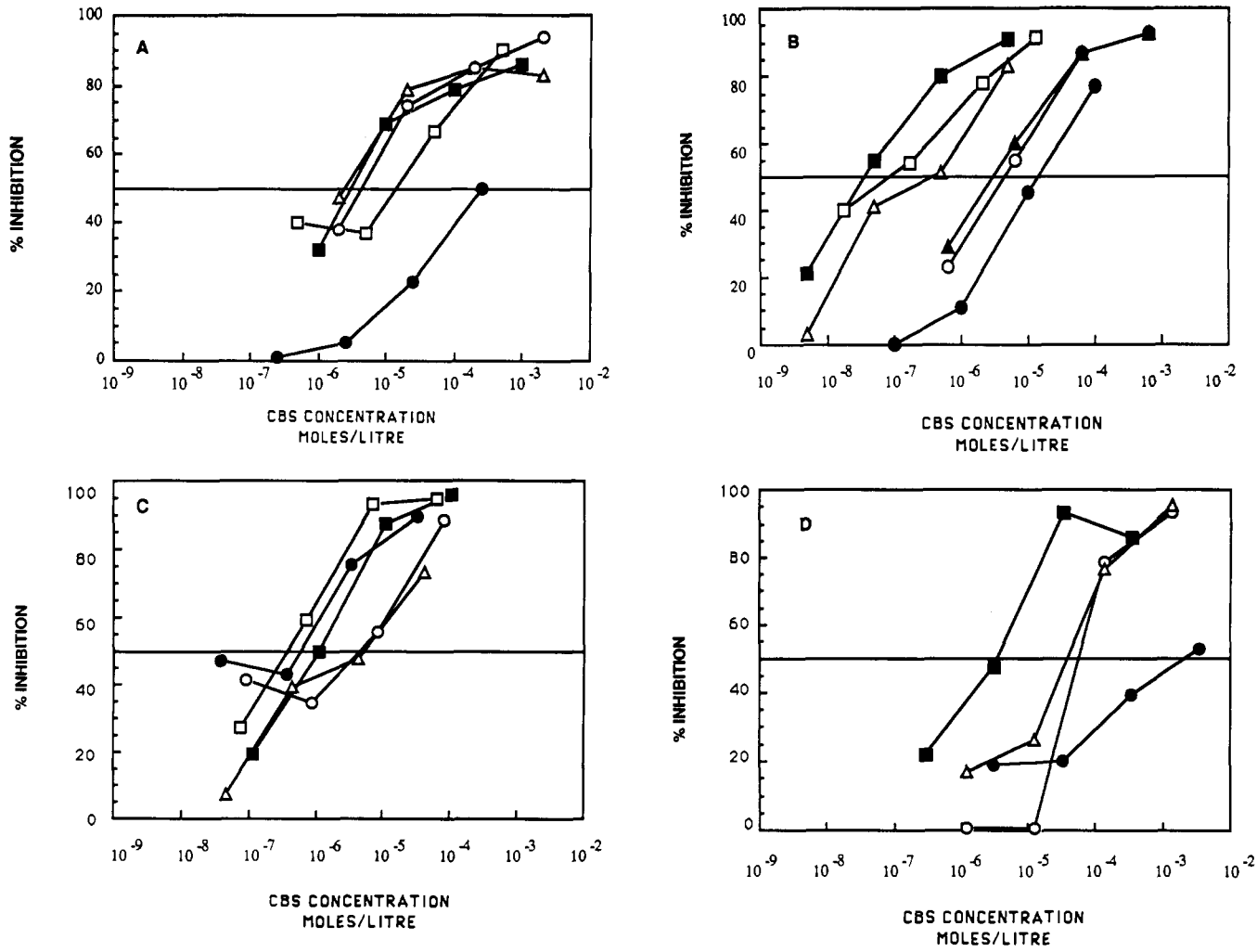

FIGURE 1: Inhibition of the lysis of liposomes containing DPPC/CHOL/LCBS (due to P antibodies) (A, B) or bSM/CHOL/LCBS (due to S antibodies) (C, D) by liposomes containing different molar ratios of CBS in DPPC or bSM. (A) Inhibition of the lysis of DPPC/CHOL/LCBS by bSM/CHOL/LCBS liposomes at CBS/bSM ratios of (△) 0.4, (○) 0.3, (■) 0.2, (□) 0.1, (●) 0.01. (B) Inhibition of the lysis of DPPC/CHOL/LCBS liposomes by DPPC/CHOL/LCBS liposomes at CBS/DPPC ratios of (○) 0.4, (▲) 0.3, (□) 0.2, (■) 0.1, (△) 0.05, and (●) 0.01. (C) Inhibition of the lysis of bSM/CHOL/LCBS by bSM/CHOL/LCBS liposomes at CBS/bSM ratios of (■) 0.4 and 0.3, (□) 0.2, (●) 0.1, (○) 0.025, and (△) 0.01. (D) Inhibition of the lysis of bSM/CHOL/LCBS by DPPC/CHOL/LCBS liposomes at CBS/DPPC ratios of (○) 0.4, (△) 0.3, (■) 0.1, and (●) 0.01. Concentrations are expressed as moles/liter CBS.

are averages for the whole antiserum. These results suggest that the conformation or structure of the antigenic epitope formed in SM vesicles must be different from that formed in DPPC vesicles.

Indeed, a difference in the organization of CBS in PC and SM was evident when the molar ratio of CBS contained in the inhibiting liposomes was varied. Reducing the CBS/bSM or CBS/DPPC ratio below the range 0.05–0.1 reduced the ability of either type of liposome to inhibit the lysis of the indicator liposomes, suggesting that a minimum antigenic density was required for optimal recognition of CBS, as had been shown for a number of other lipid haptens in liposomes (Uemura & Kinsky, 1972; Alving et al., 1974; Uemura et al., 1980) and in cells (Nores et al., 1987). Increasing the CBS/SM ratio to 0.2 improved the recognition of the antigen by P antibodies further (Figure 1A), although never to the level observed with a CBS/DPPC ratio of 0.1. However, it had little effect on the ability of the liposomes to inhibit lysis of the SM vesicles (Figure 1C). Thus, the optimal CBS/SM ratio for recognition by S antibodies was lower than for recognition by P antibodies.

Surprisingly, however, increasing the CBS/DPPC ratio above 0.2 reduced the recognition of CBS by both the P and S antibodies (Figure 1B,D), in contrast to results for other lipid haptens. In PC, it is clear that increasing the proportion of CBS altered the organization or conformation of the antigen such that recognition by both P and S antibodies was reduced below that observed at the optimal antigen density. This did not occur in bSM. This suggests that at high ratios, and possibly also at low ratios, the organization or conformation of CBS in DPPC was different from that in bSM. This may partially account for the binding to CBS in SM and PC by different populations of antibodies.

Fine Specificity of the Antibodies Binding to CBS in DPPC (P Antibodies). The antiserum had been shown to be specific for CBS with some cross-reactivity with the related glycolipid galactosylceramide (GalC), but no reactivity with DPPC/Chol vesicles (Crook et al., 1987). This was confirmed by immunoinhibition results shown in Table IV. However, the large dependence of antibody recognition of CBS on the lipid environment suggests that some structures present at the bilayer surface in addition to CBS (contributed by PC or cholesterol) might form part of the antigenic epitope recognized by the antibody. The anti-CBS antiserum was raised in rabbits by immunization with cholesterol-rich particles containing DSPC, cholesterol, and CBS. Therefore, the specificity of the P antibodies was probed further by testing the ability of various substances which share structural features with one or more

components of CBS in DPPC/CHOL liposomes to competitively inhibit the lysis of indicator liposomes containing DPPC/CHOL/LCBS. The specificity of the S antibodies was probed in a similar series of experiments using bSM/CHOL/LCBS indicator liposomes and will be considered separately.

Involvement of Charge. CBS is a negatively charged lipid, and thus it is likely that its recognition depends partly upon its charge. Inhibition by charged compounds increased in the order $Cl^- \ll SO_4^{2-} = PO_4^{2-}$ = small sulfated or phosphorylated compounds ≪ heparin = dextran sulfate ≪ CBS in liposomes (Table IV). This indicates that sulfate, either free or part of a small molecule, is part of the antigenic epitope and that cross-reaction with phosphate but not chloride occurs. Galactose 3-sulfate was not available (but see results on lyso-CBS below), but the charged monosaccharides, galactose 1-phosphate and galactose 6-phosphate, and two sulfated molecules, *p*-nitrocatechol sulfate and *p*-acetylphenyl sulfate, both inhibitors of the enzyme aryl sulfatase, were not any more effective than free sulfate or phosphate.

Inhibition by dextran sulfate and heparin occurred at a 50-fold lower concentration than required for sulfate free in solution. Dextran on the other hand did not inhibit. Both of the former macromolecules contain many sulfated carbohydrate residues. Their multivalent nature may contribute to their ability to inhibit lysis. Similarly, cholesterol sulfate in DPPC liposomes at a molar ratio of 0.75:1 was significantly more inhibitory than monovalent sulfate free in solution. Incorporation of cholesterol sulfate into liposomes permitted a multivalent form of sulfate to be presented to the antibody, and it also allowed sulfate to be presented together with phosphocholine and some features of the cholesterol molecule. However, cholesterol sulfate in DPPC/CHOL liposomes at ratios of DPPC/CHOL/CHOL-SO_4 of 1:0.65:0.1 was even less inhibitory than free sulfate.

Effect of Cholesterol and PC. Cholesterol is an important membrane constituent that is important for the recognition of many lipid haptens by their respective antibodies (McConnell, 1977). The removal of cholesterol from DPPC/CBS liposomes reduced their inhibitory ability by about 100-fold (Table IV). This effect of cholesterol was not due to a change in fluidity of the liposomes, however, since the difference in inhibition by gel-phase DPPC/CBS and liquid-crystalline-phase egg PC/CBS liposomes was not significant.

Removal of both PC and cholesterol from the liposomes also reduced the inhibitory ability of CBS by 50–400-fold, depending on the species of CBS. However, it should be pointed out that for a given CBS concentration, 18.5 times more total lipid was present when CBS was included in PC/CHOL liposomes than when by itself. These liposomes were multilayered, and thus much of the CBS was not exposed to antibody. If the size distribution and number of layers was similar for CBS by itself as when in PC/CHOL liposomes, the number of liposomes and total surface area was 18.5 times less for CBS by itself at a given CBS concentration. If the amount of antibody that can bind per liposome is limited, CBS by itself might be expected to be 18.5 times less inhibitory than DPPC/CHOL/CBS liposomes on the basis of these considerations alone. The much larger decrease observed for the inhibitory ability of pure CBS indicates that other factors are more important.

There were notable differences in the ability of CBS species with different fatty acid chain lengths to inhibit lysis in the absence of other lipids. This could be due to differences in the size of the vesicles, the tendency to form micelles, or the organization of the lipid in the bilayer, which could affect the lateral spacing between CBS head groups. Inclusion of cholesterol into CBS liposomes in the absence of PC did not markedly improve their inhibitory ability, but eliminated the dependence on the CBS chain length. Thus, both cholesterol and PC were required for maximal inhibitory ability of CBS; neither by itself was sufficient.

Lyso-CBS, which lacks a fatty acid chain and probably forms micelles in the absence of other lipids, inhibited even less than pure CBS when added alone. Indeed, it was only twice as effective as DPPC/CHOL-SO_4 and only a little more effective than dextran sulfate or heparin in spite of the fact that it contains 3-galactosyl sulfate, unlike the former two compounds. Incorporation of lyso-CBS into DPPC/CHOL liposomes reduced its recognition further. Thus, the entire ceramide structure was also necessary, in addition to PC and cholesterol, to maximize the inhibitory ability of the 3-galactosyl sulfate head group.

Recognition of Phosphocholine Moiety. Since CBS was best recognized in a DPPC/CHOL environment, inhibition by molecules which share structural features with PC was tested to determine if there is partial specificity for phosphocholine or for phosphocholine and cholesterol. However, liposomes containing only DPPC/CHOL did not inhibit (Table IV), and phosphocholine, glycerophosphocholine, or glycerophosphate inhibited at similar concentrations as phosphate (Table IV). Choline chloride, on the other hand, did not inhibit until the concentration reached 300 mM.

Inhibition by DPPE/CHOL/LCBS liposomes occurred at a 14-fold higher concentration than that by DPPC/CHOL/LCBS liposomes (Table IV). Unfortunately, it proved impossible to achieve consistent trapping of tempocholine and lysis of liposomes containing DPPE, and thus no information on concentration and affinity of the antibodies reacting with CBS in DPPE/CHOL could be obtained. Use of *N*-methyl-DPPE, which resembles DPPE more closely than DPPC, improved the recognition somewhat, but *N*,*N*-dimethyl-DPPE, which resembles DPPC more closely than DPPE, did not. These results suggest that phosphocholine could form part of the antigenic epitope, but only when CBS is present. However, CBS in SM was 3 times less inhibitory than in DPPE/CHOL liposomes, even though bSM has the same phosphocholine head group as PC. This could be due to effects of the bSM ceramide structure on CBS or a different conformation of phosphocholine in SM compared to that in PC (Barenholz & Thompson, 1980).

Fine Specificity of the Antibodies Binding to CBS in bSM (S Antibodies). The specificity of the antibodies binding to CBS in bSM was examined by comparing the ability of various substances to inhibit the lysis of bSM/CHOL/LCBS liposomes. The data are summarized in Table IV. Overall, the S antibodies were less specific for membrane-bound antigen than the P antibodies. If the decrease in total lipid concentration (resulting in a decrease in the number of liposomes) is taken into account, LCBS by itself was nearly as inhibitory as when it was in SM/CHOL liposomes. However, removal of cholesterol from SM/CHOL/LCBS liposomes reduced recognition by 63-fold, while removal of SM instead of cholesterol reduced recognition by 33-fold. These differences could not be accounted for by the reduction in the total amount of lipid. Thus, if auxiliary lipids are present, both SM and CHOL must be present together to achieve maximal recognition of CBS by the S antibodies.

The S antibodies, like the P antibodies, had only minor reactivity with sulfate and phosphate and other small sulfated

Table V: Competitive Inhibition of Liposomes Containing DPPC/CHOL/PA/DNP-PE[a] by Different Soluble and Membrane-Bound Substances

inhibitor[b]	IC_{50}[c] (μM)
Soluble Inhibitors	
DNP	1
DNP-lysine	0.01
NaCl	>150000
Na_2SO_4	>150000
heparin	10000
Membrane-Bound Inhibitors	
DPPC/CHOL/DNP-PE	1
bSM/CHOL/PA/DNP-PE	1
DNP-PE (only)	1

[a] The molar ratio of the DPPC/CHOL/PA/DNP-PE indicator liposomes was 1:0.75:0.1:0.1. [b] All inhibitors were diluted in VBS. Liposome inhibitors did not contain trapped tempocholine, and the molar ratios were the same as for indicators, except for the DNP-PE only liposomes. Heparin is given as moles of sulfate. [c] IC_{50} is the concentration of inhibitor that inhibits the lysis of the DPPC/CHOL/PA/DNP-PE by 50%.

or phosphorylated compounds. However, inhibition by lyso-CBS by itself required only a 50-fold higher concentration relative to CBS in bSM/CHOL, in contrast to the 650-fold higher concentration of lyso-CBS, relative to CBS in DPPC/CHOL, required to inhibit P antibody binding. Thus, although auxiliary lipids and intact CBS improved recognition of the 3-galactosyl sulfate head group by S antibodies, they were not as necessary as for recognition by P antibodies.

Inhibition of Lysis of DNP-PE Liposomes. Inhibition studies were performed by using the DNP-PE/anti-DNP antibody system to determine (i) if the inhibition of antibody binding to CBS observed with charged compounds could also be seen with an uncharged, glycerol-based lipid hapten and (ii) if the lipid environment influenced recognition of a lipid hapten by an antibody raised to the hapten bound to BSA rather than raised to a complex lipid mixture. Inhibition of the lysis of CBS liposomes with Na_2HPO_4 and Na_2SO_4 and other charged compounds could have resulted from nonspecific effects. However, when these inhibitors were added to the DNP-PE liposome indicator system (composed of DPPC/CHOL/PA/DNP-PE), inhibition did not occur until concentrations greater than 150 mM (Table V), much higher than required to inhibit lysis of CBS-containing liposomes. In addition, inhibition by heparin occurred only at concentrations 100-fold higher than required for CBS. This confirms that the inhibition of lysis of the CBS-containing liposomes by sulfate and heparin was specific. The only compound to inhibit lysis of the DNP-PE-containing liposomes more effectively than free DNP was DNP-lysine. Since the hapten was coupled to albumin via lysyl residues to raise the antibody, it is not surprising that both lysine and DNP form part of the antigenic determinant.

As shown in Table V, free DNP inhibited the lysis of DNP-PE-containing liposomes equally as well as DNP-PE in DPPC/CHOL/PA liposomes, when compared on a molar basis. However, if the fact that only 10–20% of the DNP-PE in the inhibitor liposomes was available to bind antibody is taken into consideration, then it can be concluded that liposomal DNP was more inhibitory than free DNP. Dispersions containing DNP-PE only also inhibited at similar DNP concentrations to DNP-PE in DPPC/CHOL/PA liposomes. However, because there was a 19.5 times larger total amount of lipid, and probably a correspondingly greater number of liposomes for the latter, these data indicate that DNP-PE was actually recognized better by itself than in the presence of auxiliary lipids, in contrast to CBS. In addition, there was no difference in the ability of DNP-PE in a SM or PC environment to inhibit lysis, also in contrast to the behavior of CBS.

Discussion

In this and an earlier study we have examined the influence of the membrane environment and ceramide composition of CBS on its recognition by antibody. Our earlier study showed that in a PC environment both the CBS ceramide composition and PC chain length influenced the recognition and that, relative to PC, the recognition of CBS was reduced in bSM (Crook et al., 1986). In the present study we investigated the difference between PC and SM in more detail. These studies, based on determination of relative k and c values for antibody binding by the Vistnes (1984) model, rely on the assumptions that the surface area of the liposomes and relationship between degree of lysis and numbers of bound antibody molecules do not vary with lipid composition. Therefore, we have also carried out immunoabsorption and immunoinhibition studies with different types of liposomes and obtained a measure of their relative surface areas. The results support our conclusions based on the k and c values and indicate that these results are not an artifact due to differences in surface area or susceptibility to lysis of these liposomes.

In addition to confirming our earlier conclusion (Crook et al., 1986) that antibodies which bound to POHCBS were higher affinity antibodies than those which bound to LCBS, the immunoabsorption studies with liposomes containing POHCBS and LCBS also showed that antibodies which bound to POHCBS had a similar specificity as those which bound to LCBS. This suggests that the restriction of antibodies which can bind to POHCBS in PC to a small pool of high-affinity antibodies is probably due to decreased accessibility rather than altered conformation of the head group, as a result of hydroxylation of the fatty acid.

The increasing degree of motional restriction of the spin-labeled fatty amine H-TEMPO with increasing chain length of PC reported in this study also supports our earlier conclusion that the reduced recognition of CBS in longer chain length PCs is due to decreased exposure as a result of an increase in bilayer thickness. Thus, relatively small changes in the lipid environment, such as a change in the fatty acid chain length of PC, can cause subtle changes in exposure of the CBS head group such that antibody populations of differing size and intrinsic affinity in the antiserum could be distinguished.

In the present study we showed further that a greater change in lipid environment, produced by exchanging SM for DPPC, caused such a large change in conformation or structure of the antigenic epitope that antibodies of significantly different specificity (P and S antibodies) in the antiserum could be distinguished. Binding of the S antibodies to CBS did not depend as much on the ceramide structure of CBS, particularly in bovine brain SM, or on the fatty acid chain length of SM, as found for P antibodies with PC. Studies comparing the specificity of the P and S antibodies revealed that both were specific for sulfogalactosyl residues but bound maximally to the membrane form of the antigen in PC or SM, respectively. Thus, the specificity differences of the two different antibody populations are not for different chemical groups but for different conformational or organizational epitopes. The ability of these antibodies to distinguish between CBS in PC and in SM could be due to (i) differences in the organization of CBS or exposure and conformation of its head group in PC and SM and/or (ii) differences in specificity for liposomal components in addition to CBS.

Altered Exposure and Conformation. Greater penetration of CBS into the bSM bilayer relative to DPPC could result in decreased exposure and/or alteration of the conformation of the carbohydrate head group. Studies with a number of other lipid haptens, including GalC, Forssman antigen, and lipopolysaccharide, have shown that their recognition also is reduced in bSM compared to that in DPPC, and this has been attributed to the longer fatty acid chain lengths of bSM (Katakoa et al., 1974; Alving et al., 1974; Alving & Richards, 1977; Kinsky & Nicolotti, 1977). The fact that the greatest motional restriction of H-TEMPO was found in bSM may support the idea that the reduced recognition of CBS and other glycolipids in bSM could be the result of reduced exposure.

Results obtained on the recognition of CBS in egg SM and rbc SM, which contain predominantly C16:0 and C24:0 fatty acids, respectively (Calhoun & Shipley, 1979), are partially consistent with this conclusion, since the antibody bound to LCBS in egg SM, which is expected to have a smaller bilayer thickness, with a higher affinity than in rbc SM. However, the degree of motional restriction of H-TEMPO in the different types of SM did not depend on the length of their fatty acid chains. Possibly the bilayer thickness of SM did not change much with fatty acid chain length in the presence of cholesterol because of interdigitation of the long fatty acid chains into the other side of the bilayer.

Alternatively, other structural features of SM such as the amide linkage and the sphingosine hydroxyl group, which may be involved in intermolecular hydrogen bonding, may make the bilayer more ordered and contribute to the greater motional restriction of H-TEMPO in bSM. These features may also allow interactions with similar groups in CBS that cannot occur in PC. This may cause the conformation of the CBS head group to differ in the two lipid environments. Differences in organization of CBS in PC and SM are supported by the fact that an increase in the molar ratio of CBS in DPPC altered the antigenic structure or organization in the bilayer such that both pools of antibody recognized the antigen only poorly, while this did not occur in SM. The importance of the structural features of CBS for differences in its organization in DPPC and SM is supported by the similarity of anti-DNP antibody recognition of the glycerol-based lipid DNP-PE in DPPC and SM.

Specificity for Multivalent Antigen or Other Liposomal Components. The P antibodies had a greater requirement for the membrane form of the antigen than S antibodies. The inhibition studies showed that the P antibodies were specific for CBS in a certain conformation together with PC and CHOL, rather than for the 3-galactosyl sulfate head group by itself. The S antibodies could recognize the CBS head group by itself more readily, although a conformation not found in DPPC/CHOL was required. These conclusions are supported by the fact that the antigenic state which was recognized by the P antibodies occurred also in bSM if the CBS/bSM ratio was increased, while that which was recognized by S antibodies was independent of the CBS/bSM ratio. The fact that CBS and lyso-CBS by themselves were more poorly recognized by P antibodies than by S antibodies also supports this notion. Fredman et al. (1988) have reported that a monoclonal anti-CBS antibody also recognized lyso-CBS poorly in comparison to intact CBS. Since lysosulfatide probably forms micelles that exchange with monomers in solution, it allows monovalent sulfated galactose to be presented to antibody in the absence of a membrane environment. For a given sulfate concentration it also allows presentation of a greater proportion of the antigen than for intact CBS by itself in multilamellar liposomes. The poor recognition of lyso-CBS in spite of its greater availability underscores the importance of the membrane environment and ceramide composition for recognition. It is difficult to see how the fatty acid chain could be part of the antigenic determinant since it is hidden in the hydrophobic portion of the bilayer. Rather, it seems more likely that the fatty acid chain is necessary for CBS to be organized properly in the bilayer or to confer the correct conformation on the head group. Indeed, the fact that lyso-CBS was recognized much less in PC (or SM)/CHOL liposomes than by itself indicates that it takes a very different conformation and organization in the bilayer than does intact CBS.

The greater requirement for the membrane-bound form of the antigen for P antibody binding could be due to the presence of several molecules of 3-galactosyl sulfate in the epitope. Studies by Kabat and colleagues (Kabat et al., 1988; Chen et al., 1987; Kabat, 1985) have shown that antibodies to carbohydrate determinants generally have antibody combining sites which are seven to nine carbohydrate residues in length. Thus, the dependence of P antibody binding to CBS on epitope density, as well as the much better inhibition observed with multivalent dextran sulfate and heparin than with monovalent charged compounds, may indicate that more than one CBS head group occupies the binding site of the antibody molecule. This may require that the carbohydrate moieties on the surface of the bilayer be appropriately spaced. The dependence of the immunoinhibitory ability of pure CBS (in the absence of auxiliary lipids) on its fatty acid chain length supports this idea. Similar results were found by Kawaguchi (1987) for binding of monoclonal anti-PC antibodies to PC liposomes when the PC chain length was varied. It has been reported that the distance between PC head groups on the surface of the liposomes depends on the fatty acid chain lengths of this lipid (Cornell & Separovic, 1983).

A number of other studies have also indicated the importance of spacing for the recognition of lipid haptens. Phase separation of glycolipids and other lipid haptens away from the auxiliary lipids and into domains enriched in the hapten often decreases their recognition by antibody or enzymes, while a more random distribution improves it (Humphries & McConnell, 1975; Utsumi et al., 1984; Masserini et al., 1988). On the other hand, the greater specificity of anti-PE antibodies for the hexagonal phase compared to the lamellar phase of PE found by Janoff and Rauch (1986) suggests that these antibodies may recognize a more densely packed configuration of the lipid head groups. Differences in the degree of phase separation of CBS in DPPC and SM may occur and affect its lateral spacing, leading to differences in recognition by the P and S antibodies. Studies of CBS–DPPC mixtures by calorimetry suggest that they are relatively miscible regardless of ceramide composition (Boggs et al., 1990) and would probably be randomly mixed in the presence of cholesterol. However, mixtures of CBS–SM have not yet been studied.

It is also possible that one or more cholesterol and phosphocholine head groups occupy the antibody combining site in addition to CBS, especially for P antibodies. This is supported by the decreased recognition of CBS in DPPE and methylated DPPE/CHOL liposomes. Epitopes consisting of a complex of two or more lipids have been found to be required for a series of anti-phospholipid monoclonal antibodies by Alving and co-workers (Banerji et al., 1982; Wassef et al., 1984; Alving, 1986) and for a monoclonal anti-glycolipid antibody from a patient with combined chronic lymphocytic leukemia and neuropathy (Freddo et al., 1986). The latter

example of a disease in which the neuropathy may be mediated by the humoral immune response indicates that such epitopes can have physiological relevance. In contrast, we found that recognition of a lipid hapten, DNP-PE, by an antibody raised against DNP bound to BSA is decreased by the presence of auxiliary lipids. This antibody recognizes single or multiple molecules of DNP rather than a complex of several different lipids.

Further studies of the organization, conformation, and disposition of CBS in these lipid mixtures by a number of techniques will be necessary to explain the dependence of antibody recognition on lipid composition.

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