EFFECT OF LIPOSOMES OF DIFFERENT SIZE, COMPOSITION, AND CHARGE ON COLONY-FORMING ABILITY OF CELLS

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Changes in the functional state of cells of prokaryotes and eukaryotes can be brought about by modification of the composition of membrane lipids and proteins with the aid of liposomes [8]. In the study of interaction between liposomes and cells, an important problem is the evaluation of their toxicity. In most investigations this has been determined on the basis of the ability of cells to take up vital dyes immediately after interaction with liposomes. Workers using this criterion have concluded that liposomes prepared from natural lipids have low toxicity [7, 9]. The viability of cells, based on their ability to take up vital dyes, however, does not reflect the integrity of their proliferative activity.

The aim of this investigation was to assess the effect of liposomes of different composition, change, and size on the ability of LL cells to multiply without limits and to compare these data with the viability of the cells based on their uptake of eosin.

EXPERIMENTAL METHOD

The following reagents were used: egg lecithin (phosphatidylcholine — PC) was from the Khar'kov Bacterial Preparations Factory and purified by the method in [13]; stearylamine was from Eastman (USA), and phosphatidic acid (PA) from Sigma (USA). Multilamellar liposomes (MLL) were prepared by the method in [10], as described by the writers previously [1]. For this purpose 50 μmoles PC (neutral MLL) or 40 μmoles PC and 10 μmoles PA (negatively charged MLL), or 40 μmoles PC and 10 μmoles stearylamine (positively charged MLL) in chloroform was transferred to a round-bottomed flask and evaporated to dryness on a rotary evaporator. To remove traces of chloroform the film was kept for 30 min in vacuo at 0.5 mm Hg and emulsified with 4 ml of 150 mM NaCl solution on the course of 15-30 min at 37°C. The emulsion was incubated for 2 h at 20°C and treated with ultrasound on the UZDN-1 apparatus at 22 kHz (70 W) for 1 min at 4°C. The microemulsion was freed from aggregates and traces of titanium by centrifugation at 5000g for 10 min. The supernatant was used as MLL. Unilamellar liposomes (ULL) were made from MLL preparations by treatment with ultrasound under the same conditions for 10 min, in 1-min sessions alternating with 1-min intervals for cooling at 4°C, in a constant stream of argon. The preparation was centrifuged for 10 min at 10,000g and 4°C, and the resulting supernatant was centrifuged at 15,000g for 4 h [12]. The second supernatant was used as the ULL preparations. The liposomes were kept for not more than 2 days under argon at 4°C. All procedures with lipids and liposomes were performed under argon protection and with observance of the conditions of sterility. The purity of the lipids was tested by thin-layer chromatography on silica-gel KSK (15-30 μ) in a chloroform—methanol—water (65:25:4) solvent system. The concentration of lipids was determined as lipid phosphorus [1]. The size of the liposomes was measured electron-microscopically and by the optical displacement method [2].

Experiments were carried out on cells of the LL line obtained from mice with NKLy ascites lymphatic leukemia [4], cultured *in vitro*. The cells were cultured in Carrel's flasks in medium 199 in the presence of 20% bovine serum. A cell suspension was prepared from an

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TABLE 1. Efficiency of Colony Formation by LL Cells after Treatment with Liposomes (in % of control)

Composition of liposomes	Concentration of liposomes, μmoles/ml									
	MLL					ULL				
	0,05	0,1	0,2	0,3	0,5	0,05 (10^{11})	0,10 ($2 \cdot 10^{11}$)	0,2 ($4 \cdot 10^{11}$)	0,3 ($6 \cdot 10^{11}$)	0,5 (10^{12})
PC	50,0	44,1	—	8,3	3,4	92,2	89,0	63,9	4,8	0
PC + stearylamine	79,5	51,3	—	4,4	0	85,0	82,2	31,0	4,0	1,0
PC + PA	100,0	100,0	94,6	57,6	—	85,6	56,9	21,0	2,7	—

Legend. Mean results of 3-6 experiments given; number of liposomes per cell given in parentheses.

asynchronously growing culture and seeded, 150 cells at a time, into flasks containing nutrient medium. After 24 h the medium was poured off, the flasks rinsed with physiological saline to remove traces of serum, and liposomes were introduced into them in a final concentration of between 0.05 and 0.5 μmole/ml in physiological saline. Incubation was carried out at 37°C for 2 h. The flasks were then rinsed with medium 199, nutrient medium was poured into them, and they were incubated at 37°C to form microcolonies. Flasks treated in the identical manner with physiological saline served as the control. The number of colonies was counted on the 8th day and the efficiency of colony formation expressed as a percentage of the control. The mean efficiency of colony formation was calculated from the results of three to six independent experiments, in each of which five flasks were treated identically. The viability of the cells in relation to vital dyes was determined after treatment of a monolayer culture (10^5 cells) with liposomes in the same concentrations and under the same conditions of incubation. Immediately after treatment with liposomes and also on the 1st and 4th days of culture, cells were removed from the glass and 0.5 ml of a suspension was kept with an equal volume of 0.2% eosin solution in physiological saline for 3 min. The viability was determined as the ratio of the number of unstained cells to the total number of cells in the Goryaev's chamber. As well as assessing viability, the total number of cells in the flask also was counted. The mean rate of survival was calculated from results of three to five experiments.

EXPERIMENTAL RESULTS

The MLL were a heterogeneous population of vesicles measuring from 50 to 1000 nm; because of the heterogeneity of the microemulsion the number of MLL per unit mass of lipid could not be counted. The ULL preparations were highly homogenous and, when determined from data of electron microscopy or by the optical displacement method used previously [2], the radius of ULL prepared from PC was 14.6 ± 0.2 nm. The number of PC molecules in one such vesicle, according to data obtained by various workers, was 2660-2800 [3]. Consequently, to 1 μmole PC there were about 2×10^{14} ULL. Addition of 20 moles % PA or stearylamine was reflected in the size and number of vesicles per unit of lipid, but this correction increased the size of the vesicle by not more than 2-3 times. It was therefore decided to accept the figure of 10^{14} vesicles/μmole as the initial level for ULL.

The efficiency of colony formation of LL cells after incubation with MLL and ULL was found to depend on concentration within a sufficiently narrow range of lipid concentrations. With a concentration of 0.3 μmole/ml colony fromation was found to be practically completely suppressed both for MLL and for ULL of all types (Table 1). In concentrations of 0.05 and 0.01 μmole ULL were less toxic in the colony formation test than MLL, except in the case of negatively charged MLL. Comparison of the degree of inhibition of colony formation by ULL and the ratio between the numbers of ULL per cell showed that the inhibition effect was manifested if the change in the ratio was about one order of magnitude (Table 1). If the total number of cells was counted after treatment with ULL made from PC (10^9 ULL per cell) in a concentration of 0.5 μmole/ml, proliferation was found to be inhibited on the first day (Fig. 1).

Assessment of survival of the cells by vital staining with eosin showed that within the above limits of lipid concentrations they had no toxic effect. The viability of the cells for all types of ULL and MLL, both immediately after treatment and at all times of culture, was 90-95% and did not differ from that in the control (93.4%).

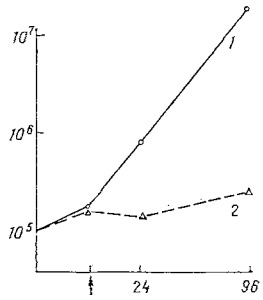

Fig. 1. Level of proliferation of LL cells after incubation with ULL made from PC. Abscissa, time (in h); ordinate, number of cells (mean of five flasks). Cells were seeded ath the rate of 10^5 per flask, treated after 24 h with ULL in a concentration of 0.5 μmole in 1 ml physiological saline; the flasks were rinsed 2 h later with medium 199, after which incubation continued in complete nutrient medium. Survival rates relative to eosin 78.6-93.7% in experiment, 92.4-93% in control. 1) Control, 2) experiment. Arrows indicate treatment with liposomes.

Analysis of the results indicates, first, the necessity for caution when assessing toxicity of liposomes during their interaction with cells. The rather widely held view that liposomes are nontoxic for eukaryote cells in a concentration of 1 μmole lipids/ml, based on the criterion of uptake of vital dyes, does not agree with concentration based on assessment of the ability of the cells to proliferate. Dunnick et al. [7] found inhibition of colony formation by EMT6 cells (mouse carcinoma cells) only with positively charged ULL in the proportion of 10^9 per cell, and they did not find inhibition of proliferation by ULL prepared from PC and PC + PA. These workers incubated liposomes with cells in the presence of serum which, as we know, reduces the effectiveness of their contact with the cell membrane [11]. Inhibition of proliferation has been shown to depend on the lipid concentration for ULL in the case of EMT6, S-49, and AE_1 cells, and these values are in good agreement with the results obtained by the present writers for LL cells [5]. There is also morphological evidence of differentiation of neuroblastoma cells under the influence of negatively charged phosphatidylserine ULL when the ratio of liposomes to cells (10^5) was quite low [6]. Analysis of data in the literature and also the close similarity between weight for weight concentrations of MLL and ULL differing sharply in the number of liposomes per unit of lipid but leading to inhibition of unlimited cell growth, indicate that modification of proliferative activity depends more on the concentration of lipid than on the number of liposomes per cell.

The possibility of modifying the proliferative activity of tumor cells by treating them with liposomes requires further investigation, but the high viability of the cells coupled with the simultaneous inhibition of their capacity for unlimited growth, may offer new prospects for the control of cell proliferation.

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