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LIPID DOMAIN FORMATION AND LIGAND-INDUCED LYMPHOCYTE MEMBRANE CHANGES

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Summary

Spectral parameters of spin-labelled phosphatidylcholine, ceramide and cerebroside in the plasma membranes of human blood lymphocytes were measured before and after treatment with various ligands, which included concanavalin-A and phytohaemagglutinin. It was found that ligand treatment led to a significant decrease in order of the hydrocarbon chains of the phospholipids. This was accompanied by a clustering of the labelled sphingolipids, as estimated by spin-spin interaction, and an increase in the order of their hydrocarbon chains. In the untreated cells the cerebroside fatty acid chain was more ordered than that of the phosphatidylcholine.

It was considered that the decrease in phospholipid order was brought about by the sequestration of the more rigid sphingolipids into the patches and caps formed by receptor-ligand complexes. The significance of these changes in lipid distribution and ordering is discussed in relation to the activation of membrane enzyme systems by mitogenic ligands.

Supplementary data to this article are deposited with, and can be obtained from, Elsevier/North-Holland Biomedical Press B.V., BBA Data Deposition, P.O. Box 1345, 1000BH Amsterdam, The Netherlands. Reference should be made to No. BBA/DD/121/78615/595 (1980) 43—56. The supplementary information includes: (1) Spectral parameters for 5-nitroxide ceramide in control and phytoheamagglutinin-treated lumin lymphocytes . (2) Effect of range of agents on the spectral parameters of 5NL and 5NC in Luman blood lymphocytes. (3) Spectral parameters of 5-nitroxide phosphatidylcholine and cerebroside in dimyristoyl phosphatidylcholine vesicles.

Abbreviations: NC, spin-labelled cerebroside; NL, spin-labelled phosphatidylcholine.

Introduction

Data from electron spin probe and immunocytochemical studies have led us to conclude that aggregation of glycosphingolipids occurs during ligand-induced capping and patching of receptors in the lymphocyte membrane [1,2]. At the same time there appears to be a decrease in lipid ordering in the bulk of the membrane. It was hypothesized that the formation of domains of higher order by the glycosphingolipids might provide a mechanism for the activation of membrane-bound nucleotide cyclases and other enzymes such as the $(Na^+ + K^+)$-ATPase.

The electron spin probes used in these studies were nitroxide fatty acids and fatty acid esters and the regions in which they were located in the membrane where lipid domain formation is suspected were inferred from the behaviour of the labels in model systems. It is clearly desirable to use spin probes whose structure more closely resembles those of the lipids actually found in the membrane. This paper reports the results of a study of the effects of various ligands on the spectral parameters of spin-labelled cerebroside, ceramide and phosphatidylcholine in the membranes of human lymphocytes.

Materials and Methods

Cells. Mononuclear cells were separated from freshly drawn defibrinated human blood in a Ficoll-Hypaque gradient (Isopaque, Pharmacia, Uppsala) by the method of Böyum [3]. The cells were washed three times and finally suspended in Eagle's minimal essential medium (Commonwealth Serum Laboratories, Parkville, Victoria, Australia). To remove phagocytes the cells were mixed with carbonyl iron (approximately 5 mg iron powder/10^7 cells in 5 ml 10% foetal calf serum/minimal essential medium) and incubated at 37° C for 45 min with gentle shaking. A magnet bar was then immersed in the cell suspension which was gently agitated for a further 10 min at 37° C.

Because lipid spin labels may be bound to serum albumin all the cells were washed twice and suspended in minimal essential medium without serum for ESR experiments. When cells were kept for more than 120 min in serum-free medium, variable changes were seen in lipid ordering and in the proportion of label remaining in the medium. For this reason all cell preparations were used within 90 min.

Lectins and other reagents. Concanavalin A, leucoagglutinin and wheat germ agglutinin were purchased from Pharmacia (Uppsala, Sweden), and phytohaemagglutinin from Difco. Succinyl-concanavalin A was prepared by the methods of Beppu et al. [4] and of Gunther et al. [5]. Cholera toxin (batch no. BZ 248) was obtained from Schwarz-Mann (Orangeburg. NJ). Neuraminidase was obtained from Behringwerke (Marburg). Phospholipase A_2 was obtained from Boehringer (Mannheim).

Spin labels. Spin-labelled cerebrosides (5NC, 12NC and 16NC) were prepared by reacting 5-, 12- or 16-doxylstearic acid with psychosine according to the method of Sharom and Grant [6]. Partition chromatography of the labelled cerebroside on glass fibre paper, eluting with $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4), gave a single spot which cochromatographed with unlabelled cerebroside. The

preparation showed bands in its infrared spectrum at 1520 and 1660 cm^{-1} characteristic of an amide linkage. Spin-labelled phosphatidylcholine (5NL, 12NL and 16NL) was prepared by reacting the anhydrides of the 5-, 12- or 16-doxylstearic acids with lysophosphatidylcholine [7]. The spin-labelled phosphatidylcholines were hydrolysed by phospholipase A_2 to yield two components which cochromatographed using the system described above with a sample of pure lysophosphatidylcholine and the corresponding spin-labelled fatty acid. 5-Nitroxide ceramide was prepared by reacting 5-nitroxide stearic acid with sphingosine under the same conditions as used in the preparation of the 5-nitroxide cerebroside. The spin-labelled fatty acids were purchased from the Syva Corporation (Palo Alto, CA, U.S.A.) and their fresh alcoholic solutions were judged to be at least 95% pure when examined by partition chromatography. Psychosine and sphingosine were obtained from the Serdary Research Laboratories, London, Ontario, Canada, and palmitoyl lysophosphatidylcholine was the gift of Dr. D. Bishop, CSIRO Division of Food Research, Plant Physiology Unit, Ryde, N.S.W. Australia. The preparations were 98% pure when examined by partition chromatography.

Spin labels were incorporated in the desired concentrations in 10% (w/v) suspensions of 70-nm diameter vesicles of dimyristoylphosphatidylcholine prepared by the method of Redwood and Polefka [8]. 2.5 μl of the liposome suspension was added to 100 μl of cells which were vortexed gently at 37°C for 20 min. The suspension was then diluted with 0.5 ml of minimal essential medium, centrifuged at 700 × g for 4 min, resuspended in the same volume of medium and centrifuged again under the same conditions. The cells were resuspended in 100 μl of medium.

Spectral measurements. All spectra were recorded immediately after the addition of the label, followed by the ligand, using a 2.5 min scan and a 0.1 s time constant. The short time scan was used because of the relatively rapid changes observed in the spectra of ligand-treated cells. By keeping the time constant short it was possible, however, to maintain good peak resolution. The order in which the ligand and the label were added did not affect the spectra. In labelled, but otherwise untreated cells the spectra were stable, linewidth and relative peak heights being unchanged after 1 h. Electron spin resonance (ESR) spectra were recorded with a Varian V4502 spectrometer fitted with a Deltron (Sydney) Model DCM 20 temperature control accessory which maintained the temperature of the sample at ±0.05°C of the temperature selected. Spectra were recorded at either 37°C or −180°C. A 100 kHz modulation amplitude was used at 2 G and a power of 6 mW. Capillary sample tubes were used each containing 10^7—10^8 cells in 50 μl.

Lipid to label ratios were calculated using a lipid value of $4.0 \cdot 10^{-15}$ mol/cell [9].

Because of the need to distinguish spectral changes due to alterations in motion from those produced by probe-probe interaction which may be facilitated by the concentration of the probes into restricted lipid domains [10,11] spectra were obtained over a wide range of membrane lipid/probe ratios. Because, except in the case of the 5-nitroxide-labelled phosphatidylcholine and cerebroside the high-field hyperfine extremum was difficult to resolve a spectral expansion method [11] was used to determine $T'_{\parallel}$ and $T'_{\perp}$. In order to

eliminate the long scanning times associated with this method (16 min for the broadest maxima) spectra were digitized, stored on magnetic tape, digitally smoothed and played back on to the *X-Y* plotter with the aid of a Hewlett Packard HP9825A calculator controller. Several successive spectral measurements upon a stable sample gave a standard deviation for the order parameter, *S*, of 0.8% which is in good agreement with deviation of 0.7% originally claimed for the spectral expansion method [11]. The order parameters were calculated [12] from the relation

$$S = \frac{T'_{\parallel}[T'_{\perp} + C]}{T'_{\parallel} + 2[T'_{\perp} + C]} \times 1.723 \tag{1}$$

where $T'_{\parallel}$ and $T'_{\perp}$ are the separation of the outer and inner extrema in gauss of the first derivative spectra and

$$C = (1.4643 - 0.053[T'_{\parallel} - T'_{\perp}])\ \mathrm{G} \tag{2}$$

It must be stressed that *S* was used to compare the relative ordering of the regions probed by the various spin labels in lymphocyte membranes before and after different treatments.

The measurement of probe-probe interaction has been discussed extensively by Sauerheber and his colleagues [11]. Two measures were used; the first was the measurement of ΔH of the mid-field (equivalent to W_0)

$$\Delta H = \Delta H_0 + \Delta H_{\mathrm{dip}} + \Delta H_{\mathrm{ex}} \tag{3}$$

where ΔH_0 is the linewidth in the absence of interaction, ΔH_{dip} is the line-broadening caused by magnetic dipolar interactions and ΔH_{ex} is contributed by spin exchange [13]. The other measure (m) is based upon the observation [11] that $T_{\perp}$ but not $T_{\parallel}$ broadens with increasing probe concentrations in liver and heart plasma membranes

$$m = \frac{2}{3}\left[\frac{T_{\parallel} - T_{\perp}}{T_{\parallel} - a_{\mathrm{N}}}\right] \tag{4}$$

where a_{N} is the isotropic coupling constant. A value of m of less than 0.95 was taken as indicative of probe-probe interaction.

Lymphocyte assays. Mitogen dose-response curves were determined by adding the desired amount of mitogen to 1 ml of 10^6 lymphocytes in minimal essential medium containing 10% foetal calf serum (Commonwealth Serum Laboratories, Melbourne), incubating for 60 h then pulsing with 1 μCi of [6-^{3}H]thymidine (The Radiochemical Centre, Amersham). The cells were incubated for a furher 4 h, collected and washed on glass fibre Millipore filters (Millipore Corp., Bedford, MA) and the radioactivity determined with the filter covered with 'Instagel' scintillant (Packard Inc., Downers Grove, IL) in a Packard liquid scintillation counter (Model 2650). Capping of lymphocytes was performed by incubating 10^7 cells with 20 μg of fluorescein-labelled concanavalin A (Calbiochem, San Diego) for 30 min at 4°C. The cells were washed twice at 4°C with minimal essential medium and then examined on the warm stage (37°C) of a Zeiss 'Ultraphot' microscope, using epi-illumination, a HBO400 lamp, 4 mm UG1 and BG38 exciting filters and a 3 mm KV48 barrier filter. Cyclic AMP levels in lymphocytes were estimated by a competitive

binding assay (The Radiochemical Centre, Amersham). Extracts for assay were prepared by sonicating 10^7 cells in 1 ml of 0.05 M of Tris/EDTA buffer, pH 7.5.

Results

Location of spin labels in lymphocytes

In labelling the cells it was noted that at least 20 min contact of cells with the spin-labelled vesicles was necessary to achieve reproducible spectra. In particular the spectra of cells labelled by short contact were more isotropic than those which had been in contact with labelled vesicles for 20 min or more. This led us to speculate whether the spectra orginated from label still contained in vesicles adhering to the cell surface, from label distributed in the plasma membrane or from label in endocytosed vesicles. To investigate this we prepared labelled vesicles containing 0.3 M $NiCl_2$, which were then suspended in minimal essential medium. This concentration of Ni^{2+} is sufficient to eliminate by broadening the signal from any spin label with which it is in contact [14]. Accordingly the signal in these vesicles measured by double integration was approximately half that of vesicles without Ni^{2+}, but containing the same amount of spin label. The cells were vortexed with the Ni^{2+}-containing vesicles as described under Materials and Methods. Aliquots were removed at 5-min intervals, washed and the ESR spectrum determined. By 20 min the signal observed from cells vortexed with the Ni^{2+}-containing vesicles was the same as that from cells vortexed with the same amount of label in vesicles which did not contain Ni^{2+}.

This was interpreted as being due to the vesicles fusing with or exchanging the label with the cell membrane, thus diluting the Ni^{2+} to a concentration where it no longer affected the spin label. This experiment was carried out with the three 5N spin labels. Further evidence against endocytosis was provided by experiments involving $^{63}Ni^{2+}$-containing vesicles vortexed with the cells which were then centrifuged and washed as described above and the supernatants pooled. The $^{63}Ni^{2+}$ was then counted and it was found that only 2% of the radioactivity added was associated with the cells, the rest was in the supernatant. This indicated that the vesicles had not passed intact into the cells. In another series of experiments the spectra of 5-nitroxide-labelled phosphatidylcholine, cerebroside and ceramide were measured in cells that were suspended in minimal essential medium containing 0.05 M $NiCl_2$ and the NaCl reduced to 0.09 M to maintain the medium's osmolality. By 60 min all of the spectra were completely broadened by the Ni^{2+}. When this experiment was repeated with 12- and 16-labelled probes no nickel-induced broadening was observed. It was found using $^{63}Ni^{2+}$ under the same conditions that Ni^{2+} did not penetrate the cells, and could be removed from the surface by two washings in minimal essential medium. These results strongly suggest that the label is located in the outer leaflet of the lipid bilayer.

Behaviour of spin-labelled phosphatidylcholine in control and lectin-treated lymphocytes

The values for S, W_0 and m at four different lipid to label ratios of 5, 12 and

TABLE I

SPECTRAL PARAMETERS FOR 5NL, 12NL AND 16NL IN HUMAN BLOOD LYMPHOCYTES BEFORE AND AFTER THE ADDITION OF PHYTOHAEMAGGLUTININ AND CONCANAVALIN A

Spectra were measured immediately after the addition of lectins. Spectral parameters are the averages of three experiments carried out on separate batches of cells from the same individual.

Probe	Lipid/probe	Control cells			Phytohaemagglutinin			Concanavalin A		
		S (± S.D.)	W_0 (G)	m	S (± S.D.)	W_0 (G)	m	S (± S.D.)	W_0 (G)	m
5NL	300:1	0.566 ± 0.008	3.8	0.97	0.459 ± 0.013	3.7	0.98	0.463 ± 0.96	3.7	0.98
	150:1	0.559 ± 0.016	3.8	0.97	0.462 ± 0.011	3.7	0.98	0.469 ± 0.012	3.7	0.97
	100:1	0.537 ± 0.012	3.8	0.98	0.445 ± 0.009	3.7	0.97	0.441 ± 0.014	3.7	0.97
	50:1	0.512 ± 0.015	4.3	0.91	0.426 ± 0.016	4.2	0.92	0.418 ± 0.018	4.2	0.91
12NL	300:1	0.405 ± 0.006	3.6	0.96	0.387 ± 0.007	3.5	0.97	0.381 ± 0.008	3.5	0.98
	150:1	0.401 ± 0.011	3.6	0.97	0.386 ± 0.006	3.5	0.97	0.380 ± 0.010	3.5	0.98
	100:1	0.391 ± 0.013	3.6	0.97	0.379 ± 0.009	3.5	0.98	0.382 ± 0.012	3.5	0.98
	50:1	0.371 ± 0.12	4.1	0.91	0.362 ± 0.006	3.8	0.91	0.357 ± 0.011	3.8	0.91
16NL	300:1	0.385 ± 0.014	3.2	0.97	0.365 ± 0.006	3.1	0.97	0.365 ± 0.005	3.1	0.97
	150:1	0.382 ± 0.016	3.2	0.98	0.363 ± 0.005	3.1	0.98	0.367 ± 0.007	3.1	0.98
	100:1	0.375 ± 0.009	3.2	0.96	0.351 ± 0.005	3.1	0.98	0.353 ± 0.007	3.1	0.98
	50:1	0.373 ± 0.012	3.2	0.96	0.361 ± 0.006	3.7	0.95	0.343 ± 0.006	3.7	0.94

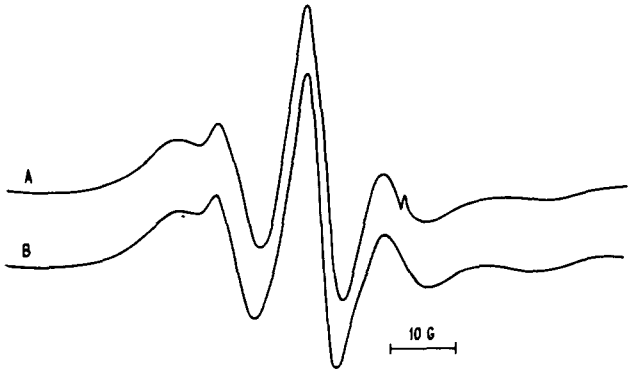

Fig. 1. ESR spectrum of 5NL in (A) untreated human lymphocytes; (B) in lymphocytes treated with 2.5 $\mu g/10^7$ cells per 100 μl phytohaemagglutinin. Spectrum of B obtained within 5 min of adding phytohaemagglutinin. Lipid : probe ratio 100 : 1.

16NL are presented in Table I. It can be seen that in both control and treated cells S is smaller in the C-12 and C-16 than in the 5-position, as would be expected. Probe-probe interaction as shown by increase in W_0 and a decrease in m is only evident at a lipid to label concentration of 50 : 1. The addition of the lectins, phytohaemagglutinin or concanavalin A to the cells caused a significant decrease in S, particularly for 5NL. A small polar component which was present in the 5NL spectra of control cells at a lipid : label ratio of 100 : 1 or less disappeared on the addition of the lectin. Washing the control cells in minimal essential medium did not remove the polar component nor result in the appearance of spin label in the wash supernatant. The appearance of such a 'fluid component' is regarded by Sauerheber et al. [11] as one of the signs of spin-spin interaction. The spectra of 5NL in control and phytohaemagglutinin-treated cells are shown in Fig. 1.

Behaviour of spin-labelled cerebroside in control and lectin-treated lymphocytes

The values for S, W_0 and m at five different lipid/label concentrations are presented in Table II for 5-, 12- and 16-nitroxide cerebrosides. It can be seen that the value for S at all positions is greater for the nitroxide cerebrosides than for nitroxide lecthins. Spin-spin interaction as judged by an increase in W_0 and a decrease in m is also evident in the control cells at a lipid to label ratio of 100 : 1.

The addition of concanavalin A or phytohaemagglutinin caused a marked increase in probe-probe interaction in cells labelled with 5-, 12- or 16-nitroxide cerebroside as judged by an increase in W_0 and decrease in m and the appearance of a polar component in the cells labelled with 5NC at a lipid : label concentration of 200 : 1 or less. It should also be noted that the height of the high-field peak of the inner hyperfine doublet was below the baseline (Fig. 2). This was characteristic of the spectra of the three probes in the lectin-treated cells and was a further indication that the probe-probe interaction was present. Washing the cells did not remove the polar component, nor result in the appear-

TABLE II

SPECTRAL PARAMETERS FOR 5NC, 12NC AND 16NC IN HUMAN BLOOD LYMPHOCYTES BEFORE AND AFTER THE ADDITION OF PHYTOHAEMAGGLUTININ AND CONCANAVALIN A

Spectra were measured immediately after the addition of lectins. Spectral parameters are the averages of three experiments carried out on separate batches of cells from the same individual.

Probe	Lipid/probe	Control cells			Phytohaemagglutinin			Concanavalin A		
		S (± S.D.)	W_0 (G)	m	S (± S.D.)	W_0 (G)	m	S (± S.D.)	W_0 (G)	m
5NC	300:1	0.671 ± 0.009	4.3	0.97	0.735 ± 0.008	4.3	0.97	0.741 ± 0.013	4.3	0.97
	200:1	0.668 ± 0.013	4.3	0.97	0.709 ± 0.006	4.5	0.94	0.713 ± 0.006	4.6	0.95
	150:1	0.659 ± 0.011	4.3	0.96	0.701 ± 0.009	4.7	0.91	0.695 ± 0.012	4.8	0.92
	100:1	0.591 ± 0.012	4.5	0.92	0.691 ± 0.005	5.1	0.89	0.687 ± 0.009	5.1	0.90
	50:1	0.556 ± 0.014	5.1	0.89	0.686 ± 0.011	5.6	0.87	0.681 ± 0.013	5.7	0.89
12NC	300:1	0.471 ± 0.008	4.0	0.96	0.501 ± 0.005	4.0	0.97	0.512 ± 0.009	4.0	0.96
	200:1	0.467 ± 0.009	4.0	0.96	0.486 ± 0.006	4.3	0.94	0.483 ± 0.007	4.3	0.94
	150:1	0.456 ± 0.011	4.0	0.95	0.483 ± 0.005	4.5	0.91	0.479 ± 0.011	4.4	0.92
	100:1	0.415 ± 0.006	4.2	0.91	0.471 ± 0.012	4.7	0.89	0.470 ± 0.006	4.6	0.91
	50:1	0.409 ± 0.009	4.4	0.90	0.457 ± 0.011	4.9	0.87	0.468 ± 0.007	4.9	0.89
16NC	300:1	0.438 ± 0.006	3.7	0.97	0.478 ± 0.015	3.8	0.97	0.470 ± 0.005	3.7	0.97
	200:1	0.436 ± 0.011	3.7	0.97	0.469 ± 0.011	3.9	0.96	0.462 ± 0.008	3.9	0.94
	150:1	0.435 ± 0.012	3.7	0.96	0.452 ± 0.006	4.2	0.95	0.456 ± 0.006	4.1	0.93
	100:1	0.391 ± 0.008	3.9	0.94	0.437 ± 0.007	4.5	0.90	0.436 ± 0.005	4.4	0.90
	50:1	0.387 ± 0.007	4.1	0.89	0.416 ± 0.009	4.7	0.89	0.407 ± 0.005	4.6	0.88

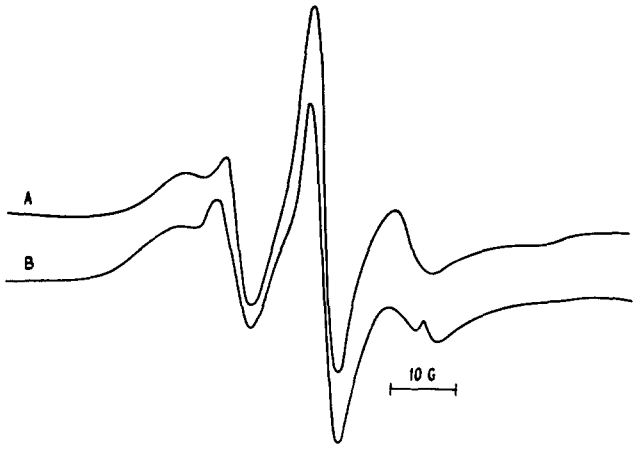

Fig. 2. ESR spectrum of 5NC in (A) untreated human lymphocytes; (B) in lymphocytes treated with 2.5 $\mu g/10^7$ cells per 100 μl of phytohaemagglutinin. Spectrum of B obtained within 5 min of adding phytohaemagglutinin. Lipid : probe ratio 100 : 1.

ance of spin label in the wash supernatant. The spin-spin interaction was absent only at the 300 : 1 lipid to label ratio. Comparing S values at this ratio it can be seen that there was an increase at all levels probed in the lectin-treated cells, compared with the control cells. This increase is, however, greatest in the 5-position.

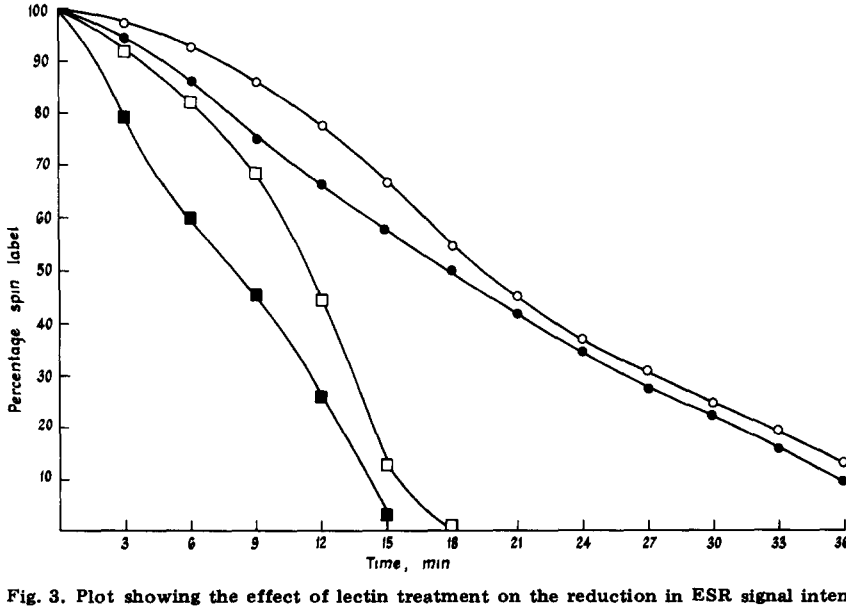

Fig. 3. Plot showing the effect of lectin treatment on the reduction in ESR signal intensity by ascorbic acid. 5NL-labelled cells: □——□, control; ○——○, phytohaemagglutinin-treated cells (2.5 $\mu g/10^7$ cells per 100 μl). 5NC-labelled cells: ●——●, control; ■——■, phytohaemagglutinin-treated cells (2.5 $\mu g/10^7$ cells per 100 μl). Lipid : probe ratio 150 : 1.

The nature of the polar component observed in spectra of 5NL in control and 5NC in lectin-treated cells

In order to determine the nature of the polar component 0.05 M ascorbic acid was added to control and phytohaemagglutinin-treated cells labelled with 5NL and 5NC lipid : label ratios, respectively, of 100 : 1 and 200 : 1. In the resulting spectra there was no sign of the polar component which had been previously observed with the control 5NL-labelled cells and in the lectin-treated 5NC-labelled cells. The spectra were then recorded at 3-min intervals and the total ESR measured by double integration. The results are plotted in Fig. 3. It can be seen that in the control 5NL-labelled cells and in the phytohaemagglutinin-treated 5NC-labelled cells the label was very rapidly destroyed by the ascorbic acid. This suggests that the components were in a more polar region which was accessible to ascorbate and that the labels in this environment were in reasonably rapid exchange with the labels in the less polar region. In contrast the labels in the 5NL-labelled, phytohaemagglutinin-treated and 5NC-labelled control cells were more stable in the presence of ascorbic acid.

Effect of increasing phytohaemagglutinin concentration on the special parameters of 5NC and 5NL

Dose-response curves for phytohaemagglutinin and [^{3}H]thymidine uptake, W_0, m and S for 5NC and 5NL are given in Fig. 4. It can be seen that up to 2.5 μg/ml, the optimal mitogenic dose, S decreased for 5NL and W_0 increased for 5NC indicating decreases, respectively, in ordering and spin-spin interaction. S for 5NC, measured at a lipid to label ratio of 300 : 1 increased, indicating increased ordering of the cerebroside fatty acid chain up to the optimal mitogenic dose of phytohaemagglutinin.

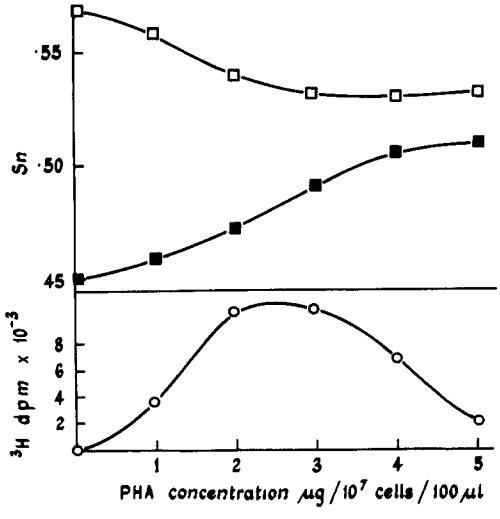

Fig. 4. Dose-response curves for phytohaemagglutinin and [^{3}H]thymidine uptake and the spectral parameters of 5NC and 5NL. ■———■, S for 5NC; □———□, S for 5NL. Spectra measured within 5 min of adding the spin label and lectin.

Effect of a range of agents on the spectral parameters of 5NC and 5NL

The spectra of 5NC and 5NL were obtained after treatment of lymphocytes with a range of agents. It was found that wheat germ agglutinin, leucoagglutinin, neuraminidase and cholera toxin had no effect on the spectral parameters whilst KIO_4 and succinyl-concanavalin A were almost as effective as concanavalin A in decreasing S for 5NC, increasing S for 5NL and causing spin-spin interaction at lipid to 5NC label ratios of 150 : 1 as judged by increase in W_0 and decrease in m.

Measurement of spectra at −180° C

Two characteristics of probe-probe interaction are broadening of the spectrum at −180° C, compared with that of the same probe in a magnetically dilute system, and increase in linewidth with temperature in contrast to a decrease in linewidth due to motional effects. We could not use the latter criterion because of instability of the cells above 40° C leading to a rapid reduction of lectin-induced broadening. When examined at −180° C linebroadening was only seen in those spectra of systems which had shown increases in W_0 at 37° C (Fig. 5). We regarded this as further confirmation that these increases in W_0 were due to probe-probe interaction.

Spectra of spin-labelled ceramide in control and lectin-treated cells

The spectral parameters for 5-nitroxide ceramide were measured in the membranes of control and phytohaemagglutinin-treated cells. It was found that the results closely parallel those for the 5-nitroxide cerebroside probe with S being increased in the lectin-treated cells at a lipid to label ratio of 300 : 1 and a marked increase in W_0, indicating spin-spin interaction occurring in these at lipid to label ratios of less than 300 : 1. A table setting all the values out has been lodged with the BBA Data Bank.

Spectra of 5NC and 5NL incorporated in phospholipid vesicles

The 5-nitroxide cerebroside and 5-nitroxide phosphatidylcholine were incorporated at a range of lipid : label ratios in dimyristoyl phosphatidylcholine

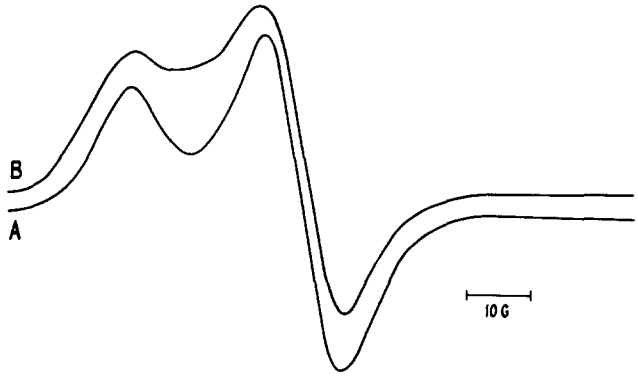

Fig. 5. Spectrum of 5NC at −180° C in (A) untreated lymphocytes; (B) cells treated with phytohaemagglutinin (2.5 μg/10^7 cells per 100 μl. Lipid : label ratio 300 : 1.

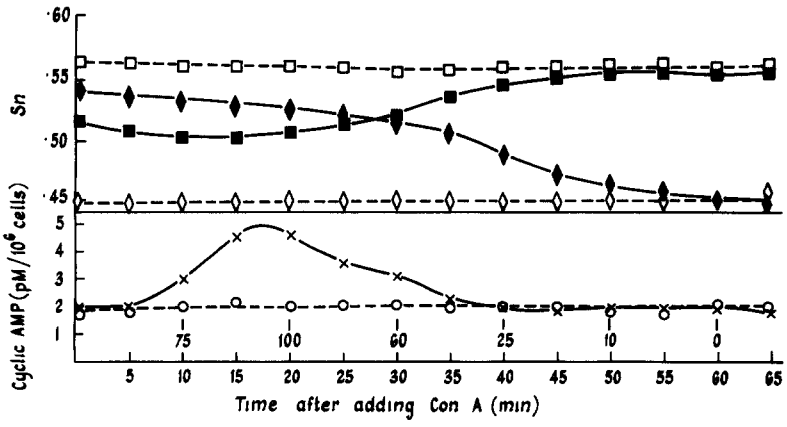

Fig. 6. Time correlation between concanavalin A receptor patching and capping, cyclic AMP production and S for 5NC and 5NL. Figures under vertical bars percentage capping. ○- - - - -○, AMP of control cells; X———X, AMP of treated cells; ◇- - - - -◇, 5NC S of control cells; ◆———◆, 5NC S of treated cells; □- - - - -□, 5NL S of control cells; ■———■, 5NL S of treated cells. Spectra obtained at a lipid : label ratio of 300 : 1.

vesicles [8]. It was found that for each position on the hydrocarbon chains S was approximately 7% more for the labelled cerebroside than for the labelled phosphatidylcholine. Both labelled cerebrosides and phosphatidylcholine showed increases in W_0 indicative of spectral broadening and spin-spin interaction at lipid : label ratios of less than 25 : 1. The addition of concanavalin A at 10 μg/ml had no effect on the spectra obtained for any of the labelled vesicles. A table setting out all the values has been lodged with the BBA Data Bank.

Correlation between concanavalin A receptor patching and capping, cyclic AMP production and the spectral parameters of 5NC and 5NL

These correlations are illustrated in Fig. 6 where it can be seen that the changes in all the spectral parameters of the treated cells return to control values within 75 min. At the end of this period all the fluorescent concanavalin A patches had disappeared from the cells, and cyclic AMP levels had returned to control values.

Discussion

Amongst the difficulties in the use of spin probes to study the structure and dynamics of the lipids of biological membranes is the fact that the latter are not homogeneous but are complex structures possessing highly specialized regions. The ideal spectroscopic probe should therefore be based upon lipid components known to occur naturally in the membrane. For this reason we used labelled phospholipids and sphingolipids in the study reported above. Even so, the nitroxide label still represents an impurity in the membrane and for this reason we were mindful to work over a range of lipid to label ratios. Because of the significance of spin-spin interaction at lower lipid to probe ratios order parameters were calculated for all ratios used. We consider, how-

ever, that the only strictly comparable S values are those obtained in the most magnetically dilute solution, i.e. lipid : probe 300 : 1.

Because of the difficulties involved in getting an adequate signal to noise ratio in the more magnetically dilute systems it was necessary to use a less than theoretically ideal modulation amplitude of 2 G. This resulted in line-broadening which increased W_0 values by approximately 15%. It, however, increased the accuracy of peak height measurement by substantially increasing the signal. Care was taken to maintain all conditions constant for each measurement so that the S and W_0 values could give a valid comparison of the properties of the membrane regions being probed and validly monitor changes induced by the action of ligands on the cell.

With these reservations our data suggest that the action of lectins on lymphocytes seems to lead to clustering or domain formation of sphingolipids as shown by the spin-spin interaction occurring between the nitroxide cerebroside probes and nitroxide ceramide in lectin-treated cells. For this to happen the probes must lie less than $1.5 \cdot 10^{-7}$ cm apart [15]. Clustering of the sphingolipids is associated with patching and capping of lectin receptors and its extent as estimated from the increase in W_0 of 5NC seems to be proportional to the mitogenic dose of lectin. The clustering is also associated with increases in the hydrocarbon chain ordering in the regions probed by all the nitroxide cerebrosides. On the other hand the order in regions probed by the phospholipid labels is decreased by the action of lectins on the cell membrane. Since the S values of the nitroxide cerebrosides are less than that of the nitroxide cerebrosides are less than that of the nitroxide phospholipids in untreated cells this may be explained by sequestration of the glycosphingolipids by lectin action, leaving the remainder of the membrane, containing the phospholipids less ordered. We have advanced this previously as an explanation of why the order parameters of spin-labelled stearic acid probes were decreased in lectin-treated lymphocytes [1]. There have been conflicting data published regarding lectins and lymphocyte membrane lipid fluidity and a full discussion of this is given in Ref. 1.

The appearance of a polar component in the spectrum of 5NC in lectin-treated cells adds support to the view that lectin-induced lymphocyte membrane changes are accompanied by rearrangement of membrane lipids in the direction of greater restriction and clustering on the part of sphingolipid. The absence of significant amounts of label in the supernatant of labelled cell suspensions suggests that label showing the polar component is still attached to cell-bound lipid. We have observed a similar polar component in the spectra of the water-insoluble methyl ester of 5-nitroxide stearic acid in lectin-treated lymphocytes [1]. However, the increase in W_0 seen at lower lipid to label ratios with the glycolipid probes in lectin-treated cells cannot be due solely to the presence of the polar component because linebroadening is still evident at $-180°$C.

Our observation that hydrocarbon chain order is still high in the glycosphingolipid clusters at the C-16 level provides a possible mechanism for the activation of enzymes such as the nucleotide cyclases which are associated with the inner leaflet of the bilayer. In the lymphocyte more than 50% of glycosphingolipid fatty acid chains contain 22 or more carbon atoms [18]. If the

relatively high ordering extends to this level then it is possible to evisage it affecting an allosteric site on the cyclase. Indeed such a bilayer leaflet coupling role has been suggested for the glycosphingolipids by Schmidt et al. [19]. There is evidence from immunocytochemical localization of cyclic AMP in human B lymphocyte membranes that active adenylyl cyclase is localized in the region of ligand-induced patches and caps [20] and that ligand, glycosphingolipids and cyclic AMP all co-cap [2]. It is therefore possible that the clustering of the glycosphingolipids is associated with adenylyl cyclase activation. The exact mechanism of this activation is unclear although hypotheses and data on the activation of membrane-associated enzymes have implicated effects ranging from compression of the enzyme by changes in lipid-film pressure [21] to alterations in the Stokes radius by changes in lipid fluidity [22]. Recently Sela et al. [23] have shown that antibodies to ganglioside GM_1 will induce mitogenic stimulation and cap formation in rat thymocytes. Because cap formation was inhibited by cytochalasin B these authors suggested that there may possibly be a direct or indirect association between surface gangliosides and submembraneous cytoskeletal assemblies. In this connection it is of interest that lectins and other ligands decrease the ordering of lipids probed by fatty acid spin probes in chicken erythrocytes which have microtubule-like structures, but not in human erythrocytes which do not [24].

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