Incorporation of phospholipids in Ehrlich ascites tumor cells[1]

J. L. Corchs, G. D. Venera, G. Mujica and R. Serrani

Cátedra de Fisiología, Facultad de Ciencias Médicas, Universidad Nacional de Rosario, Santa Fe 3100, Rosario 2000 (Argentine), 4 January 1983

Summary. Ehrlich ascites tumor cells (EATC) were incubated with unilamellar vesicles (UV) or multilamellar vesicles (MV). UV and MV were incorporated differently into EATC. The increase in ^{32}P-phospholipid in EATC in the presence of UV was 12% in 300 min. Absorption of phospholipid from MV could account for only 3%. About 50% of the UV incorporation of ^{32}P was by endocytosis and/or fusion.

Phospholipids (PL) are important constituents of cellular membranes[2-4]. The nature and distribution of phospholipids within membranes determine properties related to cellular growth and metabolism[5-8].

Liposomes are microscopic structures consisting of one or more lipid bilayer[8]. The present study concerns the incorporation of UV and MV into EATC, which are widely used as a free-floating cell model[9,10]. The findings show there are differences in the rate of incorporation of the two types of liposomes into EATC.

This work shows the possibility of modifying the phospholipid composition of membrane structures by allowing UV with a selected mixture of PL to interact with EATC, for a short period of time.

Materials and methods. EATC were injected i.p. into Rockland mice ($0.5-1\times10^7$ cells). After 7–9 days the peritoneal cavity was opened and the cells collected. The cells were separated by differential centrifugation and resuspended in phosphate buffered saline (PBS) pH 7.4 to a final concentration of 1×10^7 cells/ml[11]. The viability of cells at the end of each experiment was determined using nigrosin[12].

Phospholipids from rat liver were extracted by the addition of 200 ml of chloroform-methanol (2:1, v/v) to 10 g of tissue[13]. The extract was filtered and 100 ml of 40 mM $CaCl_2$ were added. After the solution had been thoroughly mixed, the 2 phases were separated by centrifugation. The upper phase was discarded[14]. The lower phase was stored at −20 °C. ^{32}P-labeled phospholipids were isolated as described above from the liver of a rat injected i.p. with 1 mCi ^{32}P (sodium phosphate) per 100 g b.wt 12 h before sacrifice. The phospholipid extract was evaporated under nitrogen and resuspended in 50 mM Tris-HCl buffer pH 7.4 for preparation of MV[15]. UV were made by adding sodium cholate 0.2% to the milky dispersion. The micellar solution was applied to a Sephadex G-50 column (25×1.5 cm) which was equilibrated with the phospholipid extract and eluted with the buffer. Liposomes were taken at the void volume. Fractions containing the liposomes were dialyzed against hypertonic sucrose solution for 12 h at 4 °C[16]. Vesicles with entrapped inulin were prepared by the same method (10 mg inulin per 100 mg phospholipids. Samples were taken for electron microscopy and observed by the negative-staining technique[17]. The degree of oxidation was determined by the oxidation index at 233 and 215 nm[18]. Cells were counted by the absorbance method[19]. Experiments were performed at 27 °C in a thermostatic water bath (4×10^7 cells in a final volume of 7 ml) for various time intervals up to 300 min.

In the 1st group the cells were incubated with MV (total phospholipid phosphorus: 80 µg). In the 2nd group the cells were incubated with UV (total phospholipid phosphorus: 40 µg). In the 3rd group, cells were incubated with UV containing trapped inulin. After incubation, cells of this last group were trypsinized (0.01%) for 30 min.

In order to assess nonspecific absorption, cells were suspended in a medium with UV and MV. Parallel incubations were made at 4 °C and samples were analyzed during the entire course of the experiment (0, 2, 4, 5 h).

Cells were washed 10 times with NaCl 0.9% to remove liposomes from the medium and unstable adsorbed liposomes as well as untrapped inulin for the 3rd group. Phospholipids were extracted from cells with methanol-chloroform (1:2 v/v) and ^{32}P was measured with a liquid scintillation Spectrometer (Beckman, LS-250, Cal., USA). Inorganic phosphorus from PL extract was determined by colorimetric method[20].

Phospholipid composition of EATC membranes and both types of liposomes were analyzed by 2-dimensional thin

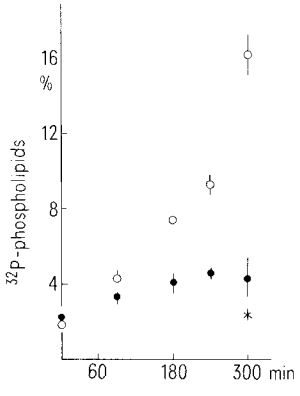

Figure 1. Radiolabeled phospholipid and phosphate incorporation from liposomes (percentage of total cell phospholipids) into tumoral cells, plotted against incubation time. ○, Unilamellar liposomes (UV); ●, multilamellar liposomes (MV); *, incorporation of ^{32}P-phosphate into cell phosphatides. Results presented are the mean of 4 separate experiments each done in duplicate.

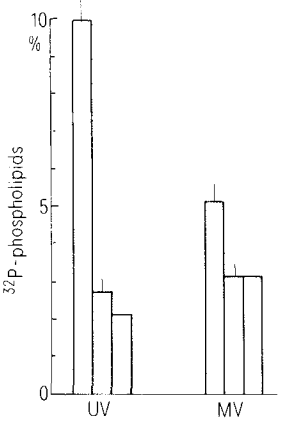

Figure 2. Effect of temperature on phospholipid incorporation from MV and UV to EATC. Left bar: 240 min, 27 °C; central bar: 240 min, 4 °C; Right bar: 0 min, 4 °C.

Separation of phospholipids by 2-dimensional TLC. Values in table represent percent of total phospholipids

	PC	PEA	Sph	PS-PI	LPC
Membrane fraction	41.41 ± 4.17 (5)	26.83 ± 1.57 (5)	8.45 ± 1.05 (5)	14.95 ± 2.13 (5)	4.30 6.00
Liposomes	42.80 ± 3.70 (12)	27.25 ± 2.40 (12)	9.30 ± 0.99 (12)	14.33 ± 1.40 (12)	4.04 4.00

Means ± SE for number of values shown in parentheses. PC: phosphatidylcholine; PEA: phosphatidylethanolamine; Sph: sphingomyelin; PS-PI: phosphatidylserine-phosphatidylinositol; LPC: lysophosphatidylcholine.

layer chromatography on plates (10×10 cm) coated with Silica Gel H (Sigma Chem. Co.) and developed with chloroform-methanol-ammonium hydroxide (13:5:1) and chloroform-acetone-methanol-acetic acid-water (6:8:2:2:1)[21].

Cell membranes were obtained by differential centrifugation with dextran and polyethylenglycol[22].

Inulin content of cells was measured by a colorimetric method[23]. The determination of phospholipids and trapped inulin in liposomes was made after aggregating the suspensions by addition of polylysine[24]. The results were expressed as percentage of the total PL cell phosphorus.

Radioactive phosphorus was obtained through the Comision Nacional de Energía Atómica of Argentina as ^{32}P sodium phosphate of high specific activity (1 mCi/ml) in sterile solution. All the other reagents were of analytical grade.

Results and discussion. The table shows the levels of phospholipids in membrane fraction and liposomes. The values for phosphatydilcholine and phosphatydil-ethanolamine agree with the findings of Wallach et al.[25] while the levels of sphingomyelin differ from those found previously. In our hands, the spots for phosphatidylinositol and phosphatidylserine were not well separated in some of the plates, so these compounds were collected together.

Electron microscopy negative staining showed an average vesicle radius of about 0.2 μm for MV and 0.1 μ for UV, although some vesicles were 2–5 times larger than the average. The oxidation index during experiments was 0.3–0.4.

Addition of liposomes to EATC in a ice-cooled medium, followed by immediate separation of supernatant and subsequent washing, led to ^{32}P uptake of 1.2% for UV and 2.5–3% for MV (nonspecific incorporation). Incubations at 4 °C showed that this nonspecific absorption was not modified significantly during the entire experiment for either type of liposome (see fig. 2, central bars).

The incorporation of ^{32}P as a function of time was different for the two types of vesicle. The attained value at 300 min (maximum time studied) considering the coresponding nonspecific incorporation at 300 min, was 3% and 12% for MV and UV respectively (fig. 1). Total ^{32}P incorporation from sodium phosphate was about 3% at 300 min (fig. 1).

Phospholipid uptake was temperature-dependent. Phospholipid uptake for UV increased 1 order of magnitude for a 23 °C increment (from 4 °C to 27 °C) during 240 min of incubation, while it increased twice as much for MV under the same conditions (fig. 2).

EATC were incubated with UV containing inulin. After trypsination the inulin that was not released from cells amounted to 57.23% ± 8.12, n = 8, compared to the paired experiments without trypsination (100%). Free inulin was not incorporated into cells under experimental conditions.

The present findings show that EATC incorporated phospholipids from liposomes. In addition, the degree of incorporation was found to depend on the type of liposomes; uptake was higher for UV than for MV.

In accordance with previous reports[26] incorporation from ^{32}P phosphate (sodium salt) to cell phosphatides amounted to 3%. Both types of liposome had a similar composition, so this factor is unlikely to have been responsible for the difference in their interaction with EATC. In spite of the greater amount of phospholipids in the medium for MV (see materials and methods, incubation conditions), the fraction of phospholipids accessible to interaction with cells is smaller for MV because of the multilayers, while most of the UV lipids are available to interact. It could be a possible explanation of the different incorporation observed in the 2 populations of liposomes.

The difference could also be explained on the basis that the smaller size of the UV would facilitate penetration into small intercellular spaces and this would increase the opportunities for fusion. Besides that, the UV have a bigger curvature and it is known that highly curved membranes have an unusual asymmetric packing of phospholipids in the outer layer that may be responsible for a better interaction[27].

The fact that inulin entrapped in UV was taken up by EATC and that only about 40% of inulin associated with cells was released by trypsin treatment indicates that fusion and/or endocytosis occurred[7,8]. The dependence of UV-EATC interaction on temperature[28] supports the last-mentioned mechanism.

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The effect of 17α-methyltestosterone on the sex of the common carp, *Cyprinus carpio* (L.)

H.N. Sathyanarayana Rao[1], G.P. Satyanarayana Rao, T.J. Varghese and H.P.C. Shetty

University of Agricultural Sciences, College of Fisheries, Mangalore 575002 (India), 11 February 1983

Summary. Dip treatment of fertilized eggs of *Cyprinus carpio* (L.) in 17α-methyltestosterone, followed by dietary administration of the same to the hatchlings over 50 days resulted in 54% males, 13% females and 33% sterile fish, while in the control group both sexes were in nearly equal numbers. The androgen-treated fish also showed better growth.

The common carp, *Cyprinus carpio*, is an important species used in composite fish culture in India. However, its 'wild spawning' in culture ponds has been found to affect the pond's yield adversely[2]. Studies conducted at the College of Fisheries, Mangalore, have revealed that by administering 17α-methyltestosterone (200 ppm) over 131 days, starting from the 2nd day after hatching, it is possible to produce a brood consisting of only male and sterile fish[3]. Since the duration of treatment in that experiment was too long for field application, an attempt was made to reduce the treatment period to 50 days.

The fish was induced, induced-bred the developing eggs in the gastrula stage were given a dip-treatment in an aqueous solution of 17α-methyltestosterone (17α-MT) at a concentration of 200 ppm for 1 h. The resultant hatchlings were maintained on a feed containing the same hormone at 200 ppm over 50 days, starting from the 2nd day after hatching. They were also given small quantities of plankton during the first week only.

The fry were reared in plastic pools during the treatment period of 50 days and were later transferred to cement cisterns (50 m^2) for further rearing over another 72 days on a hormone-free diet.

On termination of the experiment, the length and weight of all the fish were recorded and their gonads dissected out and sexed, following the methodology described elsewhere[3].

The androgen-treated group had 54% males, 33% sterile fish and 13% females, while the control group had 51% males and 49% females. The growth rate and survival of the treated fish were found to be consistently better than those of the controls. The fish classified as 'sterile' had only filiform gonads, which on histological examination did not show any germ cells. However, a few enlarged cells, resembling the germ cells, were encountered in a few filiform gonads.

The results of this investigation clearly demonstrate the possibility of reducing the period of hormonal treatment. In the earlier work on common carp, treatment with 17α-MT (200 ppm) over a period of 131 days was found to be effective in producing a population completely devoid of females[3]. Even though this could not be achieved in the present work, it has clearly indicated the possibility of reducing the period of hormonal treatment considerably. It appears highly probable that a completely female-free population could be obtained with only 50 days of hormonal treatment or even less by increasing the dosage. Another possible reason for the occurrence of females in the present study could be that the hatchlings initially fed more on the plankton given than on the hormone-containing diet. Therefore, reducing plankton food to only the first couple of days might yield better results.

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Alloxan-induced hyperglycemia increases progestin and androgen accumulation by isolated rabbit follicles in vitro[1]

E.V. YoungLai

Department of Obstetrics and Gynecology, Faculty of Health Sciences, McMaster University, 1200 Main St. West, Hamilton (Ontario, Canada L8N 3Z5), 23 April 1983

Summary. Follicles isolated from Alloxan-treated rabbits and incubated in vitro, accumulated more progesterone and testosterone than those from saline-treated rabbits. LH augmented the accumulation of these 2 steroids. By contrast, the estradiol response to LH stimulation by follicles from Alloxan-induced hyperglycemic rabbits was diminished when compared to follicles from saline-treated rabbits. Ovaries from hyperglycemic rabbits also appeared to have more cystic follicles.