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COMPARATIVE LIPID ANALYSIS OF PURIFIED PLASMA MEMBRANES AND SHED EXTRACELLULAR MEMBRANE VESICLES FROM NORMAL MURINE THYMOCYTES AND LEUKEMIC GRSL CELLS

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The lipid fluidity in purified plasma membranes (PM) of murine leukemic GRSL cells, as measured by fluorescence polarization, is much higher than in PM of normal thymocytes. This was found to be due to relatively low contents of cholesterol and sphingomyelin and a high amount of unsaturated fatty acyl chains, especially linoleic acid, in the phospholipids. PM from GRSL cells contain markedly more phosphatidylethanolamine than those from thymocytes. For both GRSL cells and thymocytes the detailed lipid composition of isolated PM was compared with that of the corresponding shed extracellular membranes (ECM), which were isolated from the ascites fluid and from thymus cell suspensions, respectively. The somewhat decreased lipid fluidity of thymocyte ECM as compared to their PM, can be ascribed to the increased cholesterol/phospholipid molar ratio (0.88 vs. 0.74). No other major differences were found between the lipid composition of these membranes. In contrast, significant differences were found between PM and ECM from GRSL cells. In this system a much lower lipid fluidity of the shed ECM was found, due to the much increased cholesterol/phospholipid molar ratio (3.5-fold) and sphingomyelin (9-fold) content, as compared to the PM. Further, the ECM contain relatively more lysophosphatidylethanolamine and less phosphatidylcholine and -inositol. ECM contain a higher amount of polyunsaturated fatty acids, especially in the phosphatidylethanolamine and lysophosphatidylethanolamine classes. On the other hand, the fatty acids of phosphatidylcholine and lysophosphatidylcholine are more saturated than in PM. In particular, ECM of GRSL cells contain less oleic and linoleic acid residues and more arachidonic acid and 22:polyunsaturated fatty acid residues than PM. The possible relevance of these differences with respect to the mechanism of shedding of vesicles from the cell surface, is discussed.

Introduction

The shedding of membrane components from the cell surface is generally a selective process which is of widespread occurrence in normal and tumor cells, both in vivo and in vitro [1]. It may be an important determinant of the cell phenotype, and may play a role in interactions between cells and in the generation and effectiveness of an immune response [1–5].

In previous papers we have reported on the shedding of membrane vesicles from the surface of normal mouse (GR/A) thymocytes [6] and the homologous thymus-derived leukemic GRSL cells [4–8] in vivo. In the latter type of cells this spontaneous formation of vesicles apparently occurs at special rigid domains of the tumor cell surface,

Abbreviations: PM, plasma membranes; ECM, extracellular membranes; DPH, 1,6-diphenyl-1,3,5-hexatriene.

which are enriched in cholesterol and tumor antigens [4–8].

The shedding of vesicles from normal and cancer cells has since been reported by many others [9–14]. However, the mechanism of this phenomenon is still largely unknown. In the present paper an attempt was made to further investigate this mechanism in normal thymocytes and GRSL tumor cells.

Since our previous results have suggested that the plasma membrane (PM) lipids might play a central role in this exfoliation of vesicles, at least in the GRSL cells [4–8], the lipid composition of these vesicles was analysed in more detail in comparison to that of the PM isolated from the corresponding cell homogenates.

The results described in the present report indicate that thymocyte PM and extracellular membranes (ECM), show only minor differences in the lipid composition. However, the ECM of the leukemic GRSL cells are significantly different from the corresponding PM, not only because of their decreased lipid fluidity (relatively high cholesterol and sphingomyelin contents; see also Refs. 4–8, 15), but also with respect to the detailed composition of the phospholipids and their fatty acyl profiles.

Materials and Methods

Cells. GRSL 18 cells from a spontaneous thymus-derived lymphoid leukemia in the GR/A mouse strain were maintained by weekly intraperitoneal transplantation in 2-months-old GR/A mice. Transplant generations 51–53 and 60–85 were used for Expts. 4 and 5, and Expts. 1–3, respectively (this may be of possible relevance in view of the differing results). The ascites fluid was routinely taken 7 days after transplantation. Cells and extracellular ascites fluid were processed as described previously [6,7].

Thymocytes were obtained from 3–5-weeks-old GR/A mice by mincing thymuses with scissors in Hanks' solution (Oxoid, London, U.K.) and isolating the single cells [6]. Supernatants of cell pellets were kept for the isolation of extracellular membranes.

Isolation of plasma membranes and extracellular membranes. Plasma membranes (PM) from GRSL cells and from thymocytes were isolated as described in detail previously [3,6,7]. Briefly, the 'nitrogen cavitation' method was used to disrupt the cells. Equilibration of the cells in the pressure vessel was performed at 600 lb/inch^2 N_2 during 17.5 min for GRSL cells and at 800 lb/inch^2 N_2 during 20 min for thymocytes. The PM was purified from $(1.95–945) \cdot 10^4 g \cdot$ min pellets of the cell homogenates, utilizing a discontinuous sucrose gradient.

Extracellular membranes (ECM) of the two cell types were purified as described previously [6,7], making use of the same centrifugation procedures and sucrose density gradient as were used for the purification of the PM. ECM from GRSL cells were isolated from the ascites fluid after spinning down the cells. Thymocyte ECM were isolated from minced thymuses and the combined cell washes, as described before [6].

Estimation of lipid fluidity by fluorescence polarization. The fluorescent hydrocarbon 1,6-diphenyl-1,3,5-hexatriene (DPH; Koch-Light Laboratories Ltd., Colnbrook, U.K.) was used as a probe for measuring the degree of lipid fluidity in the various membrane preparations. The degree of fluorescence polarization (P_{DPH}) of DPH-labelled samples was determined at 25°C with an Elscint apparatus, model MV-1A (Elscint Ltd., Haifa, Israel) as described previously [6,7].

Recently, we have presented a new and comprehensive interpretation of these measurements [15]. In brief, the steady-state fluorescence anisotropy,

$$r_s = 2P(3-P)^{-1}$$

in biomembranes is determined mainly by the degree to which rotations of the fluorophore are restricted by the molecular packing of the lipids (a static factor) rather than by its rotational rate (a dynamic factor). r_s can be resolved into a static part, r_∞, and a dynamic part, r_f. The latter contribution is related to the rotational relaxation time of the fluorophore, which is in turn proportional to the 'microviscosity'; r_∞ represents the infinitely slow decaying component, determined exclusively by the membrane anisotropy (structural order). When using the probe DPH, the lipid order parameter S_{DPH} can be quantitatively estimated from r_s measurements by using the relationships

$r_\infty = (4r_s/3) - 0.10$ (valid for the region $0.13 < r_s < 0.28$ or $0.18 < P_{DPH} < 0.37$) and $(S_{DPH})^2 = r_\infty/r_0$; $r_0 = 0.4$ is the theoretically maximal fluorescence anisotropy.

Lipid fluidity may be defined as the reciprocal of the lipid structural order parameter [15].

Analysis of membrane lipids. Lipids were extracted from the membrane preparations with chloroform/methanol (2:1, v/v) followed by Folch's partition [16].

Cholesterol was determined enzymatically, using the Merckotest cholesterol kit (Merck A.G., Darmstadt, F.R.G.), containing cholesterol oxidase and a colour reagent. To this end, a sample of the lipid extract was dried and taken up in 100 μl ethanol, followed by addition of 2 ml of enzyme reagent. After leaving at room temperature for 1 h, the absorbance was read at 365 nm.

The individual phospholipids were separated by two-dimensional thin-layer chromatography on precoated activated silicagel plates (Merck), using the solvent systems chloroform/methanol/7 M ammonia (60:60:5, v/v) (two successive runs) and subsequently chloroform/methanol/acetic acid/H_2O (50:30:8:4, v/v). Intermittant drying was performed at 40°C in vacuo. All phospholipids, except phosphatidic acid (only present in trace amounts) were quantitated by phosphate analysis of the individual spots, as described before [16].

Fatty acid analysis of the phospholipids was done by gas-liquid chromatography on a Tracor 550 apparatus, after transesterification with 0.1 M dry methanolic NaOH at 50°C or, in case of sphingomyelin, with 0.5 M methanolic HCl at 80°C. After neutralization the fatty acid methyl esters were extracted with *n*-hexane and separated on a wall-coated-open-tubular glass column, 25 m × 0.21 mm inner diameter, coated with Silar 5 CP, by splitless injection according to Grob and Grob [17] and using a temperature program (3 K/min) from 170 to 240°C. Quantitation of eluate peaks was done by a computing integrator.

Results

Comparison of plasma membranes (PM) from GRSL cells with those from thymocytes

Table I shows that the fluorescence polarization (P_{DPH}) and lipid order parameter (S_{DPH}) in the PM of GRSL cells are much lower than in the PM of normal thymocytes. The molecular and structural basis of this relatively low order parameter (high lipid fluidity) in the leukemic cell membranes can be assumed to be the following (see also Ref. 15). In sequence of importance, with respect to the lipid fluidity, these membranes exhibit (1) a relatively low cholesterol/phospholipid molar ratio (Table I), (2) a deficiency in sphingomyelin (Table I) and (3) a high amount of unsaturated fatty acyl residues, especially linoleic acid (18:2), in the major phospholipid classes, as compared to thymocyte PM (Tables II, III and IV).

Another conspicuous difference between the PM from thymocytes and GRSL cells is the relatively high phosphatidylethanolamine content (29%) in the latter type of membrane.

Comparison of plasma membranes (PM) with the corresponding extracellular membranes (ECM)

Table I shows that the fluorescence polarization (P_{DPH}) and lipid order parameter (S_{DPH}) values of ECM are higher than those of the corresponding PM, both for thymocytes and GRSL cells. However, the difference is much more pronounced in the GRSL cell system.

In the thymocytes the relatively small difference in lipid structural order between PM and ECM may be entirely ascribed to the difference in the cholesterol/phospholipid molar ratio (0.74 vs. 0.88). The thymocyte ECM contain more stearic acid (18:0) and less palmitic (16:0) and linoleic (18:2) acid in their lysophosphatidylethanolamine than do the PM (Table II). Further, no conspicuous differences in lipid constitution between thymocyte PM and ECM were found, neither in the phospholipid composition (Table I), nor in the fatty acid profiles of the phospholipids (Tables II and IV).

In contrast, in GRSL cells significant differences between PM and ECM were found with respect to lipid class composition (Table I) and fatty acid profiles (Tables III, IV and V). Generally, a much higher lipid order parameter was found in ECM, due to the 3.5-fold higher cholesterol/phospholipid ratio and the roughly 9-fold higher sphingomyelin content (see also Refs.

TABLE I

FLUORESCENCE POLARIZATION (P_{DPH}), LIPID ORDER PARAMETER (S_{DPH}), CHOLESTEROL/PHOSPHOLIPID MOLAR RATIO AND PHOSPHOLIPID COMPOSITION OF PLASMA MEMBRANES (PM) AND EXTRACELLULAR MEMBRANES (ECM) OF NORMAL GR/A MOUSE THYMOCYTES AND GRSL LEUKEMIA CELLS

Mean values are presented ± S.D. (number of experiments).

	Thymocytes		GRSL cells			
	PM	ECM	PM	ECM		
P_{DPH} value, at 25°C	0.303±0.005 (11)	0.320±0.004 (8)	0.261±0.014 (13)	0.325±0.008 (12)		
S_{DPH} value, at 25°C	0.71 ±0.01 (11)	0.74 ±0.01 (8)	0.62 ±0.03 (13)	0.75 ±0.01 (12)		
Cholesterol/phospholipid (molar ratio)	0.74 ±0.05 (4)	0.88 ±0.10 (3)	0.32 ±0.08 (7)	1.10 ±0.12 (4)		
Phospholipid composition (%)			Expts. 1–4	Expts. 1–3	Expt. 4	Expt. 5
Sphingomyelin	7.6	10.2	0.9±0.2	7.7±1.6	6.8	9.8
Phosphatidylcholine	52.0	56.0	50 ±6	39 ±5	40.5	46.6
Phosphatidylethanolamine	19.0	15.1	29 ±4	34 ±5	26	15
Phosphatidylserine	9.2	8.6	9.8±2.1	11.7±3.0	8.5	7.6
Phosphatidylinositol	6.1	4.5	6.5±0.5	3.5±0.8	4.0	2.6
Lysophosphatidylcholine	3.4	3.0	3.2±0.3	3.8±0.8	4.3	7.2
Lysophosphatidylethanolamine	2.2	2.6	2.7±1.8	3.7±0.9	10	10.6

7, 15) (Table I). Further, ECM contain decreased amounts of phosphatidylcholine and -inositol, as compared to PM (Table I).

Two out of five experiments (Expts. 4 and 5), which were related to earlier transplant generations of the GRSL cells (see Materials and Methods), gave somewhat different results. Here, ECM contained a higher amount of lysophospholipids than in Expts. 1–3 (Table I). Also, the fatty acid profiles of the ECM phospholipids, especially the lysophospholipids, of Expt. 4 (not done in Expt. 5) differed from those of Expts. 1–3 (Tables III and V). However, the differences between ECM and PM pointed in the same direction in all experiments.

Table III shows that ECM, as compared to PM of GRSL cells, contain less oleic acid (18:1) and linoleic acid (18:2) residues, particularly in sphingomyelin, phosphatidylcholine, -ethanolamine and lysophosphatidylcholine. On the other hand, arachidonic acid (20:4) and the 22:polyunsaturated fatty acid residues are increased in ECM, particularly in phosphatidylethanolamine and lysophosphatidylethanolamine. There are also significant differences in the saturated fatty acid residues of the various phospholipid classes (not in the total phospholipids) between PM and ECM of GRSL cells. ECM contain more palmitic acid (16:0) in sphingomyelin and in phosphatidylcholine, but less in phosphatidylethanolamine. Stearic acid (18:0) is increased in phosphatidylcholine and lysophosphatidylcholine, but decreased in sphingomyelin, phosphatidylethanolamine and lysophosphatidylethanolamine.

Table IV summarizes the overall differences between PM and ECM of GRSL cells and thymocytes, with respect to the total amounts of saturated, mono- and polyunsaturated fatty acyl residues. In thymocytes no significant differences were found in any of the phospholipid classes. However, in GRSL cells the total phospholipids of ECM contain a higher percentage of polyun-

TABLE II

FATTY ACID COMPOSITION OF PHOSPHOLIPIDS FROM PLASMA MEMBRANES (PM) AND EXTRACELLULAR MEMBRANES (ECM) OF GR/A MOUSE THYMOCYTES

Mean values of duplicate experiments are given, expressed as mol%. Values underlined represent major differences between PM and ECM. Fatty acids present in amounts less than 1% in the total phospholipids are not included in this table.

Fatty acid	Total phospholipids		Sphingomyelin		Phosphatidylcholine		Phosphatidylethanolamine		Phosphathidylserine		Phosphatidylinositol		Lysophosphatidylcholine		Lysophosphatidylethanolamine	
	PM	ECM	PM	ECM	PM	ECM	PM	ECM	PM	ECM	PM	ECM	PM	ECM	PM	ECM
16:0	37.9	36.4	60.9	55.8	54.2	52.0	9.6	10.0	6.3	5.6	5.9	10.0	41.4	38.9	38.9	25.8
16:1	0.9	1.0	0.4	0.4	1.2	1.2	–	0.7	–	0.3	–	–	–	0.7	–	1.2
18:0	21.0	21.8	15.4	13.8	10.4	11.4	25.2	23.7	47.7	45.3	47.9	45.0	36.3	37.1	32.5	45.3
18:1	14.2	13.7	3.8	3.5	14.9	13.3	12.0	13.0	15.7	15.7	6.1	7.1	13.8	11.8	17.1	13.4
18:2	6.2	7.6	1.0	1.0	8.4	9.5	3.8	6.2	5.9	5.8	1.5	2.2	3.3	3.4	6.9	3.8
20:3	1.6	1.4	–	–	1.2	1.1	1.9	1.5	4.4	4.4	1.9	2.0	–	0.4	–	0.4
20:4	7.7	6.5	1.8	0.9	4.3	3.5	21.2	19.2	4.6	6.6	29.3	24.2	2.3	3.3	4.3	3.5
22:3	1.0	1.2	–	–	–	0.3	2.9	3.2	1.9	2.4	1.1	1.5	–	0.3	–	0.4
22:5	1.2	1.1	–	–	0.3	0.4	4.2	3.7	3.0	2.9	1.4	2.4	–	0.3	–	0.4
22:6	3.9	3.5	–	–	1.5	1.0	16.1	14.7	8.9	7.6	2.9	3.3	1.0	1.0	–	1.5

TABLE III

FATTY ACID COMPOSITION OF PHOSPHOLIPIDS FROM PLASMA MEMBRANES (PM) AND EXTRACELLULAR MEMBRANES (ECM) OF GRSL LEUKEMIA CELLS

Data expressed as mol%, are the mean of three individual experiments (Expts. 1–3). Individual analysis did not differ by more than 10% for those fatty acids which comprised more than 5% of the total. Membrane preparations were from GRSL18, passages 60–85. Values underlined represent major differences between PM and ECM. Fatty acids present in amounts less than 1% in the total phospholipids are not included in this table.

Fatty acid	Total phospholipids		Sphingomyelin		Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylserine		Phosphatidylinositol		Lysophosphatidylcholine		Lysophosphatidylethanolamine	
	PM	ECM	PM	ECM	PM	ECM	PM	ECM	PM	ECM	PM	ECM	PM	ECM	PM	ECM
14:0	1.5	1.1	5.4	3.1	1.8	1.7	0.4	–	0.4	0.3	0.9	1.0	1.5	2.9	1.8	3.0
16:0	21.3	21.0	33.3	42.0	31.3	38.7	7.5	5.0	1.1	1.3	6.8	6.2	33	29	19.3	16.0
16:1	1.3	1.4	4.4	3.9	1.2	0.8	0.7	0.6	0.4	0.5	0.2	0.3	4.0	2.4	3.4	3.3
18:0	20.8	18.9	15.0	10.0	12.9	16.0	19.0	14.5	43	43	44	46	32	50	45	35.5
18:1	17.8	14.9	8.5	6.0	19.0	14.4	15.0	10.5	22.0	23.2	6.5	5.8	16.0	6.2	15.0	13.0
18:2	18.8	14.7	5.3	2.6	22.5	16.5	19.0	16.0	15.5	14.2	7.0	6.5	4.5	3.0	6.6	7.5
20:3	1.7	1.6	0.8	0.8	1.1	0.9	2.6	1.5	2.7	2.0	6.0	5.4	0.7	0.4	0.2	1.9
20:4	7.4	9.4	4.7	4.7	2.9	3.8	17.4	22.0	5.0	4.9	23	22	1.4	0.4	1.9	5.0
22:3	0.7	1.2	–	–	0.3	0.3	1.3	2.3	1.2	1.1	2.1	–	–	–	–	–
22:4	1.3	3.0	–	–	0.8	1.7	1.2	2.9	3.4	2.1	0.8	3.2	2.1	4.3	2.4	10.4
22:5	0.5	1.0	1.0	1.5	0.5	0.4	1.0	2.6	0.7	0.8	–	0.8	–	–	0.2	–
22:6	4.0	7.8	–	–	2.0	2.3	11.0	19.9	2.5	3.4	1.8	1.6	1.3	–	1.1	2.3

TABLE IV

SUM OF % SATURATED, MONO- AND POLYUNSATURATED FATTY ACIDS IN THE PHOSPHOLIPIDS OF PLASMA MEMBRANES (PM) AND EXTRACELLULAR MEMBRANES (ECM) OF GRSL CELLS AND NORMAL GR/A MOUSE THYMOCYTES

Data, expressed as mol%, are the mean of three (Expts. 1–3 for GRSL cells) or two (for thymocytes) individual experiments with consistent results. Only the phospholipids showing significant differences between PM and ECM are listed.

	Fatty acids	GRSL cells		Thymocytes	
		PM	ECM	PM	ECM
Total phospholipids	Saturated	44.0	42.1	59.3	59.3
	Monounsatd.	19.9	17.6	16.3	16.1
	Polyunsatd.	36.1	39.8	22.7	22.4
Phosphatidylcholine	Saturated	46.2	56.5	64.6	65.5
	Monounsatd.	21.2	16.0	17.1	16.4
	Polyunsatd.	32.0	27.3	16.9	17.0
Phosphatidylethanolamine	Saturated	27.1	19.7	34.8	34.3
	Monounsatd.	16.3	11.7	12.4	14.3
	Polyunsatd.	55.6	68.5	51.1	49.4
Lysophosphatidylcholine	Saturated	66.9	82.3	77.7	77.1
	Monounsatd.	21.2	9.6	14.6	13.5
	Polyunsatd.	11.0	8.1	7.3	8.2
Lysophosphatidylethanolamine	Saturated	66.6	54.5	71.4	73.4
	Monounsatd.	19.2	17.1	17.1	15.2
	Polyunsatd.	13.6	28.4	11.2	10.7

saturated fatty acids at the expense of saturated and monounsaturated ones. This is particularly due to the much higher amount of polyunsaturated fatty acids in phosphatidylethanolamine and lysophosphatidylethanolamine, which is not counter-balanced by the opposite distribution in phosphatidylcholine and lysophosphatidylcholine.

Tables III and IV (Expts. 1–3), clearly show

TABLE V

EXTREME DIFFERENCES BETWEEN THE MAIN FATTY ACIDS OF PHOSPHOLIPIDS FROM PLASMA MEMBRANES (PM) AND EXTRACELLULAR MEMBRANES (ECM) OF GRSL LEUKEMIA CELLS IN ONE PARTICULAR EXPERIMENT (Expt. 4)

Data, expressed as mol%, pertain to membrane preparations from GRSL18, passages 51–53. Values for phosphatidylethanolamine and phosphatidylserine are similar to those presented in Table III.

Fatty acid	Total phospholipids		Sphingomyelin		Phosphatidylcholine		Phosphatidylinositol		Lysophosphatidylcholine		Lysophosphatidylethanolamine	
	PM	ECM	PM	ECM	PM	ECM	PM	ECM	PM	ECM	PM	ECM
16:0	23	23	35.5	45	33	41	9	9	33	43	16	3.5
18:0	20	20	19	10	9.5	16	42	57	20	43	18	8
18:1	18	13	11.5	5	19	13.5	10	4.5	27	7	17	6.5
18:2	18.5	15	5.0	2.0	21	18	7	7	14	7	13	9
20:4	8.2	8.9	1.5	0.9	3.0	3.0	22	14	2.7	–	20	32
22:6	3.0	7.0	–	–	1.4	2.0	1.0	1.0	1.0	–	9	33

that in GRSL cells ECM have more saturated fatty acyl groups in phosphatidylcholine and lysophosphatidylcholine than PM, while on the other hand, they have much more unsaturated fatty acyl groups in phosphatidylethanolamine and lysophosphatidylethanolamine. These differences are even much more pronounced in Expt. 4 (Table V). In this experiment the lysophosphatidylcholine of ECM was extremely saturated (16:0 and 18:0) and the lysophosphatidylethanolamine extremely unsaturated (high 20:4 and 22:6, low 16:0 and 18:0).

One could argue that the differences between GRSL PM and ECM, described above, are the result of lipid exchange of the shedded vesicles with ascites plasma lipoproteins. This is, however, very unlikely, since e.g. the fatty acyl profiles of the various ascites plasma phospholipids are very different from, or even contrasting to those of ECM (results not shown).

Discussion

The lipid fluidity in purified plasma membranes (PM) of leukemic GRSL ascites cells, as measured by fluorescence polarization, is much higher than in PM of normal thymocytes. This is due to (a) the relatively low cholesterol/phospholipid molar ratio, (b) the virtual absence of sphingomyelin, and (c) the relatively high percentage of unsaturated phospholipid fatty acyl chains (see also Ref. 15). PM from GRSL cells show extreme values of these three parameters, also in comparison to PM isolated from other cell types, including normal and malignant lymphoid cells [9,10,15,18–23]. In regard to the degree of fatty acyl saturation in particular, the amount of linoleic acid (18:2) is extremely high (19%) in GRSL PM, especially in the phosphatidylcholine (22.5%) and -ethanolamine (19%) classes, as compared to published values (1–9%) for other lymphocyte PM [9,10,18–20].

Common characteristics of all lymphoid cell PM, including the ones studied in this paper, are the amounts of phosphatidylcholine (about 50%) [9,10,18–23], phosphatidylserine (about 10%) [9,18,21–23] and stearic acid (18:0) residues (20–25%) [9,10,18,19]. It is furthermore apparent that PM from thymocytes of various species contain a relatively high amount of palmitic acid (16:0) residues, ranging from 38% for the GR/A mouse (Table II) to as much as 56% for the rabbit [9]. This palmitic acid is predominantly contained in the phosphatidylcholine and sphingomyelin classes, amounting to 54% and 61%, respectively, in case of the GR/A thymocytes (Table II).

It has been discussed previously that extracellular membranes (ECM) in the ascites fluid of GRSL bearing mice are mainly derived from viable tumor cells by exfoliation, rather than from dying or lysed cells [7,8]. Likewise, ECM isolated from the thymus have most probably been formed in vivo by shedding from thymocytes. It is not likely that they result to a marked degree from cell death in the thymus or by disruption of cells during the preparation of single cell suspensions [9].

Table VI summarizes the differences in lipid composition between ECM and PM of GR/A mouse thymocytes and leukemic GRSL cells, in comparison with the pertinent results of investigators who have studied shed membranes in other cell systems [9–11]. In all these studies ECM are not mere random fragments resulting from the cytolysis of moribund cells, but are apparently shed from viable cells, and often originate from particular cell surface domains (see also Refs. 4–7). Generally, those parts of the plasma membrane which are more rigid (lower lipid fluidity) are preferentially shed, rabbit thymocytes [9] being an exception. Rabbit thymocyte PM show an extremely high lipid rigidity, due to the high content of saturated fatty acids, which may be the possible cause that no preferential shedding of still more rigid membrane domains can occur [9].

The molecular parameters which determine the increased rigidity of ECM relative to that of PM in the various systems (Table VI), are the increased cholesterol/phospholipid ratio (mouse thymocytes, GRSL cells), the increased sphingomyelin content (GRSL and glioma cells) or the highly increased degree of saturation of the fatty acids (calf thymocytes) [15]. It should be noted that ECM from calf thymocytes, as studied by Ferber et al. [10] were generated in vitro, either 'spontaneously' or induced by low concentrations of a detergent, whereas those from mouse and rabbit thymocytes were largely formed in situ. It is, therefore, plausible that the different results obtained in these thymocyte systems, especially with respect to

the degree of fatty acyl saturation (Table VI), may imply different mechanisms of vesicle formation.

It seems reasonable to suggest that the process of exfoliation of vesicles is promoted by factors which destabilize the lipid bilayer structure, since membrane fusion at the base of the generating vesicle is an essential step in this process. According to Cullis and De Kruijff [24] this membrane fusion requires an instability of the inner monolayer which may lead to intermediate non-bilayer configurations such as micelles and inverted micelles. The former configuration can be formed in the membrane by local concentrations of the cone-shaped lysophospholipid molecules, which are present in relatively high amounts (about 6%) in the PM of GRSL cells. Intermediary inverted micelles can be formed by local concentrations of unsaturated phosphatidylethanolamine during its bilayer-to-hexagonal polymorphic phase transition at physiological temperatures [24,25]. It seems, therefore, relevant to note that this phosphatidylethanolamine is present in relatively high amounts in PM of GRSL (29%) and glioma cells (37%), cell systems which, in contrast to thymocytes in situ (19% phosphatidylethanolamine in PM), most clearly show segregation of membrane lipids during vesicle formation (Table VI). This lipid segregation in these two cell systems is evident because the ECM exhibit a much increased sphingomyelin content (rigidity), a decreased phosphatidylinositol content and an increased unsaturation of fatty acids, as compared to PM. In addition, in the GRSL system in particular, the degree of fatty acyl unsaturation of phosphatidylcholine and lysophosphatidylcholine in ECM is much lower than in PM, whereas the reverse is true for phosphatidylethanolamine and lysophosphatidylethanolamine (Tables III, IV, V).

At this moment no satisfactory explanation can be given for this segregation of lipids during the exfoliation of vesicles. It has been suggested previously that in the GRSL system the viral precursor glycoproteins ($Pr73^{env}$ or MLr antigen) could select or assemble these specialized rigid cell surface domains, and could subsequently trigger the exfoliation process ('abortive virus formation') [4,5,7]. We still consider this as an hypothesis, without knowing the mechanism.

In conclusion, in this paper detailed information is provided on the lipid composition of membrane vesicles exfoliated from leukemic GRSL cells and normal thymocytes in situ, in comparison to the PM isolated from these cells. The results show minor differences in the thymocyte system, but significant differences in the GRSL system, which is indicative for a selective, nonrandom process. It

TABLE VI

DIFFERENCES BETWEEN PLASMA MEMBRANES (PM) AND EXTRACELLULAR MEMBRANES (ECM) IN VARIOUS CELL SYSTEMS

Cells (origin)	Cholesterol/phospholipid (M/M)		Degree of saturation of fatty acids	Phospholipid classes *
	PM	ECM		
Thymocytes				
GR/A mice	0.74	0.88	No diff.	minor diff.
Rabbit [9]	0.59	0.62	ECM < PM	no diff.
Calf (in vitro [10])	0.59	0.56	ECM ≫ PM	LPC↑
GRSL (T-leukemia in GR/A mice)	0.32	1.10	ECM < PM	LPC↑, LPE↑ Sph↑, PC↓, PI↓
C-6 glioma (cell line [11])	n.d.	n.d.	ECM < whole cells	Sph↑ PE↓, PI↓

* Abbreviations: LPC and LPE, lysophosphatidylcholine and -ethanolamine; Sph, sphingomyelin; PC, PE and PI, phosphatidylcholine, -ethanolamine and -inositol. ↑/↓, ECM contains more/less than PM; n.d., not determined.

seems evident that the exfoliation processes in these two cell systems have different, as yet unknown mechanisms, which may be important subjects for further study.

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References

1 Black, P.H. (1980) Adv. Cancer Res. 32, 75–199
2 Emerson, S.G. and Cone, R.E. (1981) J. Immunol. 127, 482–486
3 Van Blitterswijk, W.J., Emmelot, P., Hilgers, J., Kamlag, D., Nusse, R. and Feltkamp, C.A. (1975) Cancer Res. 35, 2743–2751
4 Van Blitterswijk, W.J., Hilgers, J., Feltkamp, C.A. and Emmelot, P. (1981) in Mammary Tumors in the Mouse (Hilgers, J. and Sluyser, M., eds.), Chapter 14, pp. 573–625, Elsevier/North-Holland Biomedical Press, Amsterdam
5 Emmelot, P., Van Blitterswijk, W.J., Van Beek, W.P. and Van Hoeven, R.P. (1981) in Cell Biological Aspects of Disease, The Plasma Membrane and Lysosomes (Daems, W.T., Burger, E.H. and Afzelius, B.A., eds.), pp. 37–66, Leiden University Press, The Hague
6 Van Blitterswijk, W.J., Emmelot, P., Hilkmann, H.A.M., Oomen-Meulemans, E.P.M. and Inbar, M. (1977) Biochim. Biophys. Acta 467, 309–320
7 Van Blitterswijk, W.J., Emmelot, P., Hilkmann, H.A.M., Hilgers, J. and Feltkamp, C.A. (1979) Int. J. Cancer 23, 62–70
8 Van Blitterswijk, W.J., Hilkmann, H.A.M., De Veer, G., Hilgers, J., Feltkamp, C.A. and Emmelot, P. (1980) Protides of the Biological Fluids, Vol. 27, pp. 201–204, Pergamon Press, Oxford
9 Roozemond, R.C. and Urli, D.C. (1981) Biochim. Biophys. Acta 643, 327–338
10 Ferber, E., Schmidt, B. and Weltzien, H.U. (1980) Biochim. Biophys. Acta 595, 244–256
11 Trams, E.G., Lauter, C.J., Salem, N., Jr. and Heine, U. (1981) Biochim. Biophys. Acta 645, 63–70
12 Dvorak, H.F., Quay, S.C., Orenstein, N.S., Dvorak, A.M., Hahn, P., Bitzer, A.M. and Carvalho, A.C. (1981) Science 212, 923–924
13 Liepins, A. and Hillman, A.J. (1981) Cell Biol. Int. Rep. 5, 15–26
14 Howard, S.C., Sherblom, A.P., Huggins, J.W., Carraway, C.A.C. and Carraway, K.L. (1981) Arch. Biochem. Biophys. 207, 40–50
15 Van Blitterswijk, W.J., Van Hoeven, R.P. and Van der Meer, B.W. (1981) Biochim. Biophys. Acta 644, 323–332
16 Van Blitterswijk, W.J., Emmelot, P. and Feltkamp, C.A. (1973) Biochim. Biophys. Acta 298, 577–592
17 Grob, K. and Grob, K., Jr. (1974) J. Chromatogr. 94, 53–64
18 Maler, T. and Riordan, J.R. (1980) Biochim. Biophys. Acta 598, 1–15
19 Ip, S.H.C. and Cooper, R.A. (1980) Blood 56, 227–232
20 Levis, G.M., Evangelatos, G.P. and Crumpton, M.J. (1976) Biochem. J. 156, 103–110
21 Koizumi, K., Shimizu, S., Nishida, K., Koizumi, K.T., Yamanaka, N., Ota, K. and Matsuyama, M. (1980) J. Natl. Cancer Inst. 65, 817–821
22 Koizumi, K., Kano-Tanaka, K., Shimizu, S., Nishida, K., Yamanaka, N. and Ota, K. (1980) Biochim. Biophys. Acta 619, 344–352
23 Johnson, S.M. and Robinson, R. (1979) Biochim. Biophys. Acta 558, 282–295
24 Cullis, P.R. and De Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399–420
25 Verkleij, A.J., Van Echteld, C.J.A., Gerritsen, W.J., Cullis, P.R. and De Kruijff, B. (1980) Biochim. Biophys. Acta 600, 620–624