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PECULIAR BEHAVIOUR OF RABBIT THYMOCYTES IN INTERACTION WITH LIPOSOMES OF DIFFERENT COMPOSITIONS SHOWN BY FLUORESCENCE POLARIZATION STUDIES, LIPID ANALYSIS, AND UPTAKE OF VESICLE-ENTRAPPED CARBOXYFLUORESCEIN

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In order to obtain more information on membrane phenomena occurring at the cell surface of rabbit thymocytes we have performed experiments aimed at altering the lipid composition of the plasma membrane. Thymocytes were incubated at 37°C with phospholipid vesicles of different compositions. Vesicle-cell interaction was followed by measuring the degree of fluorescence polarization and the uptake of vesicle-entrapped carboxyfluorescein. Neutral and negatively charged liposomes prepared from egg phosphatidylcholine are currently used in investigations of vesicle-cell interaction. In this report we show that these liposomes do not interact with rabbit thymocytes as is evident from unaltered lipid fluidity measured in whole cells and in isolated plasma membranes. This was confirmed by experiments with vesicle-entrapped carboxyfluorescein showing hardly any uptake of the fluorophor from neutral and negatively charged egg phosphatidylcholine liposomes. Using both techniques substantial interaction was found with positively charged egg phosphatidylcholine liposomes and with liposomes prepared from soybean lecithin which is composed of a variety of phospholipids. The results of these experiments were supported by lipid analysis of cells treated with soybean lecithin liposomes. Increase in phosphatidylcholine contents of mixed phospholipid vesicles was further shown to result in decreased vesicle-cell interaction. From measurements of the quantity of carboxyfluorescein inside cells and the total amount of cell-associated carboxyfluorescein it is concluded that adsorption plays a prominent role in interaction between liposomes and rabbit lymphocytes. The grade of maturation of lymphocytes was also found to affect vesicle-cell interaction. The more mature thymocytes took up more vesicle-entrapped carboxyfluorescein from soybean liposomes than immature thymocytes. Mesenteric lymph node cells exhibited a still stronger interaction. The role of vesicle and cell surface charge and membrane fluidity of both vesicles and cells in interaction between liposomes and rabbit thymocytes is discussed.

Introduction

In a previous study we found that plasma membranes of rabbit thymocytes exhibited an unusually high rigidity [1]. This was also found for membranes exfoliated from thymus cells in situ [2]. Because even more rigid membrane structures were expected in this latter case in view of the reports by others [3,4], we assumed that there exists a limiting value for the lipid fluidity (expressed in terms of fluorescence polarization) of plasma membranes from eukaryotic cells. In order to investigate this further we decided to manipulate the lipid composition of the plasma membranes of rabbit thymocytes.

There was another reason for carrying out these experiments. In the thymus, lymphocytes are found at various stages of maturation. It is possible to

enrich the immature and more mature cells in subclasses by density gradient centrifugation. The more mature rabbit thymocytes are more effectively stimulated by mitogenic lectins than the immature cells [5] which is in line with the observations of other authors with mouse and rat thymocytes [6–9]. Because we also found that plasma membranes of the more mature cells exhibited a higher lipid fluidity than plasma membranes from immature cells [1], we wanted to know if this difference in lipid fluidity is directly related to the (immune) reactivity of the thymocytes.

There are various means of altering the lipid fluidity of plasma membranes. The most elaborate technique consists in incubation of the cells with liposomes, preferentially unilamellar vesicles [10–13]. By application of this technique, various authors have found contradictory results with respect to the relation between lipid composition (lipid fluidity) and mitogenic stimulation [14–22]. This might be due to different liposome-cell interactions as a result of differences in cell surface properties of the various cell types used in these investigations. For this reason, we first wanted to know to what degree liposomes interact with rabbit thymocytes (and their subclasses) before testing the effect of this treatment on cell reactivity.

In the present paper we show, in the first instance from fluorescence polarization studies, that rabbit thymocytes are very unwilling to react with egg phosphatidylcholine liposomes, even when the liposomes are negatively charged. Interaction was only observed with positively charged phosphatidylcholine liposomes and with negatively charged liposomes of complex phospholipid composition as found in soybean lecithin. This is confirmed by lipid analyses of treated cells.

Because these results were at variance with data reported in the literature, we decided to verify our results with another technique. For that end we applied the technique of entrapping the fluorescent dye carboxyfluorescein in the aqueous space of liposomes, as described in detail elsewhere [23–25]. Briefly, carboxyfluorescein is encapsulated inside liposomes in a high, self-quenched concentration. Transition of the dye from cell-associated vesicles into the cytoplasm as a result of fusion or membrane perturbation causes a vast dilution of the dye giving rise to an immediate fluorescent signal. The total amount of cell-associated carboxyfluorescein including the amount of dye still entrapped in vesicles adsorbed to the cell surface, can be detected by the addition of a detergent which causes immediate desintegration of the vesicles resulting in dilution of all carboxyfluorescein in the incubation medium.

The results of the carboxyfluorescein experiments entirely support our conclusions drawn from the fluorescence polarization experiments. Incubation of thymocytes of different stages of maturation with carboxyfluorescein-containing soybean lecithin liposomes further showed a stronger interaction with the more mature cells.

From the carboxyfluorescein experiments we conclude that adsorption of liposomes to the cell surface is the most important phenomenon which may be followed by fusion and/or lipid exchange. Endocytosis was ruled out on the basis of experiments with metabolic inhibitors and cytochalasin B.

Materials and Methods

Chromatographically pure egg phosphatidylcholine, commercial grade and type IV soybean lecithin, dicetyl phosphate, stearylamine, and 2-deoxyglucose were purchased from Sigma. Phosphatidylserine was obtained from Supelco, and cholesterol from BDH. Cytochalasin B was a product of Serva. Carboxyfluorescein was purchased from Eastman Kodak, and purified by passage of a 10% aqueous solution, pH 7.4, through a Sephadex LH-20 column, dimensions 15 × 1 cm. Hanks' balanced salt solution and RPMI 1640 were from Gibco.

Cells and plasma membranes. Lymphocytes were obtained from rabbit thymus and separated into subclasses on a Ficoll/Metrizoate density gradient as described previously [1]. The subclasses with peak densities of approx. 1.067, 1.076, and 1.084 g/ml are denoted as fractions I, II, and III, respectively. The viability of the cells was determined by the eosin exclusion test. Plasma membranes were obtained using a cell-disrupting pump followed by differential and density gradient centrifugation as described [1].

Preparation of liposomes. The appropriate

amount of lipid was dissolved in a small volume of chloroform/methanol (2:1, v/v). The solvent was removed by rotary evacuation in a round-bottom flask leaving a lipid film on the glass wall. After lyophilization, the lipid was dispersed in medium (Tris-buffered saline, Hanks' balanced salt solution, RPMI 1640, or 200 mM carboxyfluorescein, all at pH 7.4) in a bath sonicator. The dispersion was then subjected to ultrasonic irradiation at 0°C under nitrogen in a MSE Mullard probe sonicator (output 60 W). For the assessment of the time needed for adequate conversion of the multilamellar vesicles into small unilamellar vesicles, the sonication was performed in Tris-buffered saline and at various times the absorbance at 300 nm was measured [26]. After sonication, the liposome dispersions were centrifuged at $100000 \times g$ for 30 min to remove metal impurities and remaining multilamellar vesicles. We found that 85–95% of the lipid was converted into small unilamellar vesicles. In the case of carboxyfluorescein-containing liposomes, non-entrapped and entrapped dye were separated by passage through a Sephadex G-75 column, dimensions 15×1 cm, equilibrated in Hank's balanced salt solution (without pH indicator). The degree of self-quenching of the vesicle-entrapped carboxyfluorescein was determined by estimation of the fluorescence intensity, before and after the addition of Triton X-100 to a final concentration of 1% and appropriate dilution with Hanks' balanced salt solution to arrive in the linear part of the calibration curve [27]. Usually, the degree of self-quenching was between 96 and 98%. Fluorescence measurements were carried out in a Perkin-Elmer 650-40 fluorescence spectrophotometer with excitation and emission wavelengths of 494 and 518 nm, respectively.

Incubation of thymocytes with liposomes. For estimation of the degree of fluorescence polarization and analysis of the lipid composition, thymocytes (10^7/ml) were incubated with liposomes at 37°C in a shaking water bath, during 30 or 60 min (see Results). After incubation, the suspensions were cooled to 0°C. The cells were sedimented at 350 g and washed twice with Hanks' balanced salt solution (without pH indicator). For fluorescence polarization measurements, the resulting cell pellets (10^6–10^7 cells) were suspended in 0.5 ml Hanks' balanced salt solution (without pH indicator). For lipid analysis, the pellets were frozen-dried and extracted as described [1,2]. The incubation experiments with carboxyfluorescein-containing liposomes were performed in principle according to the method of Van Renswoude et al. [27]. Lymphocytes (10^7/ml) were incubated at 37°C in a shaking water bath with carboxyfluorescein-containing vesicles, empty vesicles (vesicles not containing carboxyfluorescein in the aqueous space), and empty vesicles in the presence of 25 μM free carboxyfluorescein, in polystyrene tubes at a final volume of 1 ml. The phospholipid concentration was 0.5 mg/ml. At the indicated times, the suspensions were cooled to 0°C and the cells were immediately sedimented by centrifugation and washed three times, the last washing being performed in fresh polystyrene tubes [27]. The resulting pellets were suspended in 1.2 ml Hanks' balanced salt solution (without pH indicator). The fluorescence intensity was measured before and after the addition of 0.12 ml 10% Triton X-100. All incubations were carried out in triplicate. To account for cell-induced leakage of vesicle-encapsulated carboxyfluorescein, samples were taken from the supernatant after the first centrifugation of the incubated cells, and diluted 1:40 with Hanks' balanced salt solution (without pH indicator). The fluorescence intensity was measured directly (F_d) and indirectly (F_i) after the addition of Triton X-100. This yields the correction factor $(1 - \Lambda)^{-1}$ [27] with $\Lambda = (\Lambda_t - \Lambda_0)/(1 - \Lambda_0)$; here $\Lambda_t = F_d/F_i$ and Λ_0 denotes the ratio of the fluorescence readings before and after addition of detergent in a liposome sample immediately after collection of the void volume of the Sephadex G-75 column [27]. Corrections were also made for differences in fluorescence intensities at different pH values in the interior of the cell and in the medium, as described by Van Renswoude et al. [27].

Fluorescence microscopy. Cells were mounted under a coverslip and observed with a Leitz Orthoplan microscope with Ploem-Opak incident illumination, and supplied with a $95 \times (1.10–1.32)$ objective and $4 \times$ eyepieces.

Biochemical analyses and fluorescence polarization measurements. Phospholipid phosphorus, phospholipid composition, cholesterol, and fatty acid composition of the phospholipid and free fatty acid fractions were determined as described

previously [1,2]. Column chromatography of soybean lecithin was performed on silica gel [28]. The fractions were monitored by one-dimensional thin-layer chromatography on HPTLC plates (Merck) using petroleum ether (60–80°C)/diethyl ether/acetic acid (85:15:2, v/v) for elution. Steady-state fluorescence polarization measurements were performed as described before [1] in an Elscint MV-1a apparatus using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a fluorescent probe.

Results

Effect of liposome treatment on the degree of fluorescence polarization of diphenylhexatriene in thymocytes

No significant differences were found in the degree of fluorescence polarization of diphenylhexatriene in untreated thymocytes belonging to the density fractions I, II, and III: the P values at 25°C were 0.263 ± 0.005, 0.262 ± 0.004, and 0.264 ± 0.005 (mean ±S.D. of eleven experiments), respectively. Incubation with liposomes prepared from egg phosphatidylcholine, egg phosphatidylcholine/cholesterol (1:1 mol/mol), egg phosphatidylcholine/dicetyl phosphate (9:1 mol/mol), egg phosphatidylcholine/phosphatidylserine (9:1 mol/mol), and phosphatidylserine hardly affected the degree of fluorescence polarization (Table I). In considering the data from Table I and the following tables it is important to note that the accuracy of measuring the P value is ±0.005, as concluded from all experiments done. When we used positively charged liposomes of egg phosphatidylcholine/stearylamine (9:1 mol/mol) or negatively charged liposomes of a complex phospholipid composition as in commercial grade soybean lecithin (Table II, last column), the degree of fluorescence polarization was significantly lowered (Table I). No significant differences were found between the various subclasses of thymocytes, although in all experiments conducted so far the lightest density fraction always showed the greatest decrease in fluorescence polarization. From seven experiments, a mean value (±S.D.) of 0.240 (±0.005) was calculated for the P value at 25°C in unfractionated thymocytes treated with soybean lecithin liposomes, compared with 0.264 (±0.005) for untreated thymocytes. The viability and the recovery of the cells were good. When the cells were treated with higher concentrations of liposomes or for longer periods, both the viability and the recovery were affected, particularly of the heaviest fraction. This was especially the case with the stearylamine containing liposomes which already affected viability and recovery of the fraction III cells drastically after 30 min incubation

TABLE I

EFFECT OF LIPOSOME TREATMENT ON THE DEGREE OF FLUORESCENCE POLARIZATION OF DPH IN THYMOCYTE SUBCLASSES

Thymocytes were separated into subclasses on a Ficoll/Metrizoate density gradient and, after washing, incubated with liposomes for 30 min at 37°C. The data are expressed as the difference from the degree of fluorescence polarization measured in control cells. PC, phosphatidylcholine; Chol, cholesterol; DCP, dicetyl phosphate, PS, phosphatidylserine; SA, stearylamine; SLE, soybean lecithin (commerical grade).

Liposome composition	ΔP (25°C)		
	Fraction I	Fraction II	Fraction III
PC	−0.007	−0.004	−0.003
PC/Chol (9:1 mol/mol)	+0.002	−0.002	+0.001
PC/DCP (9:1 mol/mol)	+0.003	−0.003	−0.005
PC/PS (9:1 mol/mol)	−0.006	−0.009	−0.008
PS	−0.006	−0.006	−0.004
PC/SA (9:1 mol/mol)	−0.023	−0.023	−0.014
SLE	−0.028	−0.024	−0.023

resulting in a far less reduced P value compared with the other cell fractions (Table I).

Because a commercial grade preparation of soybean lecithin was used, the effect on the degree of fluorescence polarization may possibly be due to impurities. Especially free fatty acids might influence the lipid fluidity of cellular membranes markedly, even in low concentrations [29]. The soybean lecithin used contained only very small amounts of free fatty acids as determined by thin-layer chromatography (data not shown). Fractionation by column chromatography on silica gel yielded a pure phospholipid fraction. We found that the effect of this neutral lipid-free soybean lecithin on the degree of fluorescence polarization of DPH in liposome-treated thymocytes was the same as in the case of unpurified soybean lecithin (data not shown).

Lipid composition of thymocytes after treatment with soybean lecithin liposomes

Because all density fractions of thymocytes showed about the same decrease in the degree of fluorescence polarization after treatment with soybean lecithin liposomes, lipid analyses were performed on unfractionated thymocytes. As expected, the cholesterol content was decreased in cells treated with soybean lecithin liposomes (Table II). This is consistent with the decrease in the degree of fluorescence polarization of diphenylhexatrine in liposomes prepared from the total lipid fraction extracted from the cells, as is also shown in Table II. Removal of neutral lipids (mainly cholesterol) from the lipid extract by one-dimensional thin-layer chromatography resulted in a further decrease in the degree of fluorescence polarization. The remaining phospholipid fraction from soybean lecithin-treated cells showed a still lower P value compared with control cells. This might be attributed to the altered phospholipid composition of thymocytes as a result of the treatment with soybean lecithin liposomes (Table II). This change was not accompanied by a corresponding change in the fatty acid composition of the phospholipid fraction (Table III). This leads us to the assumption of a rapid acyl chain metabo-

TABLE II

DEGREE OF FLUORESCENCE POLARIZATION, CHOLESTEROL CONTENT, AND PHOSPHOLIPID COMPOSITION OF THYMOCYTES AFTER TREATMENT WITH SOYBEAN LECITHIN LIPOSOMES

Thymocytes were incubated at 37°C for 30 min with liposomes (0.5 mg/ml) prepared from neutral lipid-free soybean lecithin. SLE, soybean lecithin (commercial grade).

	Treatment		SLE
	None	SLE	
P (25°C)			
Whole cells	0.263	0.243	
Total lipid fraction [a]	0.258	0.239	
Phospholipid fraction [a]	0.244	0.231	0.163
Cholesterol/phospholipid molar ratio	0.22	0.18	
Phospholipid composition (%)			
Origin	–	0.2	5.7
Phosphatidylserine	13.3	14.6	18.8
Phosphatidic acid	0.1	0.7	8.4
Sphingomyelin	2.7	2.7	–
Lysophosphatidylcholine	0.3	1.1	2.3
Phosphatidylcholine	49.7	38.7	28.1
Phosphatidylethanolamine	28.6	32.2	30.1
Diphosphatidylglycerol	0.9	2.6	–
Unknown	4.5	7.1	6.6

[a] The lipids were first dispersed in 0.5 ml Tris-buffered saline by brief sonication in a bath sonifier and then mixed with the diphenylhexatriene solution.

TABLE III

EFFECT OF TREATMENT OF THYMOCYTES WITH SOYBEAN LECITHIN LIPOSOMES ON THE FATTY ACID COMPOSITION OF THE PHOSPHOLIPID AND FREE FATTY ACID FRACTIONS OF LIPIDS EXTRACTED FROM THE CELLS

Thymocytes were incubated at 37°C for 1 h with liposomes (0.5 mg/ml) prepared from neutral lipid-free soybean lecithin (commercial grade). The data are expressed as the means of analyses performed on two types of liquid phase: SP 2330 and SP 2340 (from Supelco). SLE, soybean lecithin.

	Lipids extracted from control and treated cells (mol%)				SLE
	Phospholipid fraction		Free fatty acid fraction		
	Control	SLE	Control	SLE	
14:0	1.0	0.5	0.7	–	0.3
15:0	0.6	0.4	1.4	1.6	0.2
16:0	45.2	47.5	39.4	31.9	38.0
16:1	2.6	2.4	3.1	2.8	1.9
17:0	0.4	0.4	–	–	–
18:0	20.0	19.7	20.2	16.7	8.8
18:1	18.7	18.1	14.2	13.7	8.8
18:2	6.8	7.6	4.0	12.5	31.6
18:3	0.5	–	–	–	2.3
20:0	0.2	0.2	4.4	3.1	0.4
20:1	–	0.9	3.1	4.9	1.1
20:4	2.3	0.7	0.7	–	–
21:0	0.6	0.6	1.7	2.9	–
22:0	0.5	0.4	3.6	4.1	0.5
22:1	–	–	3.2	5.1	1.6
22:4	0.2	0.1	0.5	0.7	3.8
24:0	0.1	0.2	–	–	1.3
Saturated	68.7	69.9	71.4	60.3	49.5
Mono-unsatd.	21.4	21.4	23.8	26.5	13.1
Poly-unsatd.	9.9	8.7	4.8	13.2	37.5

lism of phospholipids taken up by the cells. The free fatty acid fraction from lipids extracted from soybean lecithin-treated cells was found to be greatly increased compared with control cells, as concluded from one- and two-dimensional thin-layer chromatograms (data not shown). This is accompanied by a substantial increase in the linoleic acid (18:2) content which is one of the main fatty acid components of soybean lecithin phospholipids (Table III).

Degree of fluorescence polarization of diphenylhexatriene in plasma membranes isolated from liposome-treated cells

Because liposomes first adhere to the cell plasma membrane which may be followed by fusion, endocytosis, or lipid exchange [13], the change in lipid fluidity measured in intact cells would be smaller than the change in lipid fluidity measured in isolated plasma membranes. In order to check this, we isolated plasma membranes from thymocytes treated with soybean lecithin liposomes. For comparison we also isolated plasma membranes from thymocytes treated with egg phosphatidylcholine liposomes which were shown not to interact with thymocytes in such a way that the degree of fluorescence polarization measured in intact cells was affected. As shown in Table IV, the degree of fluorescence polarization measured in the plasma membrane fractions PM2-PM4 (which are considered as the purest fractions [1]) was decreased almost twice as much as the degree of fluorescence polarization measured in intact thymocytes after treatment with soybean lecithin liposomes. The ineffectiveness of treatment with egg phosphatidylcholine liposomes was again illustrated by the insignificant change in the degree of fluorescence polarization measured in isolated plasma membranes.

Effect of lipid composition and vesicle charge on the interaction between thymocytes and carboxyfluorescein-containing liposomes

As a control for light scattering and uptake of carboxyfluorescein released from the vesicles, cells were also incubated with empty vesicles in the absence and presence of free carboxyfluorescein [27]. From these experiments it could also be calculated, with the aid of a calibration curve for the relation between fluorescence intensity and pH, that the internal pH of the cells was 6.8–6.9 [25,27]. Only minor quantities of free carboxyfluorescein were taken up by rabbit thymocytes, approx. 0.04 pmol/10^6 cells after 30 min incubation in a suspension containing empty vesicles and 25 μM carboxyfluorescein. Leakage of carboxyfluorescein from the liposomes at the various incubation times was determined by taking samples from the supernatants after the first centrifugation step and measuring the fluorescence intensity be-

TABLE IV

DEGREE OF FLUORESCENCE POLARIZATION OF DIPHENYLHEXATRIENE IN THYMOCYTES AND PLASMA MEMBRANES AFTER TREATMENT OF THE CELLS WITH LIPOSOMES

Thymocytes were incubated for 30 min at 37°C with liposomes (1 mg/ml) prepared from egg phosphatidylcholine and soybean lecithin. After intensive washing, plasma membranes were isolated, and the degree of fluorescence polarization was measured. The plasma membrane fractions were named PM 1–4 as described previously [1]. PC, phosphatidylcholine; SLE, soybean lecithin (commercial grade).

	Untreated cells	PC-treated cells		SLE-treated cells	
	P (25°C)	P (25°C)	ΔP [a]	P (25°C)	ΔP [a]
Intact cells	0.264	0.260	−0.004	0.245	−0.019
Plasma membranes					
PM1	0.309	0.304	−0.005	0.297	−0.012
PM2	0.347	0.343	−0.004	0.320	−0.027
PM3	0.352	0.345	−0.007	0.315	−0.037
PM4	0.354	0.349	−0.005	0.318	−0.036

[a] Difference from untretated cells.

fore and after the addition of Triton X-100. The leakage of carboxyfluorescein from phosphatidylcholine liposomes increased in time to a value of 8.0% at 60 min, and was hardly affected by the presence of dicetyl phosphate, phosphatidylserine, or stearylamine. Soybean lecithin liposomes showed an increased leakage: 12.7% at 60 min. The leakage was most pronounced in the case of liposomes prepared from rabbit serum lipids. We found that after 30 min incubation with thymocytes 19% carboxyfluorescein was leaked out of vesicles prepared from Chinchilla rabbit serum lipids and 37% out of vesicles prepared from normal rabbit serum (Gibco) lipids. We also measured bulk leakage of vesicle-entrapped carboxyfluorescein in the absence of cells (data not shown) and found that by the addition of cells the Λ_t-values were hardly affected in the case of liposomes prepared from phosphatidylcholine with or without dicetyl phosphate, phosphatidylserine, or stearylamine, for periods up to 1 h. In the case of soybean lecithin liposomes, cell-induced leakage superimposed on bulk leakage was not observed until approx. 20 min of incubation. The situation changed with the introduction of cholesterol. Liposomes prepared from soybean lecithin/cholesterol (10:3 mol/mol), and rabbit serum lipids showed much higher Λ_t values in the presence of cells.

All incubation experiments were carried out in triplicate. From the results a mean standard deviation of 9.5% was calculated for the estimation of fluorescence intensity in cell suspensions. The fluorescence readings of the incubated cells were corrected for pH effect, leakage of bulk liposomes content, and the effect of Triton X-100 as described by Van Renswoude et al. [27].

Incubation of thymocytes with carboxyfluorescein-containing soybean lecithin liposomes resulted in a rapid uptake of vesicle-entrapped carboxyfluorescein with a plateau after 20 min (Fig. 1). The amount of carboxyfluorescein inside cells is then 30% of the total amount of cell-associated carboxyfluorescein and remains fairly constant during further incubation. Fig. 1 further shows that carboxyfluorescein is already taken up by the mere contact between cells and liposomes at 0°C for only some minutes without further incubation (the suspensions were centrifuged immediately after mixing cells and liposomes).

Because the fluorescence polarization experiments have indicated that the phospholipid composition of the liposomes might be an important factor in the interaction with rabbit thymocytes, we also carried out experiments with liposomes prepared from various phospholipid sources: commercial grade and type IV soybean lecithin, and lipid preparations obtained from normal rabbit

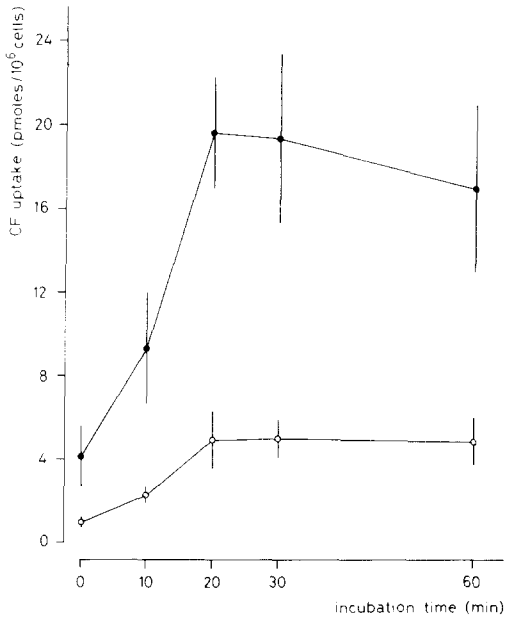

Fig. 1. Uptake of vesicle-entrapped carboxyfluorescein by thymocytes from soybean lecithin (commercial grade) liposomes. Each point represents the mean value (±S.D.) of four independent experiments, each performed in triplicate. ○——○, carboxyfluorescein inside cells; ●——●, total cell-associated carboxyfluorescein. CF, carboxyfluorescein.

serum (Gibco) and Chinchilla rabbit serum. Type IV soybean lecithin contains less phosphatidylserine and more phosphatidylcholine than commercial grade soybean lecithin (Table V). A further decrease in phosphatidylserine and increase in phosphatidylcholine contents is shown by both serum lipid preparations which also contain a fair amount of lysophosphatidylcholine (Table V). Incubation of rabbit thymocytes with liposomes prepared from these lipid mixtures showed that the change in phospholipid composition is accompanied by a decrease in uptake of vesicle-entrapped carboxyfluorescein (Table VI). In the case of the two serum lipid mixtures this decrease cannot be attributed to the presence of cholesterol, because addition of cholesterol to soybean lecithin rather promoted the uptake of vesicle-entrapped carboxyfluorescein strongly suggesting an increased vesicle-cell interaction (Table VI).

Table VI also shows that the interaction between rabbit thymocytes and phosphatidylcholine liposomes is very low compared with commercial grade soybean lecithin which is in accordance with the results from the fluorescence polarization experiments. As shown before, the application of a

TABLE V

LIPID COMPOSITION OF SOYBEAN LECITHIN AND RABBIT SERUM

Normal rabbit serum from Gibco and serum from Chinchilla rabbit (the experimental animal in the present study) were frozen-dried. Lipid extracts were prepared as described previously [1]. The phospholipid composition was determined by phosphorous analysis after two-dimensional thin-layer chromatography on HPLTC plates (Merck) [2]. Cholesterol and cholesterol ester were determined enzymatically [1].

	Soybean lecithin		Serum lipids	
	Commercial grade	Type IV	Normal rabbit serum (Gibco)	Chinchilla rabbit serum
Phospholipid composition (%)				
Phosphatidylserine	18.8	5.3	2.9	3.1
Phosphatidic acid	8.4	8.2	0.3	–
Spingomyelin	–	–	9.0	4.9
Lysophosphatidylcholine	2.3	3.7	31.6	16.4
Phosphatidylcholine	28.1	42.3	51.7	71.0
Lysophosphatidylethanolamine	–	–	1.1	0.3
Phosphatidylethanolamine	30.1	29.4	2.4	4.4
Unknown	12.3	10.2	1.1	–
Cholesterol/phospholipid molar ratio	–	–	0.34	0.25
Cholesterol ester/cholesterol molar ratio	–	–	2.41	2.12

TABLE VI

EFFECT OF LIPID COMPOSITION AND VESICLE CHARGE ON THE UPTAKE OF VESICLE-ENTRAPPED CARBOXYFLUORESCEIN BY RABBIT THYMOCYTES

Thymocytes (10^7/ml) were incubated with carboxyfluorescein-containing liposomes (0.5 mg phospholipid/ml) at 37°C and processed as described in Materials and Methods. SLE, soybean lecithin; Chol, cholesterol; NRS, normal rabbit serum; RS, rabbit serum; PC, phosphatidylcholine; DCP, dicetyl phosphate; PS, phosphatidylserine; SA, stearylamine.

Liposomes		Total cell-associated carboxyfluorescein (pmol 10^6 cells)		Fraction of cell-associated carboxyfluorescein inside cells (%) [b]
Composition	P (25°C)	0 min	20 min [a]	
SLE (commercial grade)	0.168	4.2	20	30 (28 –32)
SLE (type IV)	0.151	0.6	6.9	34 (32 –35)
SLE/Chol (10:3 mol/mol) [c]	0.282	6.3	49	26 (24 –28)
NRS (Gibco) lipids	0.256	0.6	4.3	55 (54 –56)
Chinchilla RS lipids	0.222	0.5	1.2	42 (39 –44)
PC	0.147	4.6	3.8	8 (6.3– 9.7)
PC/DCP (9:1 mol/mol)	0.174	0.1	0.6	27 (23 –29)
PC/PS (9:1 mol/mol)	0.145	0.6	1.1	22 (19 –25)
PC/SA (9:1 mol/mol	0.147	24	41	18 (14 –20)

[a] In all cases a plateau was reached after 20 min incubation.
[b] Mean of values calculated at 10, 20, 30, and 60 min intervals (range between parentheses)
[c] Commercial grade SLE was used.

negative charge to phosphatidylcholine liposomes by the addition of dicetyl phosphate or phosphatidylserine did not affect the degree of fluorescence polarization of intact cells incubated with this type of liposomes. This lack of interaction is confirmed by the experiments with carboxyfluorescein-containing liposomes (Table VI). Incubation of rabbit thymocytes with positively charged phosphatidylcholine/stearylamine liposomes lead to a rapid uptake of vesicle-entrapped carboxyfluorescein. The interaction with this type of liposomes was even stronger than with soybean lecithin liposomes (Table VI). As mentioned before, the incubations with stearylamine-containing liposomes could not be continued for longer periods than 30 min because of strongly impaired cell viability.

Involvement of the grade of lymphocyte maturation in vesicle-cell interaction as monitored by the uptake of vesicle-entrapped carboxyfluorescein

Maturation of T lymphocytes is accompanied by an increase in electrophoretic mobility of the cells [7,30] as a result from an increase in net negative charge of the cell surface [31]. This may affect the interaction with liposomes which was already suggested by the fluorescence polarization experiments with subclasses of thymocytes although no significant differences were found (Table I). For this reason, we incubated subclasses of thymocytes with carboxyfluorescein-containing soybean lecithin liposomes. The most mature thymocytes are present in the 1.067 g/ml density fraction after Ficoll/Metrizoate density gradient centrifugation and these cells reacted more readily with liposomes than cells from the 1.076 and 1.084 g/ml density fractions (Fig. 2). Another property also appears from Fig. 2. Before fractionation on the Ficoll/Metrizoate gradient, the original thymocyte population incorporated more vesicle-entrapped carboxyfluorescein than the fractionated cells, even twice as much as cells from the 1.084 g/ml density fraction which make up approx. 80% of the total lymphocyte population in the rabbit thymus [32]. This implies that contact of rabbit thymocytes with Ficoll/Metrizoate may affect their behaviour. We have obtained more indications from other experiments for the damaging effect of

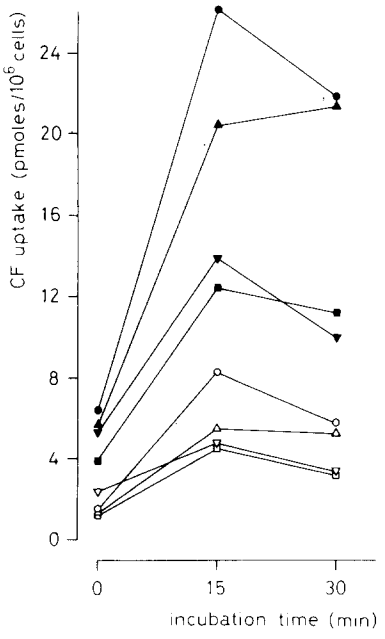

Fig. 2. Uptake of vesicle-entrapped carboxyfluorescein by subclasses of thymocytes from soybean lecithin (commercial grade) liposomes. Each point represents the average of triplicate measurements. Open symbols, carboxyfluorescein inside cells; closed symbols, total cell-associated carboxyfluorescein. ○——○, ●——●, original thymocyte population; △——△, ▲——▲, 1.067 g/ml density fraction; □——□, ■——■, 1.076 g/ml density fraction; ▽——▽, ▼——▼, 1.084 g/ml density fraction, CF, carboxyfluorescein.

the Ficoll/Metrizoate gradient. For example, incubation of thymocytes with soybean lecithin liposomes followed by fractionation on a Ficoll/Metrizoate gradient resulted in a substantial loss of cells and high cell death (data not shown).

Lymphocytes in peripheral lymphoid tissues belong to the most mature category of lymphocytes. In view of the preceding experiment illustrated in Fig. 2, it is to be expected that these cells will interact still more avidly with soybean lecithin liposomes than mature thymocytes. This is supported by experiments with mesenteric lymph node cells which took up approx. 5-times more vesicle-entrapped carboxyfluorescein than thymocytes (data not shown).

Effect of metabolic inhibitors and cytochalasin B on the interaction between thymocytes and carboxyfluorescein-containing liposomes

The first event in vesicle-cell interaction is adsorption of the vesicles to the cell surface. This may be followed by membrane fusion, lipid exchange or endocytosis with subsequent fusion of the endocytotic vacuole with lysosomes. The majority of rabbit thymocytes possess an ill-developed lysosomal apparatus (Leene, W., personal communication). Thus, we do not expect endocytosis followed by fusion with lysosomes and release of vesicle contents to be an important pathway in the interaction between rabbit thymocytes and liposomes. This can be demonstrated experimentally by the application of metabolic inhibitors and drugs which affect the cytoskeleton, because endocytotic processes require energy and the operation of the cytoskeleton [10,33,34]. We employed sodium azide as an inhibitor of electron transport, 2-deoxyglucose as an inhibitor of glycolysis, and cytochalasin B as a microfilament-disrupting drug. None of these compounds, alone or in combination, markedly affected the uptake of vesicle-entrapped carboxyfluorescein from soybean lecithin liposomes measured after 15 and 30 min of incubation (data not shown). From this we conclude that endocytosis is not an important pathway in the processing of liposomes by rabbit thymocytes.

Fluorescence microscope

The rapid uptake of vesicle-entrapped carboxyfluorescein by rabbit thymocytes from soybean lecithin liposomes could clearly be observed by fluorescence microscopy. Carboxyfluorescein inside cells was visible as an evenly distributed fluorescence throughout the cytoplasm. More prominent was the presence of a bright punctuate ring of fluorescence around the cells after longer incubation times. This is indicative of strong adsorption of liposomes to the cell surface concomitant with leakage of carboxyfluorescein from the liposomes to such a degree that the self-quenching effect is abolished and vesicle-entrapped carboxyfluorescein fluoresces strongly.

By fluorescence microscopy, thymocytes treated for short periods with phosphatidylcholine/stearylamine liposomes showed stronger fluores-

cence than with soybean lecithin liposomes, in accordance with the biochemical experiments. The beforementioned damaging effect of phosphatidylcholine/stearylamine liposomes on lymphocytes could also be observed in the fluorescence microscope. The degradation of the cells was accompanied by a strong increase in background fluorescence suggestive of strong carboxyfluorescein leakage from the liposomes and the cells.

Discussion

Effective vesicle-cell interaction will result in changes in the lipid composition of cellular membranes, in the first place of the plasma membrane. Most authors working with lymphocytes and lymphoma cells have employed uncharged phosphatidylcholine liposomes with or without cholesterol and observed the expected changes in cholesterol content and lipid fluidity of intact cells or plasma membranes, during long-term [14,16,20,22,35] as well as short-term [15,17,36–38] incubations. We have chosen for short incubation periods, up to 1 h, for the following reasons. First, long-term treatment of lymphocytes with liposomes more drastically interferes with cellular lipid metabolism which manifests itself, for instance, in an altered mitogen induced stimulation of [^{3}H]thymidine uptake [14–17,20,22]. Second, in preliminary experiments, we have found that longer incubation times resulted in increased cell death, particularly of the immature cells of the 1.084 g/ml density fraction. This vulnerability of rabbit thymocytes also appeared from experiments in which liposome-treated cells were subsequently fractionated on a Ficoll/Metrizoate gradient. Both the viability of the cells and their recovery from the gradient were considerably reduced (data not shown).

On the basis of the literature we expected that short-term treatment of rabbit thymocytes with phosphatidylcholine liposomes would results in effective decrease of the cholesterol content and, thus, of the degree of fluorescence polarization of diphenylhexatriene. No decrease of the degree of fluorescence polarization was, however, observed in intact cells, irrespective of the stage of maturation of the thymocytes. The degree of fluorescence polarization measured in isolated plasma membranes was also not significantly affected. This behaviour of rabbit thymocytes towards phosphatidylcholine liposomes was confirmed by experiments with vesicle-entrapped carboxyfluorescein. Rabbit thymocytes were hardly found to associate with carboxyfluorescein-containing egg phosphatidylcholine liposomes. From these experiments we conclude that rabbit thymocytes constitute an exception to the rule that the lipid composition of cellular membranes can effectively be manipulated by treatment with egg phosphatidylcholine liposomes.

According to Poste and Papahadjopoulos [10,33], Poste [13], and Papahadjopoulos et al. [39], vesicle surface charge and the physical state of the phospholipids within the membrane are of major importance in determining vesicle-cell interactions. For instance, fusion with cellular membranes is assumed only to occur if the liposomes are in a 'fluid' state and if a net charge is present. Definite proof for real fusion was recently obtained by Poste et al. [40] from experiments in which an integral protein was transferred from (fluid) phosphatidylserine and mixed phosphatidylserine/phosphatidylcholine or phosphatidylethanolamine/phosphatidylcholine vesicles to the plasma membrane with retention of its full functional activity in the recipient cell.

Instead of an acidic phospholipid, dicetyl phosphate can also be used to obtain negatively charged vesicles [12,41,42]. The application of a net negative charge to egg phosphatidylcholine by the addition of dicetyl phosphate or phosphatidylserine did not improve, however, the interaction with rabbit thymocytes. The degree of fluorescence polarization measured in intact cells remained unaffected and the uptake of carboxyfluorescein from negatively charged carboxyfluorescein-containing phosphatidylcholine liposomes was even decreased compared with uncharged vesicles. A number of authors have also performed experiments with positively charged liposomes prepared from phosphatidylcholine and stearylamine [12,43–45]. We observed that incubation of rabbit thymocytes with this type of liposomes lead to a strong vesicle-cell interaction, presumably by electrostatic attraction.

When dealing with negatively charged vesicles, we observed only a marked change in the degree

of fluorescence polarization and a considerable uptake of vesicle-entrapped carboxyfluorescein if rabbit thymocytes were treated with liposomes composed of a mixture of different phospholipids as present in commercial grade soybean lecithin. These effects were accompanied by appreciable changes in the cholesterol/phospholipid molar ratio and phospholipid composition of the treated cells. Commercial grade soybean lecithin contains an appreciable amount of acidic phospholipids (Table V). On the basis of this phospholipid composition a high negative ζ-potential of soybean lecithin liposomes can be expected [26,46]. We suppose that this high negative surface charge together with the presence of a variety of phospholipid species determine the interaction of rabbit thymocytes with (negatively charged) liposomes.

The influence of phospholipid composition on the interaction between liposomes and rabbit thymocytes was further demonstrated by experiments with type IV soybean lecithin, and two different lipid mixtures obtained from rabbit serum. Type IV soybean lecithin contains more phosphatidylcholine and less phosphatidylserine than commercial grade soybean lecithin; the phosphatidylethanolamine content is practically the same. This change in phospholipid composition was accompanied by a reduced vesicle-cell interaction. A further decrease was observed with liposome preparations from normal rabbit serum (Gibco) and Chinchilla rabbit serum lipids. These lipid mixtures are characterized by a high content (approx. 85%) of choline phospholipids and very low levels of phosphatidylserine and phosphatidylethanolamine, thus showing striking differences with both soybean lecithin preparations. Apparently, an increase in phosphatidylcholine content leads to a decrease in interaction between liposomes and rabbit thymocytes.

The reduced interaction with serum lipid liposomes can not be due to the presence of cholesterol, because soybean lecithin/cholesterol liposomes with approx. the same cholesterol/phospholipid molar ratio as the serum lipids exhibited a very strong interaction with rabbit thymocytes. These liposomes even associated to a 2.7-fold greater extent with the cells than liposomes prepared from soybean lecithin alone. This effect might be due to the increased rigidity of the vesicles caused by the addition of cholesterol (increase in P value from 0.168 to 0.282), in accordance with Szoka et al. [45] who found that 'solid' neutral and negatively charged vesicles associated to a 3-fold greater extent, mainly by adsorption, with a variety of cultured fibroblasts than 'fluid' vesicles.

As mentioned before, it has been shown by various authors that the interaction between liposomes and cells is not affected solely by the charge of the vesicle membrane but also by its fluidity (for review, see Ref. 13). The fluidity of soybean lecithin liposomes is not appreciably different from that of egg phosphatidylcholine, egg phosphatidylcholine/dicetyl phosphate (9:1 mol/mol), and egg phosphatidylcholine/phosphatidylserine (9:1 mol/mol) liposomes, however, to account for the substantial different interaction with rabbit thymocytes. Nevertheless, membrane fluidity may play a possible role in this case. It may be hypothesized that the different behaviour of rabbit thymocytes in comparison with other cell types is in some way related to the exceptionally high rigidity of the thymocyte plasma membrane with a P value (at 25°C) of 0.350 that is not found in other normal eukaryotic cells (see Ref. 1). Apparently, in the case of negatively charged vesicles their phospholipid composition should match the phospholipid composition of these rigid thymocyte plasma membranes which contain relatively high levels of phosphatidylethanolamine and phosphatidylserine beside phosphatidylcholine as the main phospholipid [2]. We hope to solve this question by first treating the thymocytes with soybean lecithin (commercial grade) liposomes to lower the P value of the plasma membrane by approx. 10%, and subsequently with carboxyfluorescein-containing phosphatidylcholine liposomes to measure the extent of vesicle-cell interaction.

When considering the mode of interaction between thymocytes and soybean lecithin liposomes, it is evident from our experiments that only about a third of the cell-associated vesicles react with the plasma membrane in such a way that vesicle-encapsulated carboxyfluorescein is transferred to the cytoplasm. This transfer may be the result of endocytosis (followed by fusion of the endocytotic vacuole with lysosomes and release of liposome contents), fusion of vesicle and plasma mem-

branes, or increase in permeability of vesicle and plasma membranes due to perturbation of the lipid bilayers [13,33,39]. Endocytosis was ruled out by us in experiments with metabolic inhibitors and cytochalasin B. The carboxyfluorescein technique can not be used to discriminate between the other possibilities mentioned [13,40], but application of this technique did show that adsorption of liposomes to the plasma membrane of rabbit thymocytes is the most prominent process in vesicle-cell interaction. From the adsorbed vesicles, lipids may exchange with lipids of the thymocyte membranes leading to the observed changes in lipid composition of soybean lecithin-treated cells.

Beside the supposed influence of plasma membrane fluidity on vesicle-cell interaction, cell surface charges may also play an important role. This is suggested by our experiments with subpopulations of thymocytes and mesenteric lymph node cells. Association with carboxyfluorescein-containing soybean lecithin liposomes was found to increase in the same order as lymphocyte maturation. In the mouse, maturation of T lymphocytes has shown to be accompanied by an increase in the net negative charge of the cell surface [30,31]. There are indications that this is also true in the rabbit [5] but it has still to be proven conclusively.

Observations by various authors [25,27,35] as well as by ourselves (this study) have indicated cell-induced leakage of vesicle-entrapped carboxyfluorescein due to increased membrane permeability of both vesicles and cells after adsorption of vesicles to the plasma membrane. Recently, Van Renswoude and Hoekstra [47] investigated cell-induced leakage of liposome contents more closely and arrived at the conclusion that vesicles are subject to cell-induced leakage (superimposed on bulk leakage in the absence of cells) only when they contain more than 30 mol% of cholesterol or more than 20 mol% of cholesterol plus at least 10 mol% of negatively charged lipid. For the initiation of the leakage induction a transient or lasting physical contact between vesicles and cells is required. We observed the same phenomenon as Van Renswoude and Hoekstra with respect to the introduction of cholesterol into liposomes: bulk leakage in the absence of cells was reduced, but cell-induced leakage was increased (data not shown). In our opinion this effect may be explained by the observed much stronger interaction (mainly adsorption) of cholesterol-containing soybean lecithin liposomes with the cells. Ralston et al. [48] have found that incorporation of lysophosphatidylcholine in the liposomal bilayer increased leakage of liposome contents. This is also shown in our experiments with liposomes prepared from Chinchilla rabbit serum and normal rabbit serum (Gibco) lipids which contain 16.4% and 31.6% lysophosphatidylcholine, respectively.

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