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RELATION OF CYTOLYTIC ACTIVITY OF AN ETHER-DEOXY LYSOPHOSPHATIDYLCHOLINE ANALOG TO AVAILABLE MEMBRANE SURFACE

COMPARISON OF NORMAL AND TUMOR CELLS FROM MICE

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Summary

1-Hexadecylpropanediol-3-phosphorylcholine, an ether-deoxy analog of lysophosphatidylcholine, has been employed to study the sensitivity of various types of mouse cells with respect to changes in membrane permeability induced by lysophosphatidylcholine. Cells used included erythrocytes, thymocytes, spleen cells and macrophage, as well as 4 different tumors (2 lymphomas, 1 Ehrlich ascites and 1 methylcholanthren-induced fibrosarcoma). The sensitivity to the lysophosphatide (on a per-cell basis) of the above cell types varied by a factor of 65. When lytic concentrations were related to available membrane surface, this variation was reduced to a factor of 2.5. No principal difference was observed between the sensitivity of normal versus tumor cell membranes with respect to lysophosphatidylcholine lysis.

Membrane surface, available for lysophosphatidylcholine, has been estimated from binding equilibria of ^{14}C-labelled deoxy-lysophosphatidylcholine to the cells under standardized conditions. This method is based on the finding that binding equilibria of lysophospholipids to cells are predominantly determined by the available membrane surface.

Introduction

The cytolytic activity of lysophosphatidylcholine (1-acyl-glycero-*sn*-3-phosphorylcholine) has, in the past, been studied primarily with erythrocytes [1—8]. Red cells of various species were found to differ in their sensitivities to

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synthetic lysophosphatidylcholine analogs, in spite of having identical binding affinities for these lipids [7—8]. Recent findings of antitumor [9] and adjuvant [9—12] activities of lysophosphatides in vivo have therefore revived the interest in reports on selective sensitivity of tumor cells vs. normals cells to lysophosphatidylcholine [13—15].

Using a 1-alkyl-2-deoxy lysophosphatidylcholine analog (1-hexadecylpropanediol-3-phosphorylcholine), we have investigated lytic sensitivities and binding characteristics of a variety of non-erythrocytic cells, mainly from mice, including several tumor cell lines. An attempt has been made to relate these data to the cellular membrane area, available to the lysophosphatide.

The synthetic deoxy-analog of lysophosphatidylcholine was selected for these experiments because of its resistance to the action of lysophospholipases and acyltransferases. In our assay systems, this leads to greatly diminished degradation of the molecule.

Materials and Methods

1-Hexadecylpropanediol-3-phosphorylcholine (ether-deoxy lysophosphatidylcholine)

Chemical synthesis and radiolabelling with ^{14}C in the choline moiety of ether-deoxy lysophosphatidylcholine analogs have been described previously [5,8,16].

Cells

The following cell types were used in this study: erythrocytes from citrate-stabilized blood of mice (C57Bl/10), rat (Lewis), cattle, sheep, goat and man. Thymocytes of calf and C57Bl/10 mice. Spleen cells of C57Bl/10 mice, after removal of erythrocytes by lysis in 0.1% aqueous ammonium chloride solution [17]. Bone marrow-derived macrophage of C57Bl/10 mice after 9 days in culture according to Meerpohl et al. [18]. Tumor cells: <5178Y lymphoma from DBA/2 mice (in following abbreviated as Y78), El-4 lymphoma of C57Bl/10 mice, Ehrlich ascites tumor from NMRI mice and a methylcholanthren-induced fibrosarcoma, grown as ascites in BalB/c mice (abbreviated in the following as Meth A).

Before use, all cells were washed 3 times with phosphate-buffered saline (0.01 M phosphate, containing 0.85% NaCl, pH 7.2).

Cytolysis

In standard experiments 10^6 cells per ml of phosphate-buffered saline, pH 7.2, were mixed with increasing amounts of lysophosphatide. After 15 min at 37°C, the mixture was centrifuged and the supernatant assayed for potassium, ATP, hemoglobin or lactate dehydrogenase. Previous data on lysophosphatidylcholine-mediated hemolysis [5,8] had shown lysis, as well as binding of C_{16}-alkyl-deoxy lysophosphatidylcholine to the cells to be in equilibrium after that time.

Potassium was determined in a Perkin Elmer model 290 B atomic absorption spectrophotometer at 766 nm. Sensitivity limit of the method is approx. $2 \cdot 10^{-8}$ mol K^+/ml or approx. 10^6 cells/ml. For ATP determinations, supernatants

were kept in ice to avoid degradation. Aliquots were mixed with an extract of desiccated firefly tails (Sigma Chem. Co.) as described by Frish et al. [19], and luminescence was measured in a liquid scintillation counter (Packard, Tricarb 3000). Hemoglobin was measured colorimetrically at 546 nm and lactate dehydrogenase (EC 1.1.1.27) was determined according to Bergmeyer and Bernt [20].

Binding of ^{14}C-labeled lysophosphatidylcholine to cells was determined as previously described for red cells [7,8,21].

Cell volumes were measured in an MSE Super Minor centrifuge equipped with a micro-hematocrit head.

Results and Discussion

Lysophosphatide-induced cytolysis

Four different methods have been employed to study lysophosphatide mediated changes in membrane permeability:

(a) Determination of cell-free ATP, using the firefly luciferin-luciferase system [19]. Due to its extreme sensitivity this test is particularly useful when little cell material is available.

(b) Permeability of cells to trypan blue; this is usually taken as an indication of cell death.

(c) Determination of cell-free potassium by flame photometry. This method is slightly less sensitive than method (a) but combines simplicity with great accuracy. Difficulties may arise from spontaneous potassium release by certain cells. Cells were therefore transferred to potassium-free buffer only immediately before use.

(d) Determination of lactate dehydrogenase for non-erythrocytic cells and of hemoglobin for red cells, was used to follow release of macromolecules. Both methods are less sensitive than K^+ or ATP determinations by a factor of 20—100. Permeability changes induced by the deoxy lysophosphatidylcholine analog in calf thymus membranes do not discriminate between small ions, such as K^+, and molecules such as ATP or trypan blue. At both 0° and 37°C methods (a)—(c) lead to overlapping 'lysis' curves. 50% 'lysis' concentrations of the lysophosphatide (L_{50} values) at $1 \cdot 10^6$ cells/ml for ATP, K^+ or trypan blue, were determined at 0°C to be 2.3, 3.0 and 2.8 nmol/ml and at 37°C, 7.8, 7.1 and 8.0 nmol/ml, respectively. In order to relate these permeability changes to actual cell lysis, i.e., loss of macromolecular components, we have compared K^+ permeation with the release of hemoglobin from human and bovine erythrocytes and of lactate dehydrogenase from calf thymocytes. L_{50} concentrations of the lysolipid are given in Table I.

It is apparent from these data that at 0°C there are no significant differences between the release of K^+ and of macromolecules. At 37°C, slightly higher lysophosphatide concentrations (varying with the cell type) are needed for the release of macromolecules than for K^+. The maximum difference between the two values, however, is only a factor of 1.8. From these findings we deduce that K^+ release may be taken as a valid indication of the lower limit of cytolysis caused by C_{16}-alkyl-deoxy lysophosphatidylcholine. Thus, all the following

TABLE I

COMPARISON OF DEOXYLYSOPHOSPHATIDYLCHOLINE-INDUCED RELEASE OF POTASSIUM AND MACROMOLECULES FROM RED CELLS AND THYMOCYTES

All assays in 0.01 M phosphate buffer, pH 7.2, containing 0.85% NaCl.

Cell type	Cell count (cells/ml)	Temperature (°C)	Lysophophatide concentrations (L_{50}) in nmol/ml for 50% release of		
			Potassium	Hemoglobin	Lactate-dehydrogenase
Human red cells	$3.8 \cdot 10^7$	0	6.9 ± 1.0 *	8.9 ± 0.8	—
Human red cells	$3.8 \cdot 10^7$	37	4.2 ± 0.7	5.6 ± 0.8	—
Bovine red cells	$6.4 \cdot 10^7$	0	6.4 ± 0.8	5.9 ± 0.7	—
Bovine red cells	$6.4 \cdot 10^7$	37	6.0 ± 1.0	6.8 ± 0.7	—
Bovine thymocytes	$1.2 \cdot 10^8$	0	18 ± 3	—	20 ± 3
Bovine thymocytes	$1.2 \cdot 10^8$	37	32 ± 8	—	55 ± 15

* Maximal deviation in 3 experiments.

data on the cytolytic activity of the deoxy lysophosphatide have been derived from potassium determinations.

When different mouse cells are tested in this manner for their sensitivity to lysis by the deoxy lysolipid, L_{50} concentration at $1 \cdot 10^6$ cells/ml vary from 0.2 nmol/ml for red cells to 10—13 nmol/ml for macrophage and Meth A tumor cells, i.e., by a factor of 65 (see Fig. 1 for representative data).

Binding of lysophosphatide to cells

Three factors may be involved in creating the different sensitivities of cells to deoxy lysophosphatidylcholine: (a) distinct differences in membrane resistance to lysolipids, (b) differences in size and (c) different binding affinities for the lysophosphatide. As far as binding affinities are concerned, the data presented in Fig. 2 indicate that, at least in the mouse system, there are no

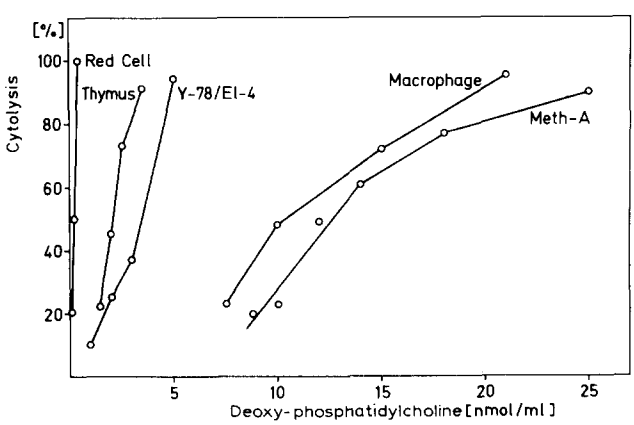

Fig. 1. Cytolytic activity of ether-deoxy lysophosphatidylcholine towards various mouse cells at 37°C. 10^6 cells/ml were incubated with increasing lysophosphatidylcholine concentrations at 37°C. After 15 min, cells were centrifuged and potassium concentrations in the supernatants determined by flame photometry (see Methods).

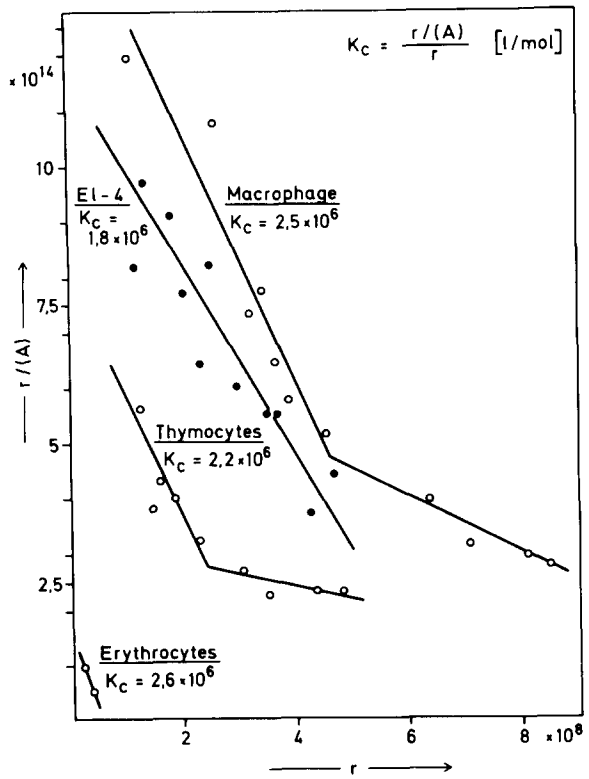

Fig. 2. Scatchard plots of binding data of ^{14}C-labeled deoxy lysophosphatidylcholine to various mouse cells at 37°C. *r*, bound lysophosphatidylcholine (mol/cell), (A), free lysophosphatidylcholine concentration [mol/l]. Constant amounts of cells ($5 \cdot 10^6$ cells/ml) in phosphate-buffered saline were incubated with increasing (but sublytic concentrations of ^{14}C-labeled 1-hexadecylpropanediol-3-phosphorylcholine for 15 min at 37°C. After centrifugation, radioactivity in sediment and supernatant was determined as described previously for red cells [7,8,22].

significant differences in binding constants of the lysolipid to a variety of cell types, as determined from the slopes of Scatchard plots [21]. This is in full agreement with our observations on erythrocytes of different species [7,8].

Membrane surface determinations

The fact that the binding affinity of deoxy lysophosphatidylcholine to cells seems to be largely independent of the cell type implies that the extent of lysophosphatide binding under standardized conditions will be primarily controlled by the available membrane surface area. Conversely, we propose that the distribution of the lysophosphatide between cells and medium may be used as a measure of membrane surface. This hypothesis was checked with a variety of red cells, which, because they possess exclusively outer membrane, have the advantage of the membrane surface equalling the cell surface. Moreover, data on shape, size, and in many instances, on the surface area of erythrocytes of various species are available in the literature [23,24].

When $1-15 \cdot 10^7$ cells/ml are incubated with hexadecyl-deoxy lysophosphatidylcholine at a concentration of 1.5 nmol/ml, the relationship of distribution (bound over free lysophosphatide) to cell count is close to linear (see

Fig. 3). The slopes of these curves relate well to the calculated surfaces. This is illustrated in Table II, which is a compilation of data on red cells of 6 different species with greatly varying lipid composition and size. The first part of this table shows: (a) surface values for red cells, available in the literature [24], (b) surfaces calculated from published values for cell dimensions [23], assuming spheres, having the radius of the biconcave cells. These values are generally larger than accurately calculated data. (c) Surfaces calculated from cell volume determinations (see Methods), again assuming spherical shape. These values are generally smaller than accurately calculated ones. (d) Slopes of bound-over free lysophosphatidylcholine concentration vs. cell count (see Fig. 3).

In the second part of Table II all values for human red cells are normallized to 100 and the others adjusted accordingly. From these data it is apparent that, within the range of error of the method (±20%), the relative relationships of binding equilibria for different red cells compare well with relative surface values obtained by other methods. We thus conclude that, at least for red cells, lysophosphatidylcholine binding equilibria can be used for rather accurate determinations of cellular surfaces. It is also evident from these data, that even for smooth cells, such as erythrocytes, surface determinations via cell volume measurements necessarily lead to values which are significantly smaller than the actual surface area. This latter problem is of particular interest for non-erythrocytic cells, many of which have highly irregular surface structures when studied by scanning electron microscopy. Any method for surface determinations of those cells that includes the assumption of smooth surfaces, may thus lead to extremely missleading results. As described above for erythrocytes (Fig. 3 and Table II), we have also determined the average values for bound-over free lysophosphatidylcholine concentrations per 10^7 cells for various nucleated mouse cells (Table III) and compared these data to the value for

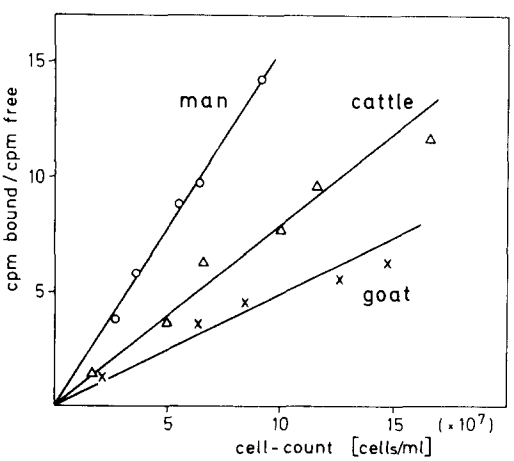

Fig. 3. Distribution of ^{14}C-labeled deoxy lysophosphatidylcholine as a function of cell counts for 3 different types of red cells. Increasing cell numbers of human, bovine and goat erythrocytes in phosphate-buffered saline were mixed with 1.5 nmol of ^{14}C-labeled 1-hexadecylpropanediol-3-phosphorylcholine per ml and kept for 15 min at 37°C. Cells were then centrifuged and radioactivity determined in sediment and supernatant as described previously [7,8,22].

TABLE II

COMPARISON OF DIFFERENT SURFACE DETERMINATIONS WITH LYSOPHOSPHATIDYLCHOLINE DISTRIBUTION (BOUND-OVER FREE PER 10^7 CELLS) FOR A VARIETY OF RED CELLS

	Red cell species					
	Goat	Sheep	Mouse	Cattle	Rat	Man
I. Surfaces in μ^2/cell						
(a) Data from ref. 25		62		95		163
(b) Surface calculated for spheres, using the radii [24] of the biconcave cells	50	72	113	109	145	176
(c) Surface calculated from cell volume (see text), assuming spherical shape	35	44	76	73	81	107
(d) Bound/free lysophosphatidylcholine per 10^7 cells *	0.47	0.62	0.70	0.79	0.9	1.5
II. Data normalized for human = 100						
(a) Data from ref. 25		38		58		100
(b) Surface from radii for spheres	28	41	64	62	82	100
(c) Surface from cell volume	33	41	71	68	76	100
(d) Bound/free lysophosphatidylcholine *	31	41	47	53	60	100

* Determined with constant amounts of 1.5 nmol of ^{14}C-labeled deoxy lysophosphatidylcholine per ml.

TABLE III

COMPARISON OF CYTOLYTIC ACTIVITY OF DEOXY LYSOPHOSPHATIDYLCHOLINE TO CELL SURFACE FOR VARIOUS MOUSE CELLS

Cell type	Surface determined from cell volume (μ^2/cell)	Relative cell surface from		L_{50} per 10^6 cells (nmol)	L_{50} per surface units (nmol)
		Volume determination	Lysophosphatidylcholine binding equilibrium		
Erythrocyte	76	1.0	1	0.2 ± 0.05	0.2
Thymocyte	147	2.0 (1)	8 (1)	2.5 ± 0.8	0.31
Spleen cells	209	2.7 (1.35)	11 (1.38)	2.5 ± 0.8	0.23
Macrophage	405	5.3 (2.65)	20 (2.5)	10.0 ± 2	0.50
E1-4	304	4.0 (2.0)	12 (1.5)	3.0 ± 1	0.25
Y-78	458	6.0 (3.0)	15 (1.9)	3.0 ± 1	0.20
Ehrlich ascites	n.d.	n.d.	28 (3.5)	10.0 ± 2	0.36
Meth A ascites	468	6.4 (3.2)	36 (4.5)	13.0 ± 2	0.36

n.d., not determined.

mouse erythrocytes, normalized to 1 (see column 3 of Table III). In comparison, we have determined cell volumes (see Methods) and, assuming spherical shape, calculated the respective surfaces (see column 1 of Table III). In column 2 of Table III these data are also normalized for a relative red cell surface of 1. When the data of columns 2 and 3 are compared, it is apparent that cell surfaces determined by the adsorption method are significantly higher (by a factor of 3—4) than those derived from volume measurements. However, when standardization is related to thymus cells, both methods result in rather similar surface relations (see data in parentheses in columns 2 and 3 of Table III), indicating that the adsorption method leads to principally meaningful results also in the case of non-erythrocytic cells. On the other hand, it appears that as far as relative surface relations are concerned, the determination via cell volumes is not as inaccurate as had been anticipated.

The question of whether deoxy lysophosphatidylcholine binds exclusively to surface membranes or equilibrates also with intracellular membrane structures, cannot at present be conclusively answered. In the first case, binding data could be directly related to cell surface, while in the second they would give a measure of the total membrane area, available to the lysolipid. As indicated above, surface determinations from cell volumes will necessarily yield surface values lower than the actual value. The finding that surfaces determined by the lysophosphatidylcholine absorption method are larger only by a factor of 3—4, may, with some caution, be taken as an indication that lysophosphatidylcholines interact primarily with the surface membrane of intact cells.

With regard to the principal question of this paper, namely the determination of lytic activity of lysophosphatidylcholine per available surface area, the above mentioned problem is of only secondary importance. Whatever the nature of the determined membrane area it will participate in the uptake of lysophosphatidylcholine and, hence, allow an accurate measure of lysophosphatidylcholine concentration per unit membrane surface. When these kinds of data are compared for various mouse cells (Table III, last column), it is apparent that for most of them no significant differences can be detected. The

extreme values of red cells and macrophage differ by a factor of only 2.5.

The finding that all four tumor lines do not exhibit sensitivities dramatically different from other cells of the same species (including red cells) implies that the results of Butterworth [13,15] and Klibanski et al. [14] concerning the high sensitivity of tumor cells to lysophosphatidylcholine lysis can certainly not be generalized. On the other hand, our data do not exclude the possibility, that the observed in vivo tumor rejection by synthetic lysophosphatidylcholine analogs [9] may involve cytotoxic effects caused by these compounds other than direct cytolysis.

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