Fusion and Endocytosis of Anionic Liposomes with Ehrlich Ascitic Carcinoma Cells

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Polyethylene glycol 300 and low pH of the incubation medium initiate fusion of anionic lipid liposome membranes with Ehrlich ascitic carcinoma cell membranes. Some liposomes are endocytosed in cells and are distributed near the inner surface of the membrane. Liposome membranes spontaneously fuse with the cells under certain conditions.

Key Words: *Ehrlich ascitic carcinoma cells; membrane fusion; fluorescent probes; liposomes*

The interactions between liposomes composed of different lipids and cells remains an important problem due to wide practical use of liposomes as vehicles for the delivery of various drugs to human organs and tissues. Liposomal transfection of eucaryotic cells is often compared with viral infection, which is realized by fusion with cell plasma membrane and subsequent endocytosis into the cytoplasm. Cationic liposomes can effectively fuse with anionic liposomes or erythrocyte ghosts used as model membranes [13]. Endocytosis remains the most probable route of penetration of various cationic agents into the cytoplasm [6,7,10-12,14,15].

Two methods are mainly used for studies of membrane fusion process. One of them is based on measurement of fluorescence intensity of fluorescent stains, which changes in the membrane when lipids are mixed or in vesicular contents [4,8]. The other method is based on measurement of electric current through flat membranes [2]. The fluorescent method is usually preferred, because of high sensitivity and the possibility of continuous observation of the process, which permits quantitative estimation of the initial parameters of fusion kinetics.

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We investigated fusion of liposome membranes formed from anion and neutral lipids with Ehrlich ascitic carcinoma (EAC) cells by the fluorescent method.

MATERIALS AND METHODS

Large unilamellar liposomes up to 1000 Å in size (according to electron microscopy) were obtained by the reverse phase method with subsequent extrusion through polycarbonate membrane filters with 0.1-0.2 μ pores in a medium containing 100 mM NaCl and 10 mM HEPES (pH 7.4). We used liposomes consisting of palmitoyloleoyl phosphatidylcholine, palmitoyloleoyl phosphatidylserine, and cholesterol (2:2:1 molar ratio). Fluorescent-labeled liposomes contained 1 mol% of N-NBD-PE (N-(7-nitrobenz-2-oxa-1,3-diasol-4-yl)phosphatidylethanolamine) and N-Rh-PE (N-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine), in which the labels (nitrobenzoxadiasole and rhodamine B) were covalently bound to the polar group of phosphatidylethanolamine molecule. Summary content of lipids in the resultant dispersions was about 10 mM. Each liposome with diameter of about 1000 Å contained 400-500 molecules of N-NBD-PE and N-Rh-PE fluorophores (1% of all lipids). Gel filtration of lipid dispersions was performed on columns packed with Sephadex G15, Sephadex G25, sepharose 2B, and other gels.

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EAC cells were isolated from mice on day 7 after transplantation. After collection of the suspension from the abdominal cavity the cells were washed twice in Hanks solution containing 20 mM HEPES (pH 7.4).

All lipids were from Avanti Firm, N-NBD-PE and N-Rh-PE from Molecular Probes, NaCl and HEPES from Sigma, polyethylenglycol 300 (PEG-300) from The British Drug Houses Ltd., morpholinoethane sulfonic acid (MES) from Serva.

Changes in the fluorescence intensity during interactions of labeled liposomes with EAC cells were recorded on a Perkin-Elmer MPF-44B spectrofluorometer at 37°C and constant stirring. N-NBD-PE stimulation and radiation wavelengths were 475 and 530 nm.

The distribution of the probe molecules in cells was monitored on an LSM 510 NLO laser scanning confocal microscope (Carl Zeiss). Argon and He-Ne lasers (λ=488 nm and λ=543 nm) were used for excitation of fluorescence. Registration was carried out via three channels. The images were processed using LSM 510 software.

RESULTS

Fusion of liposomal membranes with cells was recorded by the method of resonance energy transfer between a couple of fluorescent probes. Fusion of membranes led to dilution of labeled lipid molecules, which reduced the efficiency of energy transfer to the acceptor (rhodamine B), while the intensity of donor (N-NBD) fluorescence increased (Fig. 1).

The interactions between anionic liposomes and cells, their collision, and subsequent fusion were impeded because of analogous electrical charge. Appropriate conditions had to be created for initiation of membrane fusion. For this purpose, fusogen (PEG-300; Fig. 1) was used or pH of the incubation medium was reduced (Fig. 2) [3,5].

Changes in fluorescence intensity (Fig. 1, *2*) reflected the increase in light diffusion in the incubation medium as a result of partial aggregation of cells (Fig. 1, *4*) and decreased the efficiency of resonance energy transfer from N-NBD molecules to rhodamine B molecules as a result of liposomal membrane fusion with cell membranes (Fig. 1, *3*).

The minimum concentration of PEG-300 initiating liposome fusion with cells in the sample under experimental conditions was 10%.

Reduction of pH of solution with EAC cells and labeled liposomes increase in the intensity of N-NBD probe fluorescence due to fusion of liposomal membranes with cell membrane (Fig. 2, *1*, *2*). Low pH of the incubation medium could par-

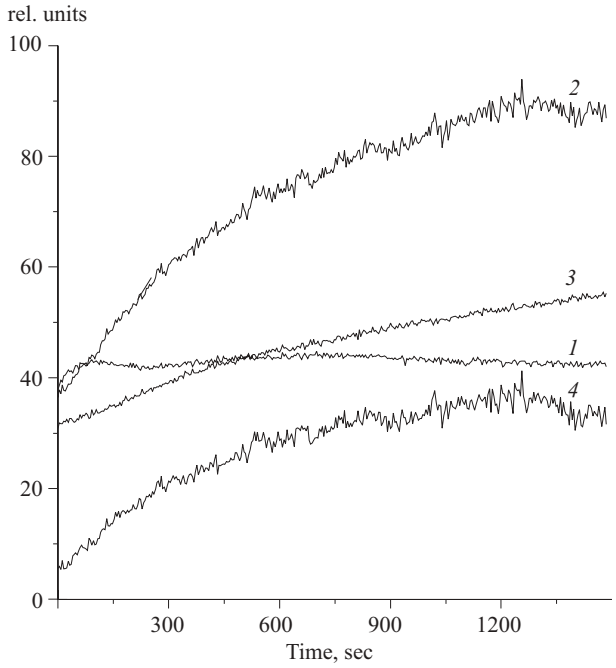

Fig. 1. Changes in N-NBD probe fluorescence intensity (*2*, *3*) and light scattering (*4*) in the incubation medium with EAC cells after addition of PEG-300. EAC cell concentration 10^7/ml. Lipid concentration in liposomes labeled by N-NBD and rhodamine B 6 μM. *1*) control (EAC cells+labeled liposomes); *2*) EAC cells+labeled liposomes+15% PEG-300; *3*) difference between curves *2* and *4*; *4*) changes in light diffusion intensity in EAC cell incubation medium with 15% PEG (cells+unlabeled liposomes+15% PEG-300).

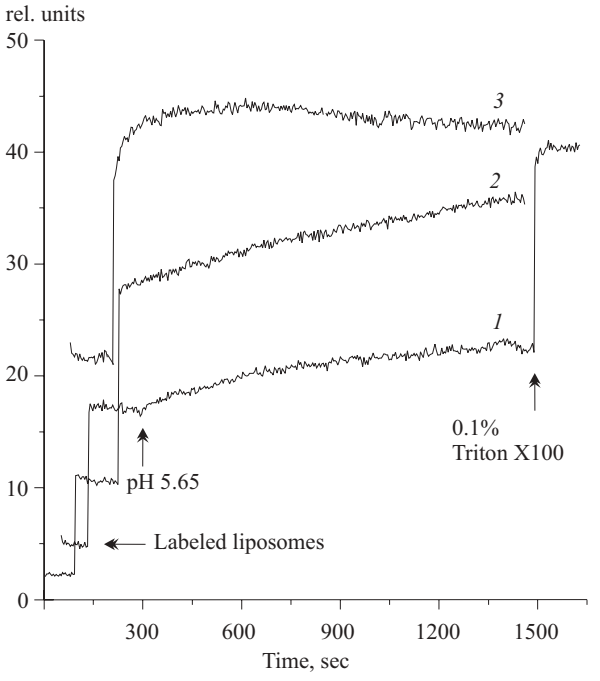

Fig. 2. pH-Induced changes in N-NBD fluorescence intensity in medium with EAC cells. *1*) pH of the solution changed from 7.4 to 5.65 (shown with an arrow) after addition of EAC cells and labeled liposomes; *2*) pH of the solution was changed from 7.4 to 5.65 before addition of EAC cells and labeled liposomes into the incubation medium; *3*) control sample.

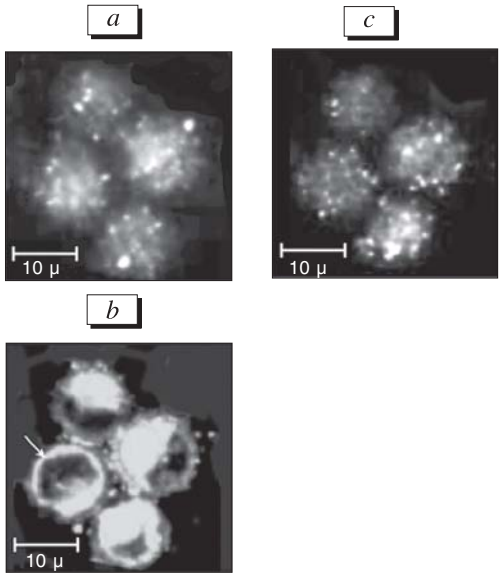

Fig. 3. Ehrlich ascitic carcinoma cells after treatment with labeled liposomes (N-NBD and Rh) in the presence of 15% PEG-300. Optical section of EAC cells at a depth of 1 (*a*), 6 (*b*), and 12 (*c*) μ. Excitation and emission wavelengths are 514 and 565-615 nm, respectively; scale 10 μ. Arrow shows membrane rim.

tially neutralize the negative charge of liposomes and cell surface, which presumably increased the probability of their collision and subsequent fusion of membranes. EAC cells became better permeable for ions, due to changed potential on the cell membrane. These changes seemed to promote membrane fusion.

EAC cells can spontaneously fuse with labeled liposomes, *e.g.* after long-term storage of cells at 5°C under anaerobic conditions (metabolically exhausted cells). This can be due to the formation of phospholipid cleavage products and changed permeability and structure of the plasma membrane.

By analogy with cell interaction with various particles and of artificial membranes between each other [1], the fusion and endocytosis are possible ways of liposome interaction with EAC cells.

Confocal laser microscopy showed these processes in the cell at different depths (Fig. 3). Fluorescence of the probe molecules was observed along the membrane rim (optical section at a depth of 6 μ), where liposomes fused with EAC cells, and in the cytoplasm closer to the periphery (Fig. 3). In addition, there were areas of probe distribution in the plasma membrane, between which resonance energy transfer was observed.

Hence, PEG-300 or reduction of incubation medium pH initiated fusion of liposome membrane consisting of anionic lipids with cell plasma membranes and endocytosis of liposomes in EAC cells.

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