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INFLUENCE OF INCORPORATED CEREBROSIDES ON THE INTERACTION OF LIPOSOMES WITH HeLa CELLS

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Summary

The presence of synthetic *N*-palmitoyl dihydrolactocerebroside in preparations of neutral multilamellar liposomes enhances their fusion interaction with cultured HeLa cells some 5—7-fold. The effect appears to be carbohydrate-specific, and is probably not attributable to a change in the physical characteristics of the liposome bilayer due to the presence of the cerebroside.

Interest in the role of cell membrane in controlling cellular activities, especially with regard to neoplastic changes, has sharply increased in recent years. One experimental approach to this problem is systematic modification of the composition of the membrane by fusion with artificial bilayer vesicles (i.e. liposomes) of definite composition, followed by correlation of changes in cellular activity with these variations in the membrane [1—3]. In the course of studying variables influencing the rate and mechanism of uptake of liposomes, we have found that the incorporation of certain synthetic cerebrosides stimulates fusion of neutral liposomes with HeLa cells in culture some 5- to 7-fold.

Liposomes were prepared by the method of Bangham [4] from egg phosphatidylcholine, cholesterol (obtained from Sigma), and synthetic cerebrosides (Miles Laboratories) in the mol ratio 7:2:1, respectively. Cerebrosides are glycolipids containing a long-chain fatty acid linked to the C-2 amino group of sphingosine and a sugar linked glycosidically to C-1. The three synthetic cerebrosides used here contain dihydrosphingosine instead of sphingosine, with glucose, galactose and lactose, respectively, as the sugar component. Egg phosphatidylcholine was purified by the method of Singleton [5], and trace amounts of [4-^{14}C]cholesterol (New England Nuclear), with or without [9, 10-^{3}H]dipalmitoyl phosphatidylcholine

(Natterman) were incorporated into each liposome preparation to allow quantitative determination of uptake.

Liposomes were incubated with $3-4 \cdot 10^5$ HeLa cells in Holme's A3 medium [6] supplemented with 10% heat-inactivated fetal calf serum (GibCo), 100 I.U./ml of penicillin, and 100 μg/ml of streptomycin. Incubations were carried out in 60 mm tissue culture dishes for 3 h at 37°C under a 5% CO_2 atmosphere. Medium containing free liposomes was removed and cells washed twice with 4 ml of 0.05 M phosphate-buffered physiological saline, pH 7.6. Cells were resuspended by 30 min incubation at 37°C with 3 ml of phosphate-buffered saline containing 0.1% EDTA, harvested by centrifugation at $120 \times g$ for 5 min, and finally suspended in 1.0 ml of phosphate-buffered saline. Aliquots were taken for radioactive counting and cell number determination by the method of Oyama and Eagle [7].

Data on the uptake of [4-^{14}C] cholesterol from liposomes of various compositions are presented in Table I. Results from two separate experiments are shown, the principal difference in protocol being the amount of lipid preparation added per plate (see footnotes to the table). Several conclusions may be drawn from the data:

(a) The incorporation of galactose-terminal cerebrosides into liposomes brings about the preferential incorporation of [^{14}C] cholesterol into the HeLa cells. (Compare the uptake seen with lipsomes containing galacto- and lactocerebrosides with the uptake of liposomes containing glucocerebroside or no cerebroside.)

(b) Lactocerebroside has approximately twice as much effect on incorporation as galactocerebroside, suggesting that the glucosyl moiety of the disaccharide allows better interaction of the terminal galactose with the putative cell-surface receptor.

(c) The increased uptake is probably not due to a change in the physical characteristics of the liposome membrane, since the phenomenon appears to be carbohydrate specific, since the uptake of lipid from *N*-palmitoyl dihydrosphingosine-containing liposomes is essentially the same as from liposomes made with phosphatidylcholine and cholesterol alone, and since the introduction of neutral glycolipids into liposomes appears to have little effect on membrane fluidity as measured by ESR [8] or on glycero-monooleate planar membrane conductance [9].

The data in Table I provide little information regarding the mechanism of uptake. The following observations relate to this question:

(a) Since the inclusion of lactocerebroside in the liposomes has no effect on the cytochalasin B sensitivity of cholesterol uptake (both controls and tests are 10—20% sensitive), enhancement of endocytosis can be eliminated. (Preliminary experiments established that the concentration of cytochalasin B used was sufficient to inhibit endocytosis of [^{14}C] sucrose 90—95% for this cell line under identical experimental conditions.)

(b) Since no more than 25% of the [^{14}C] cholesterol apparently incorporated in HeLa cells can be displaced by post-incubation of the cell suspension with lactose (Table II), it may be concluded that this represents the upper limit of uptake attributable to adsorption.

(c) Using liposomes doubly labeled in the membrane with [^{14}C] chol-

TABLE I

EFFECT OF GLYCOLIPIDS ON LIPOSOMAL LIPID UPTAKE BY HeLa CELLS

Liposome preparation	dpm of $^{14}C/10^5$ cells	
	Expt. 1*	Expt. 2**
Control (78% phosphatidylcholine, and 22% cholesterol)	140, 170	—
70% phosphatidylcholine, 20% cholesterol and 10% *N*-palmitoyl dihydrosphingosine	160, 170	430, 470
70% phosphatidylcholine, 20% cholesterol, 10% glucosyl dihydrosphingosine	140, 155	750, —
70% phosphatidylcholine, 20% cholesterol, 10% galactosyl dihydrosphingosine	—	1000, 1170
70% phosphatidylcholine, 20% cholesterol 10% lactosyl dihydrosphingosine	1740, 1610	1880, 2270

*0.19 mg lipid and $1.17 \cdot 10^5$ dpm [4-^{14}C]cholesterol/plate. (In another experiment using these conditions, we found that the phosphatidylcholine/cholesterol control gave a value of 291 ± 28 dpm/10^5 cells (n = 4), while the lactocerebroside-containing liposomes gave a value of 2194 ± 253 dpm/10^5 cells (n = 6).)
**0.27 mg lipid and $1.17 \cdot 10^5$ dpm [4-^{14}C]cholesterol/plate.

TABLE II

POST-INCUBATION DISPLACEMENT OF LACTOCEREBROSIDE LIPOSOMES FROM HeLa CELLS

Liposome preparation	dpm ^{14}C taken up/ 10^5 cells	dpm ^{14}C displaced/ 10^5 cells*	% of ^{14}C dpm displaced
Phosphatidylcholine/cholesterol (78:22)	(a) 250	3 (phosphate-buffered saline)	1.2
	(b) 220	0 (250 mM lactose)	0
Phosphatidylcholine/cholesterol/*N*-palmitoyl dihydrosphingosine (70:20:10)	(a) 1450	216 (phosphate-buffered saline)	14.9
	(b) 1180	273 (250 mM lactose)	23.1

*An aliquot of the final cell suspension was diluted to 1.0 ml with phosphate-buffered saline or lactose, incubated for 0.5 h at 37°C, centrifuged at 270 × g for 10 min, and a portion of the supernatant was counted.

esterol and [9,10-^{3}H]dipalmitoyl phosphatidylcholine, and examining HeLa cells for both radioisotopes, the ratio of $^{14}C/^3H$ was found to remain unchanged over a 6-h incubation period when lactocerebroside liposomes were used, while in control liposomes the ratio drops initially but then climbs past its initial value, suggesting preferential exchange (or net transfer) of individual lipids from liposomes to cells (Table III).

Although it is difficult in the control case to apportion quantitatively [^{14}C]cholesterol incorporation into cells via each contributory mechanism (because of the observed $^{14}C/^3H$ change in controls), it is estimated that fusion uptake is enhanced some 7-fold by the presence of lactocerebroside, based on the data in Table I.

Our observations regarding mechanism are largely in accord with those of Papahadjopoulos et al. [10] and Pagano and Huang [11], although these studies were done with unilamellar vesicles and used different cell lines and different incubation media. There is, however, one discrepancy between

TABLE III

UPTAKE OF DOUBLY MEMBRANE-LABELED LIPOSOMES BY HeLa CELLS[a]

Liposome preparation	Incubation time (h)	[^{14}C]Cholesterol/[^{3}H]-dipalmitoyl phosphatidylcholine uptake ratio
Phosphatidylcholine/cholesterol (78:22)	0	1.00
	3	0.80
	6	1.40
Phosphatidylcholine/cholesterol/lactocerebroside (70:20:10)	0	1.13
	3	1.15
	6	1.10

[a]A 6-fold enhancement of [^{14}C]cholesterol uptake was seen with lactocerebroside liposomes at 3 h. 0.19 mg lipid added per plate based on specific activity of cholesterol. Data is corrected for background and channel crossover.

our work and that of Poste and Papahadjopoulos [12], who found that neutral 'fluid' liposomes are incorporated into cells in culture largely via a cytochalasin B-sensitive mechanism. This difference might be attributed to one or more of the experimental differences mentioned above, or to the presence of cholesterol in our liposome preparations.

Galactose receptors have previously been implicated in triggering certain cellular activities, namely, the uptake of plasma glycoproteins by mammalian liver parenchymal cells [13], and cellular adhesion [14]. If galactose receptors are widely distributed in a variety of cell types, it would suggest that the interaction of this receptor with its effector can result in stimulation of different processes, depending on such variables as lipid composition of the membrane, number of interaction events per unit time, stage of development of the tissue, the particular point in the cell cycle at the time of interaction, and cell type itself. Our system would seem to be well-suited for evaluating the distribution and character of these receptors with respect to membrane function. The system is less complex than in vivo systems, and free of potential complications due to glycoprotein recognition factors other than galactose.

In summary, the approach described here using purified lipid vesicle preparations with cultured cells demonstrates enhanced fusion incorporation of liposomes containing synthetic galactose-terminal cerebrosides with a concomitant blockage of lipid molecule exchange. It may be feasible to selectively amplify the incorporation of exogenous lipids into cellular membranes, or to increase the efficiency of delivery of liposome-entrapped materials to specific cell types: *Escherichia coli* L-asparaginase, for example, in the case of certain leukemias.

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