INTERACTION BETWEEN VESICLES CONTAINING GANGLIOSIDES AND LIMULIN (*LIMULUS POLYPHEMUS* LECTIN)

Fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene

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1. Introduction

Conflicting results have been reported concerning the influence of bound lectins on the fluidity of membrane lipid bilayers [1–3]. The correlation between membrane lipid fluidity and the mobility of receptors on the cell surface is still obscure and controversial [4–8] more so as it was shown recently [8–11] that apparently no direct correlation exists between malignancy and membrane lipid fluidity. A correlation between malignancy and cell agglutinability with lectins is well documented, though [12].

In order to answer the question whether the binding of a lectin to its specific membrane receptors affects the fluidity of the lipid bilayer, we chose a simple system consisting in limulin, a mitogenic lectin [13] and phospholipid vesicles containing gangliosides as lectin receptors [14].

In the present study we monitored the degree of fluorescence polarization of the fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH) embedded in the lipid bilayer of vesicles containing gangliosides in the presence and in the absence of limulin, as well as the life-time of the DPH-excited state in all these systems.

2. Materials and methods

Limulin was obtained from the hemolymph of *Limulus polyphemus*, by affinity chromatography [15]. Phosphatidylcholine from egg yolk was isolated as in [16]. Cholesterol (Prolabo) was recrystallized twice from methanol. Gangliosides (HEG) were extracted from horse erythrocyte membranes as in [14] and partially purified by chromatography on a column of silicagel G (Merck, 70–230 mesh) [17]. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was from Koch-Light.

2.1. *Preparation of lipid vesicles*

Vesicles containing egg lecithin, cholesterol and gangliosides were generated by sonication of the dry lipids for 10 min in 0.1 M NaCl, 0.05 M Tris buffer, pH 8.5. The dispersion was further centrifugated at 130 000 × *g* for 60 min. Only the clear supernatant which contained vesicles homogeneous in size (about 30 nm) was collected. The translucent supernatant which was above the pellet was discarded in order to remove larger vesicles.

The final molar ratio of phosphatidylcholine to cholesterol was 4:1. The molar ratio of gangliosides (expressed as sialic acid) to phosphatidylcholine was 0.14. This ratio was 0.02 in the life-time experiments.

2.2. *Chemical determinations*

Lipid-bound phosphorus was estimated by the method in [18], cholesterol as in [19] and sialic acids as in [20] after acid hydrolysis (0.1 N H_2SO_4, 60 min, 80°C). All these determinations were done on the vesicle dispersion. However, because phospholipids

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give a coloration with the Aminoff's reagent [20], the actual concentrations of sialic acids were calculated after subtracting the contribution of phospholipids.

2.3. *Optical studies*

Vesicles containing gangliosides were aggregated upon binding limulin. This aggregation process was followed by dispersion A_{500nm} measurements. The lectin solution was added directly to the vesicle dispersion in quartz cells [14].

2.4. *Fluorescence labeling [6]*

A stock solution of DPH (2×10^{-3} M) in tetrahydrofuran was diluted 1000-fold by injection to a vigorously stirred buffer (0.05 M Tris–HCl, 0.1 M NaCl, 0.02 M $CaCl_2$, pH 8.5). One volume of this dispersion was added to 1 vol. vesicle suspension, and incubated 1 h at room temperature. The mixture (lecithin 2 mg/ml in 10^{-6} M DPH, 0.05 M Tris–HCl, 0.1 M NaCl, 0.01 M $CaCl_2$) was then diluted in order to have different concentrations of lecithin with a constant DPH concentration. The molar ratio of phospholipid to DPH was in the range 500–2000. The incorporation of DPH in the lipid membrane was completed within 1 h.

2.5. *Fluorescence measurements*

Static measurements of the degree of polarization $\bar{P}$ were performed with a fluoropolarimeter Elscint MV-1. This instrument was equipped with a 100 W mercury arc, a 366 nm excitation filter, Glan-Thompson polarizers, two emission cut-off filters to measure at wavelengths > 390 nm and a constant temperature chamber.

The fluoropolarimeter simultaneously monitored the fluorescence intensities through two polarizers oriented parallel ($I_{||}$) and perpendicular ($I_{\perp}$) to the direction of the polarized excitation beam, respectively. The accuracy of the fluorescence polarization was ± 0.003. Fluorescence lifetimes were measured with a single photon counting instrument, equipped with Ortec electronics and an Ortec multichannel analyzer and an Applied Photophysics optical unit with a gated airfilled flash lamp (λ_{exc} = 360 nm). The fluorescence decay d(t) and the instrument response function $g(T)$ (scattering light) were stored in 256 channels. The mean life times $\langle\tau\rangle$ were calculated by fitting a single exponential function:

$$f(t) = a\, e^{-t/\langle\tau\rangle}$$

which is related to the experimental fluorescence decay and the scattering light signal by the function:

$$d(t) = \int_0^t f(t-T)\, g(T)\, dT$$

3. Results

3.1. *Fluorescence polarization of DPH embedded in vesicles*

In the range of lecithin concentration 0.2–5 mg/ml used in our experiments the A_{500} values ranged from 0.01–0.1. A net decrease of the DPH fluorescence polarization as a function of the absorbance was observed. The fluorescence polarization of DPH was strongly increased by the presence of cholesterol and slightly increased by the presence of gangliosides, but was not affected by the presence of calcium (0.01 M) in the buffer. The average fluorescence lifetime of DPH was not dependent upon the presence of gangliosides but was slightly higher in the presence than in the absence of cholesterol (table 1).

3.2. *Time course of aggregation and modification of fluorescence polarization of DPH after addition of lectins*

We recently showed that vesicles containing horse erythrocyte gangliosides (HEG) were aggregated by limulin. The absorbance of the vesicle suspension increased after addition of low concentrations of limulin (14 μg/ml, 28 μg/ml or 42 μg/ml). The effect was reversed by addition of specific inhibitors of the lectin: ethylene diamine tetracetate for the activity of limulin is calcium-dependent; or bovine submaxillary mucin (bound sialic acid 10 μM) which is one of the best inhibitors of limulin [15]. The reversion showed that no fusion occured during the aggregation process.

The fluorescence polarization of DPH embedded in vesicles was measured time to time after addition of limulin. The $\bar{P}$ values decreased rapidly and then reached a plateau (fig.1). Both absorbance increase and decrease of DPH fluorescence polarization were a function of the lectin concentration. No change

Table 1
Fluorescence data of DPH embedded in vesicles

System	$A_{500\ nm}$	$\bar{P}_{20°C}$	$\bar{r}$[a]	$<\tau>$ ns	$<\rho>$[a]
Lecithin	0.022	0.152	.106	8.1	3.3
Lecithin–cholesterol 1:0.25	0.018	0.189	.132	8.8	5.0
Lecithin–gangliosides 1:0.17	0.028	0.163	.115	8.2	3.8
Lecithin–cholesterol–gangliosides 1:0.25:0.02	0.040	0.202	.144	8.8	5.3
Lecithin–cholesterol–gangliosides 1:0.25:0.02 + limulin 38 μg/ml	0.040[b]	0.205	.147	8.5	5.8

[a] $\bar{r} = 2\bar{P}/(3-\bar{P})$; $<\rho> = \tau\bar{r}/(r_0-\bar{r})$; $r_0 = 0.362$ [21]
[b] 30 min after the addition of limulin, the suspension was diluted with buffer in order to obtain an absorbance value similar to the initial one
$\bar{P}$ values are ± 0.003; $<\tau>$ values are ± 0.4 ns; $<\rho>$ values are ± 0.6 ns

Lecithin concentration: 0.5 mg/ml

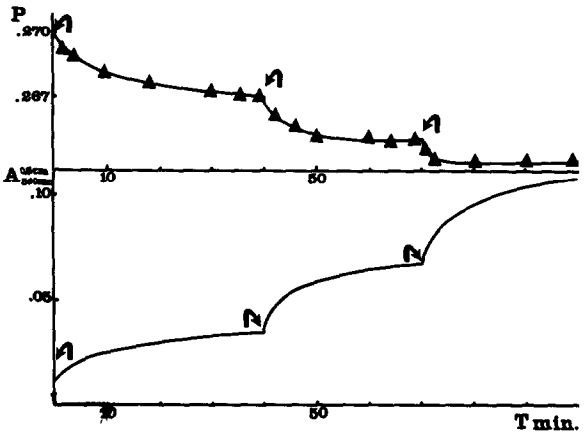

Fig.1. Time course of aggregation of egg lecithin vesicles containing gangliosides upon adding limulin. Limulin concentrations: (a) 14 μg/ml; (b) 28 μg/ml; (c) 42 μg/ml. $A^{0.5\ cm}_{500\ nm}$, absorbance at 500 nm, light-path; 0.5 cm. P, fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene. Egg lecithin–cholesterol vesicles containing horse erythrocytes gangliosides (molar ratios: PC : cholesterol : NAN; 1 : 0.25 : 0.14). Lecithin concentration : 0.5 mg/ml. The vesicles were suspended in 0.1 M NaCl, 0.01 M $CaCl_2$, 0.05 M Tris–HCl buffer, pH 8.5, at 25°C.

was observed when limulin was added to a suspension of ganglioside-free vesicles. The addition of a small amount (14 μg/ml) of limulin to a suspension of vesicles containing cholesterol and gangliosides induced a slight aggregation process: the absorbance increased 3-fold therefore the number of scattering particles was only slightly decreased and so the average size of the scattering particles was only slightly increased. The relationship between absorbance and fluorescence polarization was linear (fig.2) and similar to that of fluorescence polarization versus the concentration of vesicles.

The addition of a larger amount of limulin to a suspension of vesicles containing cholesterol and gangliosides induced a dramatic aggregation process: the absorbance increased 12-fold; the number of scattering particles was very decreased and their average size very increased. The slope of the curve absorbance versus fluorescence polarization became smaller than that of the curve absorbance versus vesicle concentration. So, the relationship (fluorescence polarization versus absorbance) relevant for free vesicles, is usable for moderate aggregated particles but not for highly aggregated particles. This point is also supported by the findings that the fluorescence polarization of multibilayers was constant in a large

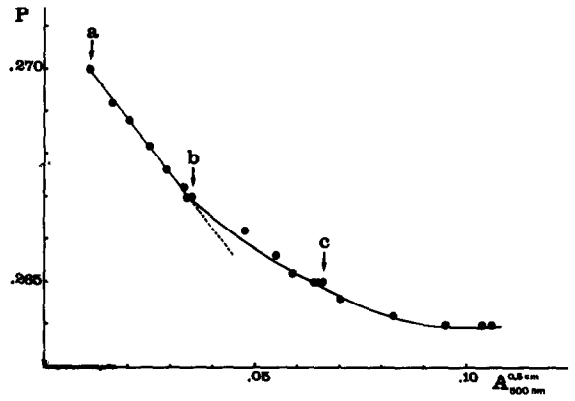

Fig.2. Changes of fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene related to the increase of absorbance of the vesicle suspension upon adding limulin. Egg–lecithin–cholesterol vesicles containing horse erythrocyte gangliosides (molar ratios: PC : cholesterol : NAN; 1 : 0.25 : 0.13) were suspended in 0.1 M NaCl, 0.01 M $CaCl_2$, 0.05 mM Tris–HCl buffer, pH 8.5, at 25°C. Lecithin concentration: 0.5 mg/ml. Limulin adducts, final concentrations: (a) 14 μg/ml; (b) 28 μg/ml; (c) 42 μg/ml. $A^{0.5\,cm}_{500\,nm}$, absorbance at 500 nm, light-path 0.5 cm. *P* fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene.

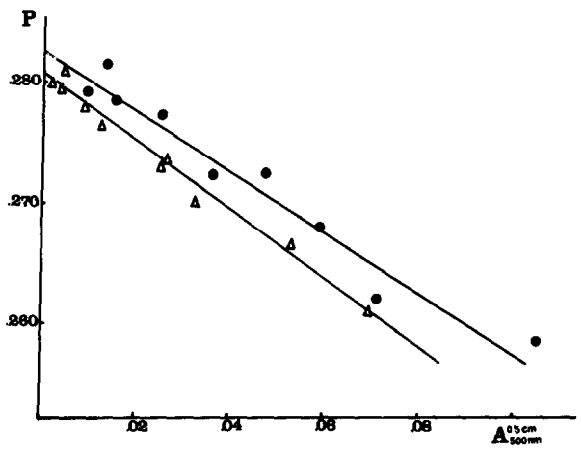

Fig.3. Changes of fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene in the absence (–△–) and in the presence (–●–) of limulin (40 μg/ml) related to the absorbance of the vesicle suspension. Egg lecithin–cholesterol vesicles containing horse erythrocyte gangliosides (molar ratios: PC : cholesterol : NAN; 1 : 0.25 : 0.13) were suspended in 0.1 M NaCl, 0.01 M $CaCl_2$, 0.05 M Tris–HCl buffer, pH 8.5. Lecithin concentration: 0.25–2 mg/ml. The measurements were registered 60 min after addition of limulin, at 25°C.

concentration range (0.25–1 mg/ml of lecithin corresponding to absorbances 0.13–0.8, respectively).

3.3. *Changes of fluorescence polarization related to the aggregation process*

In order to determine if the measured fluorescence polarization was either a unique consequence of light scattering effect or a consequence of a change in the dynamics of the bilayer, a same amount of limulin was added to different concentrations of vesicles containing similar gangliosides/phospholipid ratios. For each vesicle concentration, the absorbance and the *P* were determined before and 60 min after the addition of limulin. As shown by plotting *P* versus absorbance, the fluorescence polarization (in the presence of limulin) extrapolated to zero absorbance was very close to the extrapolated value of *P* in the absence of limulin (fig.3). Therefore, the measured change of *P* has to be related to a light scattering process. To ascertain this conclusion, pulse fluorimetry experiments were conducted using a monoexponential analysis. The average fluorescence lifetime of DPH was found to be insensitive to the presence of gangliosides in the vesicles and to be very slightly decreased by the aggregation induced by adding limulin.

4. Discussion

No attempt was made to express the fluorescent measurements as the microviscosity of the bilayer. Indeed, the lipid bilayer is not an isotropic medium and DPH may be in several different environments, so the observed fluorescence polarization, and the monoexponential fluorescence lifetime are average values [4,22–27]. However, if the average polarization obtained under continuous excitation cannot be expressed quantitatively as a change of viscosity, it does reflect qualitatively a change in the dynamic of the lipid bilayer.

Fluorescence polarization by light scattering in turbid solutions was extensively studied [28]. The main source of error in the measurement of fluorescence polarization is due to the scattering of the exciting light which induces an artificial increase of the polarization. This error was eliminated by using

bandpass interference filters between the light source and the sample and between the sample and the photomultipliers.

Indeed, with multibilayers, large aggregates or cells, fluorescence polarization was found to be not or only slightly dependent upon the turbidity. Conversely, with small vesicles, fluorescence polarization was found to decrease in relation with an absorbance increase. This result is consistent with that recently reported in the case of plasma membrane vesicles [29]. On this basis, the decrease of fluorescence polarization that we observed should mainly be attributed to a light-scattering depolarization, because the addition of limulin to suspensions of vesicles containing gangliosides induces an aggregation process and therefore an absorbance increase. To take into account the light-scattering depolarization, either the aggregated solution was diluted in order to reach the initial absorbance value, or the polarization measurements were done at several vesicles concentrations, and extrapolated to absorbance zero. In both cases, it appeared that there was no significant change in fluorescence polarization relative to the presence of limulin. These results associated with those of the pulse fluorescence experiments give informations on the dynamical properties of the lipid bilayer. Indeed, the static fluorescence polarization is directly related to the fluorescence anisotropy $\bar{r}$, which depends upon both the mean lifetime $\langle\tau\rangle$ of the probe and its mean rotational correlation time $\langle\rho\rangle$ according to $\bar{r} = r_0/(1 + \langle\tau\rangle/\langle\rho\rangle)$. So, $\bar{r}$ is expected to be constant either when $\langle\tau\rangle$ and $\langle\rho\rangle$ are constant, or when a change of $\langle\tau\rangle$ is compensated by an opposite change of $\langle\rho\rangle$. Then, because $\bar{P}$ (and therefore $\bar{r}$) after correction for the light scattering process, and $\langle\tau\rangle$ were not significantly affected by the aggregation process of vesicles containing gangliosides, induced by limulin, it can be concluded that no dynamical change occurs in the lipid bilayer related to the gangliosides–limulin interaction.

Similarly concanavalin A, another mitogenic lectin, at low concentration (2 μg/ml) did not affect the fluorescence polarization of DPH incorporated in membrane vesicles [30]; to the contrary, concanavalin A at high concentration (2 mg/ml) was reported to increase the fluorescence polarization [30]; but at such a very high concentration, concanavalin A also interacted with pure phospholipid vesicles [31].

However, changes in the dynamics of the membrane have been reported mainly with lymphocytes upon adding various lectins. A 3% decrease of fluorescence polarization was found with erythrocytes upon adding *Ricinus communis* agglutinin [32]. A similar result was obtained upon adding (3 μg/ml) succinyl concanavalin A [33]. An important decrease was obtained by addition of *Wistaria floribunda* lectin, a mitogenic lectin, but not with non-mitogenic lectins [34]. These changes occured within 1 h after the lectin addition.

Of course, in the simple model we were using, the expected change could only be ascribed as a physicochemical change of the bilayer, whereas with living cells such as lymphocytes, many biological changes may occur. For instance, the increased biosynthesis of phospholipids observed within 30–60 min after the addition of a mitogenic lectin [35–37] may change the lipid composition of the membranes and so the fluorescence polarization of DPH may be affected.

The lack of change in static fluorescence polarization and in average lifetime of DPH embedded in vesicles containing gangliosides interacting with limulin must be interpreted either in terms of an absence of any dynamical changes or in terms of a sensitivity lack of the probe to detect minute changes of lipid bilayers.

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