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Association of gangliosides with the lymphocyte plasma membrane studied using radiolabels and spin labels

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Gangliosides are known to act as potent suppressors of lectin-stimulated lymphocyte activation when added to the culture medium. Since this effect may be mediated via ganglioside association with (or insertion into) the plasma membrane, we have used ^{3}H- and spin-labelled derivatives of mixed gangliosides to probe the nature of this interaction. Gangliosides bind rapidly to the lymphocyte membrane and show no preference for association with either inside-out or right-side-out membrane vesicles. Around 20% of the bound gangliosides can be removed by repetitive washing, and a further 22–28% by treatment with pronase for 1 h, suggesting that this fraction is tightly bound to membrane proteins at the cell surface. The ESR spectrum of membrane-bound gangliosides did not resemble the spin-exchanged spectrum of micellar spin-labelled gangliosides in aqueous solution, but was similar to that seen for 5 mol% ganglioside spin label in liposomes of egg phosphatidylcholine. This suggests that the bulk of the membrane-bound gangliosides are inserted and molecular dispersed in the lymphocyte membrane. Binding of wheat-germ agglutinin to lymphocyte-associated gangliosides results in specific immobilization of the carbohydrate headgroup, while concanavalin A and other lectins have little or no effect on oligosaccharide mobility. Membrane-inserted gangliosides show a response to lectin binding which is qualitatively different from that seen for gangliosides in bilayers of phosphatidylcholine.

Introduction

The gangliosides are a family of complex glycosphingolipids containing the acidic sugar sialic acid, which are ubiquitous in the plasma membranes of eucaryotic cells. They act as receptors for a variety of external agents, such as cholera toxin, viruses, interferons, hormones and lectins, and have been implicated in a variety of important biological processes including the control of cell morphology and differentiation, contact inhibition, neoplastic transformation, synaptic transmission and lymphocyte activation [1–4]. More recently, exogenous gangliosides have been observed to be potent suppressors of lectin- and MLC-induced lymphocyte activation in vitro, in both murine and human systems [5–7]. These in vitro observations have been linked to in vivo studies where certain cancer patients and tumor-bearing animals were found to have high circulating levels of gangliosides, presumably shed from the surface of tumor cells [8]. These patients were also found

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ESR, electron spin resonance; PC, phosphatidylcholine; Tempamine, 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl; TIBS, 2,4,6-triisopropylbenzenesulfonyl chloride.

to be highly immunosuppressed and it has been suggested that this immunosuppression, which may play a major role in various tumor-induced pathological states, is most probably due to the presence of circulating gangliosides [6–8].

Gangliosides are well-known for their ability to transfer from the external medium, where they exist in micellar form, to the outer leaflet of lipid bilayer vesicles and the plasma membrane of intact cells [9–11] where they appear to retain their receptor-binding functions [12–14]. Gangliosides are also capable of binding to the surface of intact lymphocytes, and it has been suggested that ganglioside-induced immunosuppression requires insertion of exogenous gangliosides into the plasma membrane, followed by later events, either on the membrane surface or within the cell, which block the activation process [5,7,15].

At present, the exact nature of the association between exogenously added gangliosides and the lymphocyte plasma membrane is not clear. We have attempted, in this study, to use the technique of spin labelling to answer some questions concerning the nature of this association, and to shed more light on the molecular behavior and dynamics of these associated gangliosides within the lymphocyte membrane.

Materials and Methods

Lymphocyte plasma membrane was isolated from pig mesenteric lymph nodes as described by Maeda et al. [16]. Approx. 30 g of tissue was processed during one preparation and the resulting plasma membrane vesicles were stored frozen in aliquots at −70°C. Plasma membrane vesicles used for spin-labelling experiments were thawed only once. Assays for 5′-nucleotidase at various stages of the lymphocyte membrane isolation were carried out by the method of Michell and Hawthorne [17], and released inorganic phosphate was quantitated as described by Kates [18]. The 5′-nucleotidase specific activity of the plasma membrane preparation was enriched more than 12-times with respect to lymphocyte homogenates.

Membrane protein was determined by the method of Peterson [19] using bovine serum albumin (Sigma, crystallized and lyophilized) as a standard.

Concanavalin A, wheat-germ agglutinin, α-methylmannoside and *N*-acetylglucosamine were obtained from Sigma.

Mixed bovine brain gangliosides were isolated and purified as described previously [20].

Synthesis of ^{3}H-labelled mixed bovine brain gangliosides. Mixed bovine brain gangliosides were radiolabeled on sialic acid residues with NaB^3H_4 (Amersham, specific activity 228 mCi/mmol) using the method of Veh et al. [21]. The final specific activity of the radiolabelled ganglioside preparation was approx. 22 mCi/mmol.

Synthesis of spin-labelled gangliosides. Mixed bovine brain gangliosides were spin-labelled randomly on sugar primary hydroxyl groups with 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl (Aldrich) using 2,4,6-triisopropylbenzenesulfonyl chloride (Aldrich) as described by Sharom and Grant [20]. The final product was purified by silicic acid chromatography (Bio-Sil A, Bio-Rad) eluting with 20–70% $CHCl_3$/MeOH.

Gangliosides were spin-labelled on sialic acid residues using the method of Lee et al. [22]. This involves specific activation of sialic acid side-chains with sodium metaperiodate, followed by reductive amination with Tempamine (Aldrich) and sodium borohydride. The extent of labelling of the final product was around 0.5–0.6 spin labels per ganglioside.

Both ^{3}H-labelled and spin-labelled gangliosides were prepared for addition to lymphocyte plasma membrane by mixing appropriate amounts of each with unlabelled ganglioside (if required) in $CHCl_3$/MeOH and pumping extensively under vacuum to remove traces of solvent. Dried gangliosides were redissolved in buffer using a vortex mixer and small-diameter glass beads.

Liposomes containing gangliosides were prepared by mixing appropriate amounts of labelled gangliosides with egg PC (Sigma, purity greater than 98–99%) in $CHCl_3$/MeOH, pumping extensively under vacuum to remove organic solvents and resuspending in buffer using a vortex mixer and small-diameter glass beads.

Incorporation of labelled gangliosides into lymphocyte plasma membrane. Washed lymphocyte plasma membrane vesicles (25–30 mg protein in 600 μl 10 mM Hepes-buffered saline, pH 7.4) were mixed with 2 mg of spin-labelled mixed bovine

brain gangliosides containing approx. 2 μCi of ^{3}H-labelled material (if required). The mixture was incubation at 37°C for 2 h and the membrane was washed repeatedly with Hepes-buffered saline (pH 7.4) (30 min spins at 4°C at $50000 \times g$ in the ultracentrifuge) to remove unbound gangliosides. Following each wash, aliquots of the resuspended membrane vesicles were removed for ESR spectroscopy and liquid scintillation counting. To investigate the effects of repetitive washing on ganglioside association with the plasma membrane, a total of six washes was carried out, while for ESR experiments in general, four washes were routinely used. Lymphocyte membrane samples were resuspended in Hepes-buffered saline (pH 7.4) at a concentration of around 20–30 mg/ml protein and aliquots of this were used for ESR spectroscopy.

To quantitate ganglioside release by various agents, aliquots of membrane containing both ^{3}H- and spin-labelled gangliosides (50 μl, 1 mg protein) were incubated with 500 μl of Hepes-buffered saline (pH 7.4) containing either pronase (Sigma) at 1% or 5% of the weight of membrane protein, or 1 unit of neuraminidase (*Clostridium perfringens*, Sigma). After 1 h at 37°C, the membrane vesicles were sedimented by rapid centrifugation and the pellet resuspended in Hepes-buffered saline (pH 7.4). Pellets and supernatants were analysed by both liquid scintillation counting and ESR spectroscopy.

Time-course of ganglioside binding to lymphocyte plasma membrane. Lymphocyte plasma membrane vesicles in Hepes-buffered saline (pH 7.4) were added to ^{3}H-labelled gangliosides as described above. The mixture was incubated at 37°C and aliquots were removed for analysis at various times between 0 and 120 min after mixing. Ganglioside micelles were separated from the plasma membrane vesicles by rapid centrifugation through a column of Sepharose CL-4B (Pharmacia) as described by Felgner et al. [24]. Vesicles containing associated ganglioside were collected at the bottom of the column, while ganglioside micelles remained trapped on the column. No labelled gangliosides could be detected in the column effluent in the absence of membrane vesicles. Recovered vesicles were analysed for both total protein and radioactivity and the association of ganglioside with the vesicles was expressed as cpm per mg membrane protein recovered.

Separation of inside-out and right-side out membrane vesicles. Lymphocyte membrane vesicles containing ^{3}H- and spin-labelled gangliosides (7 mg protein in 500 μl) were applied to a 25 ml column of concanavalin A-Sepharose 4B (Pharmacia) in Hepes-buffered saline (pH 7.4) at 4°C and eluted with the same buffer. Fractions (500 μl) were collected and the absorbance at 280 nm and the protein content were determined for each. The void-volume fractions containing the inside-out membrane vesicles were pooled and the total spin-labelled ganglioside, ^{3}H-labelled ganglioside and protein in this peak were determined.

Effect of lectins on lymphocyte membrane-associated gangliosides. Lymphocyte membrane vesicles containing incorporated spin-labelled gangliosides (25 μl, about 625 μg protein) were mixed with 25 μl of the required concentration of the chosen lectin in Hepes-buffered saline (pH 7.4). Binding was allowed to take place for 30 min at room temperature before recording the ESR spectrum.

Alternatively, 25 μl of membrane vesicles containing spin-labelled gangliosides were mixed with 250 μl of the required concentration of lectin and incubated for 30 min at room temperature. The membrane vesicles were then sedimented and resuspended in Hepes-buffered saline (pH 7.4) to give a final volume of 50 μl, prior to recording the ESR spectrum.

ESR measurements. ESR spectra were recorded on a Varian E-104 spectrometer at room temperature (23°C) using 50-μl glass capillary sample tubes. Spin label motional frequency was characterized by calculation of a rotational correlation time τ_c as described previously [23] using the equation:

$$\tau_c = 6.5 \cdot 10^{-10} W_0 \left[(h_0/h_{-1})^{1/2} - 1 \right]$$

where W_0 is the width of the midfield line and h_0 and h_{-1} are the heights of the mid- and highfield spectral lines respectively. This equation is valid only for fast, isotropic motion. The overall linewidths of the membrane-associated ganglioside label suggest that there may be slow motional contributions and there is also the possibility of anisotropic motion, so that the experimental τ_c

values are only apparent values. These apparent τ_c values are useful for comparative purposes, even though they may not reflect absolute values of motional frequency.

Results and Discussion

Association of labelled gangliosides with lymphocyte plasma membrane

In order to investigate the association of gangliosides with the lymphocyte plasma membrane, we synthesized one radiolabelled and two different spin-labelled derivatives. Mixed gangliosides from bovine brain, which consist of a complex mixture of mono-, di- and trisialo species, were specifically labelled on sialic acid residues with either a ^3H label or a spin label, as outlined in Fig. 1. An alternative method for spin labelling gangliosides randomly on the oligosaccharide chain involved the linking of a 5-membered ring spin label carboxylate to sugar primary hydroxyl groups, using TIBS as a coupling agent. Both these ganglioside spin labels have been studied extensively in the past using lipid bilayer systems, where they made possible the probing of ganglioside behavior at the molecular level (for example, Refs. 20 and 22).

Incubation of lymphocyte plasma membrane vesicles with ^3H-labelled mixed gangliosides at 37°C results in relatively rapid uptake of the glycolipid, as shown in Fig. 2. The bulk of the association occurred during the first 20–30 min of incubation, and was followed by a slower uptake phase which levelled-off after about 2 h. All further ganglioside-membrane association experiments were carried out at 37°C for a 2-h time period, to ensure close to maximal incorporation of the glycolipids.

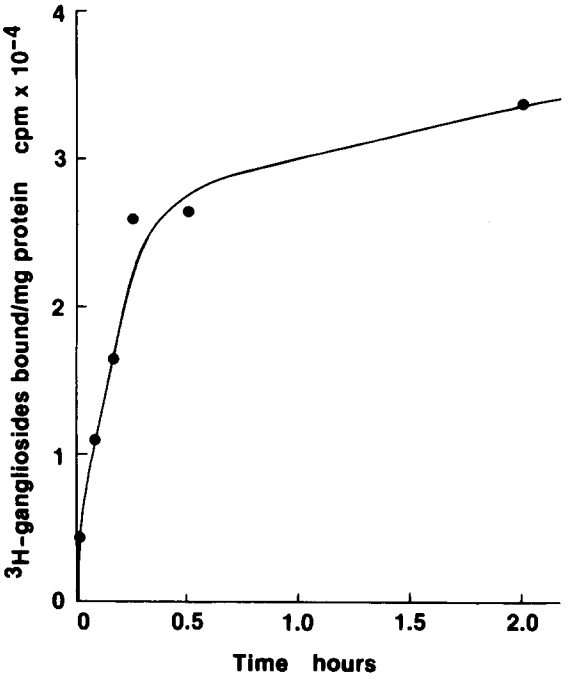

Fig. 2. Time-course of association of mixed bovine brain gangliosides with lymphocyte plasma membrane. Lymphocyte plasma membrane vesicles (25–30 mg protein in 600 μl Hepes-buffered saline, pH 7.4) were mixed with 2 mg of gangliosides containing approx. 2 μCi of the ^3H-labelled derivative. Following incubation at 37°C, aliquots were removed at various times and the ganglioside micelles separated from the membrane vesicles by rapid centrifugation through a Sepharose CL-4B column. Association of the ^3H-labelled ganglioside with the membrane is expressed as cpm per mg membrane protein recovered.

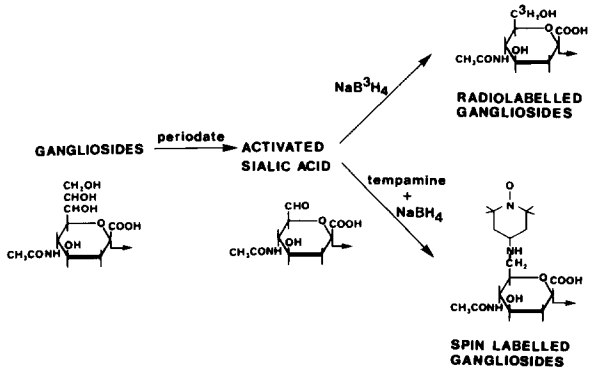

Fig. 1. Outline of the scheme used for specific labelling of mixed bovine brain gangliosides on sialic acid residues, using either a radiolabel or a spin-label tag.

Incubation of 2 mg of labelled gangliosides at a concentration of 3 mg/ml, with lymphocyte plasma membrane vesicles (25–30 mg protein) for 2 h at 37°C results in around 48–58% association of both spin label and radiolabel markers with the membrane following the first wash. Thus, under these conditions a relatively high fraction of the labelled ganglioside can be incorporated into plasma membrane vesicles, and the presence of a spin label

'tag' appears to have little effect on this process. Assuming the plasma membrane has a lipid/protein ratio of around 1:1, w/w, this corresponds to an initial added ganglioside/membrane lipid weight ratio of around 0.07 and a modified membrane composition of an additional 3–4%, w/w gangliosides following incubation. These observations are consistent with the results of Felgner et al. [24] who showed that for low weight ratios of added ganglioside to PC (less than about 0.05–0.08 depending on the ganglioside species) almost all of the ganglioside could be incorporated into preformed PC vesicles by simple addition to the buffer. Addition of increasing amounts of ganglioside resulted in decreasing proportions of ganglioside incorporated, and the limiting final ganglioside composition of these modified vesicles was 25–30%, w/w (12–15 mol%).

The lymphocyte plasma membrane used in our studies consists of a mixture of inside-out and right-side-out unilamellar vesicles, so we investigated whether micellar gangliosides showed any preference for association with right-side-out vesicles which possess a surface glycocalyx. The right-side-out vesicle subpopulation may be removed from the membrane preparation by passage down a column of concanavalin A-Sepharose, to which they bind irreversibly [25]. Concentrations of methyl α-mannoside as high as 1.0 M fail to elute a significant fraction of the right-side-out vesicles. The inside-out vesicle population is recovered in the void volume of the column, and may be monitored using either absorbance measurements (light scattering) or protein recovery (see Fig. 3). Control experiments on unmodified plasma membrane preparation showed that about 45–55% of the membrane protein was recoverable in the inside-out fraction, which agrees with previous observations by Walsh et al. [25]. When plasma membrane which had been previously treated with ^{3}H- and spin-labelled gangliosides was subjected to concanavalin A-Sepharose 4B chromatography, about 50% of the label was recovered in the inside-out vesicle fraction. Thus, it appears that gangliosides show no particular preference for transfer to vesicles with an existing glycocalyx over those without such a surface coat.

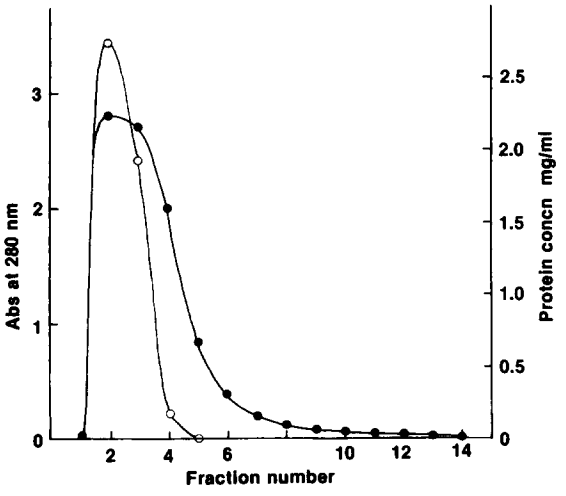

Fig. 3. Separation of inside-out membrane vesicles using concanavalin A-Sepharose 4B affinity chromatography. Lymphocyte plasma membrane vesicles containing ^{3}H- and spin-labelled mixed gangliosides (7 mg protein in 500 μl) were applied to a 25 ml column of concanavalin A-Sepharose 4B in Hepes-buffered saline (pH 7.4) at 4°C and eluted with the same buffer. The absorbance at 280 nm (●———●) and the protein content (○———○) were measured for each fraction and the total ^{3}H-labelled and spin-labelled ganglioside in the void volume peak was determined.

Nature of the association between gangliosides and the lymphocyte plasma membrane

There has been much interest in recent years in examining the molecular details of the association between gangliosides or ganglioside analogues and the plasma membranes of several different cell types [11,26–29]. We have used a variety of approaches to attempt to shed some light on the nature of the association between lymphocyte plasma membrane and exogenous gangliosides. Repetitive washing of lymphocyte plasma membrane preparations following a 2-h treatment with exogenous gangliosides produced a gradual decline in the amount of associated ganglioside. Around 20% of the initial associated label could be removed by six successive high-speed washes of the membrane in ganglioside-free buffer (see Fig. 4). The results suggest that a significant fraction of the membrane-associated ganglioside is only loosely bound to the cell surface. Labelled membrane vesicles (washed four times) were also subjected to treatment with both proteolytic enzymes, such as pronase, and *Clostridium perfringens* neuraminidase. Table I shows that a 1 h incubation of

membranes and associated gangliosides with pronase results in a maximum of 22–28% release of ^3H and spin label into the supernatant. These observations suggest the existence of a second class of membrane-associated gangliosides which is more tightly bound (and thus not removed easily by washing) but which is releasable by proteinases, indicating that the glycolipid is bound to proteins at the surface of the membrane vesicles. The proteinase-sensitive fraction of the associated ganglioside is considerably smaller in this case than that previously reported for a mouse fibroblast cell line treated with ganglioside analogues, where 65–75% of the associated glycolipid could be removed by trypsin [11,27]. These discrepancies may reflect the differences in molecular structure between naturally occurring gangliosides and the synthetic ganglioside analogues used in these previous studies. The remaining membrane-associated gangliosides are suggested to form a third class of molecules which is inserted into the hydrophobic bilayer region of the lymphocyte plasma membrane.

Neuraminidase treatment of the ganglioside-labelled washed membrane produced interesting results. A large fraction of the ganglioside-bound ^3H-labelled sialic acid is released by neuraminidase as expected. However, no additional spin-labelled sialic acid was released by the enzyme relative to the control (see Table I). The most likely explanation for this observation is that sialic acid which has been chemically modified by addition of a bulky Tempo ring is an unacceptable substrate for neuraminidase. Radiolabelling of the sialic acid moiety involves less drastic structural changes (loss of two carbons from the side-chain) and presumably the tritiated derivative can still be cleaved by the enzyme.

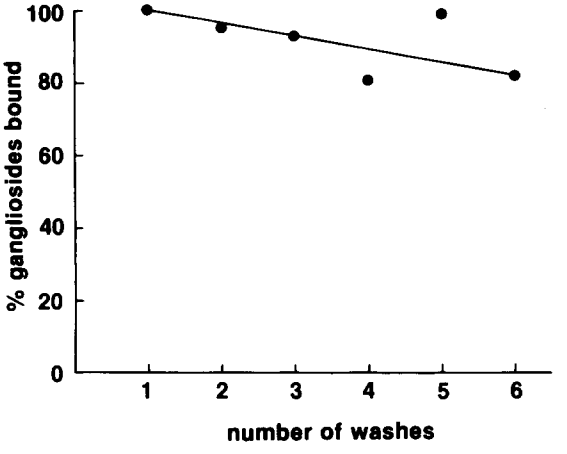

Fig. 4. Effect of repetitive washing on the amount of lymphocyte membrane-associated gangliosides. Washed lymphocyte plasma membrane (25–30 mg protein in 600 μl Hepes-buffered saline, pH 7.4) was mixed with 2 mg of spin-labelled mixed bovine brain gangliosides containing approx. 2 μCi of ^3H-labelled material. Following a 2 h incubation at 37°C, the membrane was washed repeatedly in the ultracentrifuge with Hepes-buffered saline (pH 7.4) to remove unbound gangliosides. Following each wash, aliquots of resuspended membrane were removed for ESR spectroscopy and scintillation counting.

TABLE I

EFFECT OF VARIOUS TREATMENTS ON ASSOCIATION OF MIXED BOVINE BRAIN GANGLIOSIDES WITH LYMPHOCYTE PLASMA MEMBRANE

Lymphocyte plasma membrane was treated with ^3H- and spin-labelled mixed bovine brain gangliosides and aliquots of labelled membrane (50 μl, 1 mg membrane protein) were incubated with 500 μl of 10 mM Hepes-buffered saline (pH 7.4) containing either pronase or 1 unit of neuraminidase for 1 h at 37°C. Membrane vesicles were sedimented by rapid centrifugation and the pellet resuspended in Hepes-buffered saline (pH 7.4). Pellets and supernatants were analysed by both liquid scintillation counting and ESR spectroscopy.

Treatment	% Ganglioside removed	
	^3H label	spin label
Buffer only	5	13
1% pronase	16	20
5% pronase	22	28
Neuraminidase (1 unit)	31	12

Further details of the molecular nature of the tightly bound ganglioside fraction can be obtained by studies using the headgroup spin-labelled ganglioside derivative. Fig. 5 shows the ESR spectra of sialic acid spin-labelled gangliosides in organic solvent (1 : 1, v/v, $CHCl_3$/MeOH) and aqueous buffer at a concentration of 3 mg/ml. The spectrum seen in $CHCl_3$/MeOH is relatively homogeneous, and does not show any evidence of strong spin-exchange broadening. Thus, the aggregate size of headgroup spin-labelled gangliosides in this solvent is probably fairly small.

In contrast, the ESR spectrum seen for

spin-labelled gangliosides in buffer shows evidence of both a heterogeneous environment and strong spin-exchange broadening. The spectrum (see Fig. 5) consists of a very broad spin-exchanged type triplet, with a small amount of a sharp 3-line spectrum superimposed on it. The very broad, highly spin-exchanged component is undoubtedly due to the formation of high-molecular-weight ganglioside micelles in aqueous buffers. Gangliosides are known to have a rather low critical micelle concentration (in the μM range) and should exist as micelles at concentrations above this value. The steric and packing restraints of the micellar environment would be expected to force spin-labelled headgroups into rather close proximity to each other, resulting in extensive spin-exchange interactions.

The superimposed sharp signal is probably due to the presence of some gangliosides which are either 'free' (monomeric) or present in aggregates of a very much smaller size, so that spin-exchange interactions are minimized. It should be noted that based on the approximate areas under the two types of ESR signal, only a very small fraction of the spin-labelled gangliosides are in this 'free' state. The fact that ESR spectroscopy allows us to discern these two different environments presumably means that exchange of spin-labelled ganglioside between the bulk solution and the micelles is slow on the ESR time scale. Possible contamination of the spin-labelled ganglioside preparation with another ESR-active species (such as unbound label) seems unlikely as a source of the 'sharp' component, since rechromatography of the spin-labelled ganglioside on silicic acid (Bio-Sil A) and subsequent recovery of the labelled glycolipid yielded a preparation which gave ESR spectra identical to those shown in Fig. 5. Ganglioside analogues bearing a spin-labelled fatty acid chain also show micellar and 'free' populations of glycolipids with a different environment for each [28], which the authors interpret in terms of a monomer-micelle equilibrium. The critical micelle concentrations estimated for these chain spin-labelled analogues were in the 10^{-4}–10^{-3} M range, much higher than the 10^{-6}–10^{-5} M values found for di- and trisialogangliosides [30]. It is possible that addition of a spin label heterocyclic ring may modify both the amphiphilic nature of the ganglioside molecule and its packing properties enough to substantially change the critical micelle concentration.

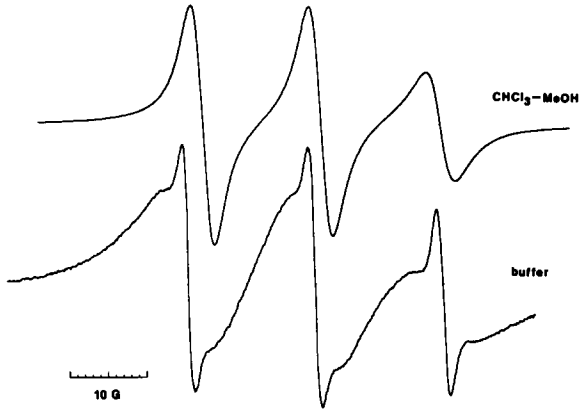

Fig. 5. ESR spectra of sialic acid spin-labelled mixed gangliosides in organic solvent and aqueous buffer. Top spectrum shows the spin-labelled glycolipid in 1:1, v/v, $CHCl_3$/MeOH at 3 mg/ml. Bottom spectrum shows the label in Hepes-buffered saline (pH 7.4) at 3 mg/ml.

When micellar gangliosides are allowed to associate with lymphocyte plasma membrane and the ESR spectrum of the washed membrane is examined, it is evident that a dramatic change in the molecular environment of the gangliosides has taken place. As shown in Fig. 6 (top spectrum), the ESR spectrum is now homogeneous, with no evidence of spin-exchange broadening, but with features due to restricted motion now visible. The ESR spectrum of the tightly bound membrane-associated gangliosides thus bears no resemblance in terms of shape or spectral parameters to the spin-exchanged micelle spectrum shown in Fig. 5. In fact, the spectrum of membrane-associated gangliosides bears a striking resemblance to that seen for 5 mol% sialic acid-labelled gangliosides in bilayers of egg PC (see Fig. 6 middle spectrum). Identical results were obtained using the second type of spin-labelled gangliosides (TIBS-labelled), where the spin label tags are randomly distributed among the headgroup sugar residues. Again, the ESR spectrum of the tightly bound gangliosides was very similar to that seen for the label at low dilution in lipid bilayer membranes and totally unlike that seen for aqueous micelles. Thus, the gangliosides have been removed from an aggre-

gated micellar environment to one in which they are molecularly dispersed in (or on) the lymphocyte membrane. The washed (four times) membrane used in these experiments should consist of two different ganglioside populations; bilayer-inserted gangliosides (the majority) and other molecules which are tightly bound to proteins at the membrane surface. These results show that the bilayer-inserted gangliosides are molecularly dispersed, rather than aggregated, and are presumably free to diffuse laterally in the plane of the membrane. Other groups using ganglioside analogues bearing spin labels in the fatty acid chain have come to similar conclusions for fibroblast and erythrocyte membrane associations [11,28,29]. It should be noted that the ESR spectra of the lymphocyte membrane-bound gangliosides are extensively motionally averaged so that they cannot be considered as diagnostic of insertion into the plasma membrane lipid bilayer as those using chain-labelled ganglioside analogues. Our results suggest that the protein-bound surface ganglioside population is also molecularly dispersed, and that it does not consist of micelles simply 'stuck on' to the outside of the membrane. It is possible that specific interactions between gangliosides and the cell surface are involved here; macrophages have been shown to bear specific ganglioside 'receptor' proteins on their plasma membrane [31].

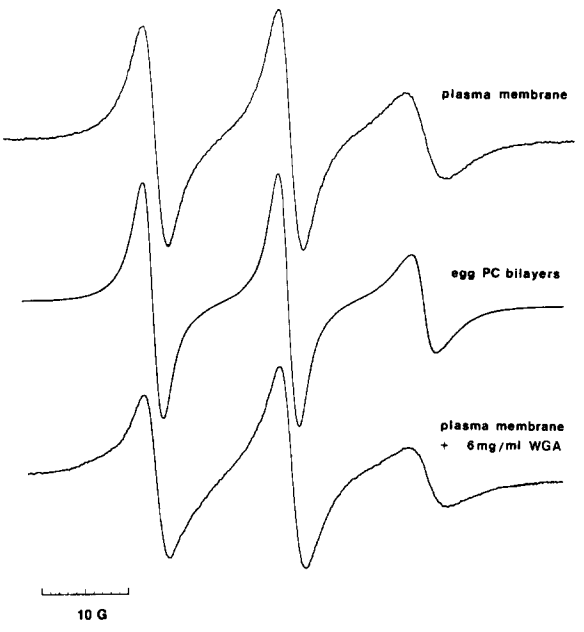

Fig. 6. ESR spectra of sialic acid spin-labelled gangliosides bound to lymphocyte plasma membrane and in egg PC liposomes. Top spectrum shows washed lymphocyte plasma membrane (500 μg membrane protein in 50 μl Hepes-buffered saline, pH 7.4) containing tightly associated sialic acid-labelled mixed bovine brain gangliosides. Middle spectrum shows bilayers of egg PC containing 5 mol% sialic acid-labelled gangliosides (2 mg lipid in 50 μl Hepes-buffered saline, pH 7.4). Bottom spectrum shows lymphocyte plasma membrane-associated gangliosides following treatment with 6 mg/ml wheat-germ agglutinin (WGA).

Effect of lectins on membrane-associated gangliosides

At this point, we were interested in whether the membrane-inserted gangliosides were capable of acting as receptors and if so, how their response to an external agent compared to that seen for gangliosides in lipid bilayers. We chose the lectin wheat-germ agglutinin as the external ligand, since it has specificity for binding sialic acid residues, and is known to bind to spin-labelled gangliosides in model membrane systems [32]. Addition of lectin to lymphocyte membrane containing bound gangliosides was carried out by two different methods. The first involved addition of 25 μl of lectin solution to 25 μl of membrane suspension (625 μg protein) followed by ESR spectroscopic analysis of the entire 50 μl sample. The second approach was to incubate 25 μl of membrane suspension with 250 μl of lectin solution at the required concentration. The membrane with bound lectin was then sedimented, resuspended in buffer to give a final volume of 50 μl, and finally subjected to ESR analysis. The amount of wheat-germ agglutinin which could be added to 25 μl of membrane suspension was limited in the first approach by the solubility of wheat-germ agglutinin in aqueous buffers, with a ratio of $\approx 2:1$, w/w membrane protein/wheat-germ agglutinin being the maximum attainable. To ensure that the wheat-germ agglutinin-binding sites on the lymphocyte membrane were fully saturated at these levels, excess lectin at the same concentration was used in the second method. Results obtained by the two techniques were almost identical, suggesting that site saturation had indeed been achieved in the 1 : 1 mixing approach.

Fig. 7 shows the dynamic response of lympho-

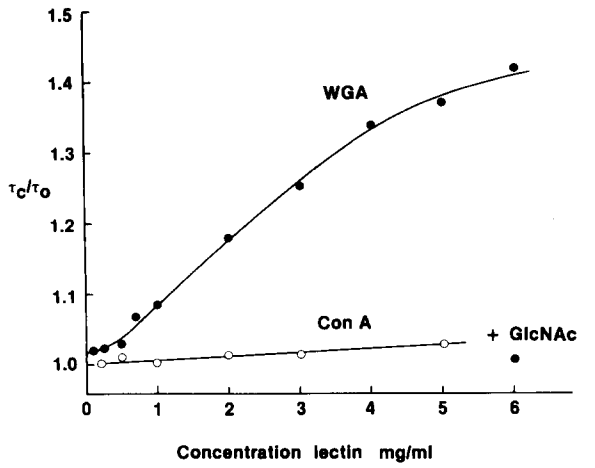

Fig. 7. Effects of lectin binding on oligosaccharide dynamics of lymphocyte membrane-associated gangliosides. Washed lymphocyte plasma membrane containing associated sialic acid spin-labelled mixed bovine brain gangliosides was incubated with the lectin wheat-germ agglutinin (WGA) (●———●) and concanavalin A (Con A) (○———○) at the required concentration. Following 30 min to allow binding, apparent τ_c values were determined and compared to that of an untreated control. *N*-Acetylglucosamine (GlcNAc) was added at a concentration of 0.1 M to assess specificity of binding of wheat-germ agglutinin.

cyte membrane-associated gangliosides to binding of the external agent wheat-germ agglutinin. The apparent rotational correlation time τ_c increased by more than 40% over that of the control as the lectin concentration was increased up to 6 mg/ml. Thus, membrane-associated (mainly bilayer-inserted) gangliosides respond to a receptor-binding event by immobilization of the carbohydrate headgroup. This motional restriction is highly specific and is completely reversed by the addition of 0.1 M *N*-acetylglucosamine, the appropriate sugar inhibitor for wheat-germ agglutinin. The response curve has some sigmoidal character, with a 'lag' in response observed at low lectin concentrations, followed by a large increase in τ_c and levelling-off at high lectin concentrations as saturation is approached. The ESR spectrum of the membrane-associated spin-labelled gangliosides following addition of saturating quantitites of wheat-germ agglutinin is shown in Fig. 6 (bottom spectrum).

Sigmoidal increases in τ_c following lectin binding to glycosylated species seem to be the most common effect noted and have been reported for lectin binding to spin-labelled gangliosides [32] and human erythrocyte glycophorin [33,34] in aqueous buffers. This sigmoidal response has been interpreted as indicating a positively cooperative interaction between the spin-labelled receptor and the lectin. However, even if τ_c was a true correlation time (which is not true in this case, see Materials and Methods), there appears to be no physical or theoretical reason why τ_c/τ_o should be a linear function of the amount of lectin bound. The sigmoidal shape of the curve may simply result from a nonlinear response of the τ_c/τ_o spectral parameter. Deducing positive cooperatively from such sigmoidal responses may thus be an over-interpretation of the experimental data. Resolution of this point must await careful spin label and binding studies on known cooperative and non-cooperative systems. Our previous studies on lymphocyte plasma membrane spin-labelled on (mainly) glycoprotein headgroups found that the overwhelming response of spin-labelled glycoconjugates to lectin binding is one of immobilization [35]. However, another group has reported an increase in motional freedom (decrease in τ_c) following binding of wheat-germ agglutinin to sialic acid spin-labelled glycophorin in human erythrocyte ghosts [36].

It should be noted that the response seen here for lymphocyte membrane-inserted gangliosides is qualitatively quite different from that seen in lipid bilayer membranes. Previous studies on spin-labelled gangliosides in bilayers of egg PC and dioleoyl-PC demonstrated an initial sharp drop in τ_c at low wheat-germ agglutinin concentrations, followed by an increase similar to the one we have noted in this study [32]. This was interpreted as indicating the presence of preexisting ganglioside clusters in the bilayer, which were disrupted by binding of small amounts of lectin. As additional lectin molecules became bound, the effect of headgroup immobilization became evident, and τ_c values increased as expected. We do not find that initial drop in τ_c following wheat-germ agglutinin binding to membrane-inserted gangliosides. That we are in fact detecting the dynamic response of the inserted gangliosides (and not simply that of the smaller subfraction which is tightly bound to surface protein) is highly likely based on the mag-

nitude of the τ_c increase seen, which is larger than the increase seen (after the initial drop) in model systems [32]. Thus, it appears that membrane-inserted gangliosides show a response to lectin binding which is qualitatively very different from that seen for gangliosides in bilayers of phosphatidylcholine. The possible origins of these differences are currently unknown.

The effect of adding large amounts of a lectin which binds extensively to the lymphocyte plasma membrane, but which does not bind directly to gangliosides is shown in Fig. 7. Concanavalin A produces an extremely small indirect effect on τ_c, with increases of only a few percent noted over the concentration range 1–5 mg/ml. A large number of other lectins were tested in a similar fashion (succinyl-concanavalin A, lentil, pea, soybean, peanut, *Phaseolus vulgaris, Ricinus communis* agglutinin) and none produced any significant increase in τ_c relative to the control. Thus, it appears that 'indirect' effects on gangliosides of extensive lectin binding to the membrane surface are minimal. Only specific binding of a lectin directly to the spin-labelled ganglioside produces an immobilization response. Previous studies on lymphocyte plasma membrane spin-labelled on glycoprotein sialic acid and galactose residues demonstrated a reciprocal response between wheat-germ agglutinin and *Ricinus communis* agglutinin, whereby wheat-germ agglutinin binding to sialic acid affected galactose dynamics and *R. communis* agglutinin binding to galactose affected sialic acid dynamics [35].

Spin-label studies can thus provide very useful information at the molecular level on membrane component behavior and interactions. We have shown that mixed gangliosides can associate with lymphocyte plasma membrane in three distinctly different ways, but that the majority of the glycolipid is bilayer-inserted and molecularly dispersed. The inserted gangliosides are fully capable of acting as receptors, but show fundamentally different behavior in response to a binding event than do gangliosides in bilayers of pure phospholipids.

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