INTRODUCTION OF FOREIGN GENETIC MATERIAL INTO CULTURED MAMMALIAN CELLS BY LIPOSOMES LOADED WITH ISOLATED NUCLEI

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1. Introduction

Efficient transfer of genetic information to a relatively large population of cells can be attained by the use of liposomes. Delivery of mRNA molecules [1,2] and metaphase chromosomes [3] into tissue cultured mammalian cells and expression of the transferred genetic information has been demonstrated.

Here we report that chicken erythrocyte nuclei, entrapped in phospholipid liposomes were introduced into CHO/Pro$^-$ cells and Pro$^+$ transformants were selected.

2. Materials and methods

2.1. *Cells*

Proline-requiring Chinese hamster ovary cells, CHO/Pro$^-$, isolated [4], were grown in BOB MEM supplemented with 10% FCS and 1 mM proline. CHO/Pro$^+$ cells were selected in proline-free BOB MEM containing 10% dialysed FCS.

2.2. *Nuclei*

Fresh chicken red blood cells were washed free of plasma proteins and the packed cells were lysed with 5 vol. 30 mM $NaHCO_3$, 50 μM EDTA, 5 μM 2-mercaptoethanol (pH 6.4 at 0°C). After 2 min 50 vol. 20% glycerol, 5 mM Mg-acetate, 50 mM Tris–HCl, 5 mM dithiothreitol, 100 μM EDTA (pH 8.0) were added and nuclei were repeatedly sedimented.

Abbreviations: PBS, phosphate-buffered saline (pH 7.2); FCS, fetal calf serum; AZCA, L-azetidine-2-carboxylic acid; TES, triethylamino-ethanesulfonic acid; BOB MEM, Eagle's MEM supplemented with non-essential amino acids and sodium pyruvate

2.3. *Liposomes*

Phospholipids were purified from beef brain according to [5]. The composition of the individual lipid fractions was checked by two dimensional thin-layer chromatography [6].

Liposomes were prepared by a modification of the methods in [1,7,8]. Briefly, 35 mg phospholipid mixture (composed of 60% phosphatidyl serine, 25% phosphatidyl inositol and 15% phosphatidyl ethanolamine) was suspended in 5 ml 0.1 M NaCl, 2 mM histidine, 2 mM TES, 0.4 mM EDTA (pH 7.4) (NHTE) by vortexing. The suspension was sonicated under N_2 for 10 min at 25°C, then Ca^{2+} was added to 20 mM final conc. and the mixture was incubated for 1 h at 37°C. The resulting precipitate was sedimented at 2500 × *g* for 10 min, vortexed and mixed with 100 μl PBS or PBS containing 10^8 nuclei. Then 150 mM EDTA in NHTE was added to 120 mM final conc. Liposomes were formed after 30 min incubation at 37°C. EDTA was removed by passing the liposomes through a Sephadex G-25 column, equilibrated with PBS.

2.4. *Fusion of liposomes with CHO/Pro$^-$ cells*

Monolayers of CHO/Pro$^-$ cells, grown in 60 or 140 mm petri dishes, were rinsed with PBS and liposomes loaded with nuclei, in 1 or 2 ml PBS, respectively were added. Subsequently the cells were incubated at 37°C for 60 min and then BOB MEM containing 10% FCS, 1 mM proline was added to the cells which was replaced by proline-free BOB MEM after 24 h.

2.5. *Fluorescence microscopy for direct visualization of liposomes*

Isolated nuclei were stained with ethidium bromide

(1 mg/ml) and liposomes loaded with stained nuclei were prepared. For fusion experiments unbound ethidium bromide was removed by passing the nuclei and the liposomes through Sephadex G-25 columns. The fluorescence of nuclei was examined in a Leitz Orthoplan darkfield fluorescence microscope.

2.6. *Resistance of CHO cells to AZCA*

Concentrations of AZCA resulting in a 50% decrease in the plating efficiency of CHO/Pro$^-$ and Pro$^+$ cell lines were determined by according to [9].

3. Results

The entrapment of chicken erythrocyte nuclei into liposomes was demonstrated by fluorescence microscopy using ethidium bromide stained nuclei. The free and entrapped nuclei differed in the intensity of red fluorescence and the entrapped nuclei were surrounded by a green lipid layer. The use of the phospholipid mixture, described in section 2.3., always resulted in 95–100% entrapment of nuclei. The explanation of this unusually high efficiency of entrapment might be that the presence of nuclei during the preparation of liposomes facilitated the formation of large vesicles and also the aggregation of small ones around the nuclei.

To introduce nuclei into CHO/Pro$^-$ cells, liposomes loaded with stained nuclei were incubated with cell monolayers (2–4 nuclei/cell) for 60 min. The cells were then washed free of unfused liposomes and examined under fluorescence microscope. As seen in fig.1, stained nuclei were taken up by cells indicating that spontaneous fusion of liposomes with cells occurred. This was a fairly frequent event since in repeated experiments it was found that 20% of the cells contained stained nuclei.

In further experiments unstained chicken erythrocyte nuclei were used to demonstrate transformation of CHO/Pro$^-$ cells to Pro$^+$ phenotype. 8×10^6 Cells grown on 140 mm plastic petri dishes were treated with 8×10^6 entrapped nuclei. After 60 min incubation, non-selective medium (BOB MEM/FCS, pro) was added to the cells and 24 h later the medium was changed to selective, proline-free BOB MEM supplemented with 10% dialysed FCS. In a 4 week incubation period Pro$^+$ cells grew into colonies while Pro$^-$ cells detached and died. Pro$^+$ colonies arose at a frequency of $3.75–6.25 \times 10^{-5}$. Under the same conditions, treatment with empty liposomes or nuclei did not result in higher transformation frequency than the spontaneous reversion, which ranged between $6.2–10 \times 10^{-7}$. We suggest that the increased number of Pro$^+$ cells was due to transformation of Pro$^-$ hamster cells by chicken erythrocyte nuclear material.

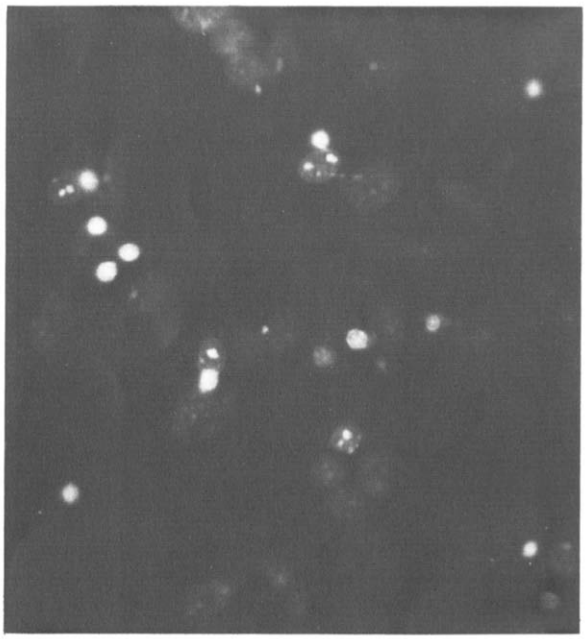

Fig.1. CHO/Pro$^-$ cells after fusion with ethidium bromide-stained chicken erythrocyte nuclei entrapped in liposomes.

Dependence of the number of Pro$^+$ colonies on the dose of loaded liposomes was tested by treating 1.5×10^6 cells with entrapped nuclei at different concentrations. As seen on fig.2 the number of Pro$^+$ colonies per dish increased with the increasing number of loaded liposomes between 0.3–4 of multiplicity (nuclei entrapped in liposomes/cell). Higher amounts of loaded liposomes seemed to be toxic for the cells.

For further analysis Pro$^+$ colonies were ring isolated [10] from petri dishes containing <5 colonies/dish. Only one colony was picked from each dish and the cells were grown in proline free medium for at least 100 generations.

Karyotypic analysis of six Pro$^+$ cell lines with G banding [11] showed a chromosomal pattern characteristic to CHO/Pro$^-$ cells and did not reveal any intact chicken chromosome or identifiable fragment.

According to [9] the number of functioning proline genes can be tested by determining the level of

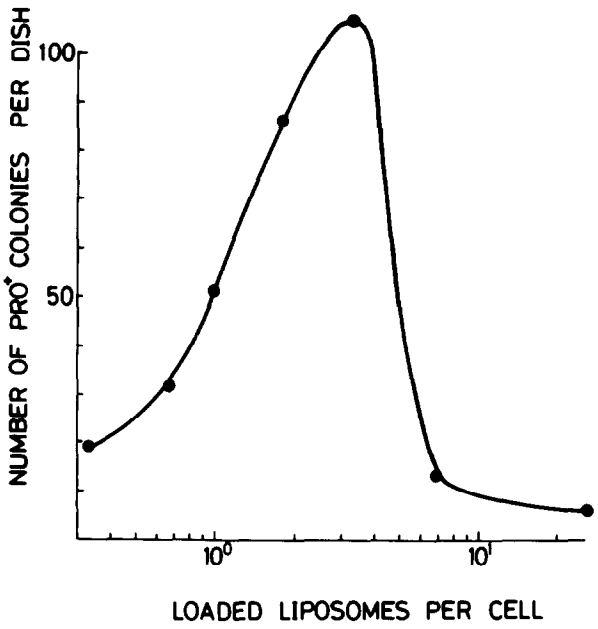

Fig.2. The number of arising Pro$^+$ colonies was in correlation with the applied dose of loaded liposomes/cell. Above a certain ratio cells were negatively affected by the complex events of treatment. From the total number of Pro$^+$ transformant colonies we subtracted the sum of the numbers of Pro$^+$ colonies arisen by treatment with empty liposomes, free nuclei and by spontaneous reversion. Treating 1.5×10^6 cells/dish with 5×10^6 liposome-entrapped nuclei, non-entrapped nuclei or empty liposomes the actual number of arising Pro$^+$ colonies were 114, 2.6 and 0.8/dish, respectively. Untreated cultures yield 1.6 spontaneous revertants/dish.

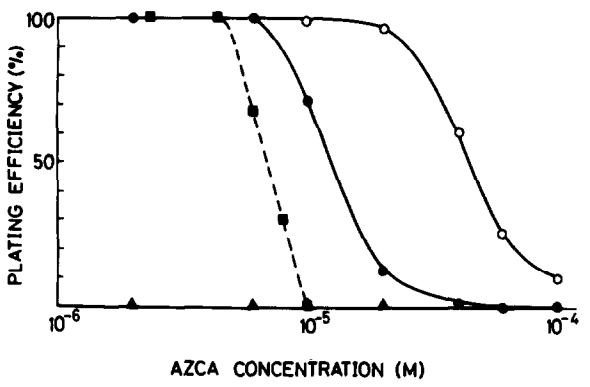

Fig.3. AZCA resistance of Pro$^+$ and Pro$^-$ cell lines. 6.9, 18 and 31 μM AZCA allowed 50% relative plating efficiency of CHO or CHL cells with 1, 2 and 4 active proline genes, respectively [9]. None of our clones showed resistance identical with that of the spontaneous revertants (1 active proline gene/genome) (■). Five Pro$^+$ cell lines (●) showed resistance to 13 μM and one (○) to 45 μM AZCA. Pro$^-$ cells (▲) had zero plating efficiency.

resistance of cells to a proline analoque, AZCA. This biochemical titration was carried out with six Pro$^+$ cell lines as well as the parental Pro$^-$ cells (fig.3). The AZCA concentrations required to decrease relative plating efficiency to 50% were 1.3×10^{-5} M in the case of 5 transformants and 4.5×10^{-5} M for one Pro$^+$ cell line. These values would correspond to 2 and 4 active hamster proline genes, respectively, according to [9].

4. Discussion

We demonstrated that isolated chicken erythrocyte nuclei can be entrapped in liposomes. The liposomes, as a consequence of their lipid composition, fused spontaneously with mammalian cells, allowing efficient transfer of erythrocyte nuclei into hamster cells.

The entrapped nuclei retained at least part of their biological activity, since Pro$^+$ cell lines appeared at an increased frequency depending on the concentration of liposomes. The frequency of transformation ($6/10^5$ cells) was higher than the frequency obtained with isolated metaphase chromosomes ($1/10^6$–10^8 cells) and about the same as that obtained with lipochromosomes ($1/10^5$) [3].

Karyotyping with G banding did not reveal the presence of any chromosomes or fragments of chicken origin. This was not surprising because in transformation studies with lipochromosomes [3] or in most cases with isolated metaphase chromosomes [12] no foreign chromosomal fragment was detected. However, the level of AZCA resistance suggests 2 or 4 functioning proline genes/hamster genome in the selected Pro$^+$ cell lines.

Since this procedure utilizes complete genome for transformation, and fusion takes place spontaneously, it might be considered as an improvement of cell fusion techniques, overcoming the disturbance of cytoplasmic elements (of one of the parent cells) and the introduction of other factors (viral proteins and nucleic acids, polyoxyethylene) used to provoke cell fusion.

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