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Flow Cytometric Analysis of Liposome-Nuclei Interaction: Transfer and Intranuclear Release of Carboxyfluorescein

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Flow cytometric analysis of the transfer of liposome-encapsulated carboxyfluorescein to isolated rat liver nuclei indicated that the fluorophore is actively taken up in this form, while negligible amounts are transferred when the dye is free in the reaction medium. The kinetic analysis of the uptake indicated a time- and dose-dependent reduction of the slope in the absence of transport saturable sites on the nuclear surface and of quenching phenomena. The comparison between entire and membrane-deprived nuclei demonstrated that the initial rate of uptake was higher in the absence of the complete nuclear envelope. The intranuclear binding sites were considered on the basis of the fluorescence distribution and of quantitative estimates of the amount of linked dye. The possibility of employing flow cytometry to monitor the interactions between liposomes and isolated nuclei by means of a fluorescent probe is discussed.

Key terms: Isolated rat liver nuclei, phospholipids, multiparameter analysis

Lipid vesicles, first used as model membranes, have been increasingly employed as a powerful technique for introducing biologically active materials into cells (7). Meanwhile, the interactions between liposomes and cells have been widely investigated, and much evidence is now available concerning the mechanisms involved (15).

In contrast, little attention has been devoted to the possible interactions between vesicle phospholipids and isolated subcellular constituents, which are significant in regard to the effects that alterations of the lipid content can exert on the cell. By employing isolated nuclei, it has been demonstrated that the incubation with phospholipid vesicles affects the endogenous transcriptional activity and the arrangement of the chromatin (10,12).

These data, while indicating a possible involvement of phospholipids in the control of gene structure and function, suggest that, although liposomes meet many of the criteria of an ideal carrier system, vesicle-derived lipids and/or their degradation products might induce changes of some cellular metabolic pathways.

In a preliminary report, we have shown that the modifications induced by phosphatidylcholine (PC) vesicles on isolated rat liver nuclei in terms of RNA synthesis, nuclease accessibility, and ultrastructure are not due to changes of the medium but depend on the actual interaction between vesicles and nuclei (16).

In this paper, we analyze by flow cytometry the mechanism of interaction of liposomes with nuclei by means of a fluorophore transfer technique. Specifically, we employ isolated rat liver nuclei and PC unilamellar vesicles containing quenched concentrations of carboxyfluorescein (CF).

MATERIALS AND METHODS

Liver nuclei were prepared as described by Widnell and Tata (20) from male adult Sprague-Dawley rats (120–150 g). Membrane-depleted nuclei were obtained by adding 0.1% Triton X-100 to the sucrose density buffers. The CF (Eastman Kodak, Rochester, NY) was purified by ethanol crystallization and gel filtration through a hydrophobic resin. Briefly, the material—precipitated by ethanol-water, dessicated, and made up to 250 mM in water at pH 7.4—was applied to a 0.75-×-50-cm column of Sephadex LH-20 (Pharmacia, Uppsala, Sweden), equilibrated and eluted with distilled water.

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One-milliliter fractions were collected and analyzed for purity on silica gel plates developed with chloroform-methanol-water, 65:25:4. The purified material was then pooled and adjusted to pH 8.0 and 100 mM in 50mM Tris-Cl, on the basis of the extinction coefficient at 492 nm (17).

Preparation of the Vesicles

Small unilamellar CF-containing vesicles (CF-SUV) were obtained by probe sonication of a 50 mg/ml suspension of PC (Type VII-E, Sigma Chemical Co., St. Louis, MO) in 50 mM Tris-Cl, pH 8.O containing 100 mM purified CF, with a Branson sonifier B15 operating intermittently at 60 W and 22 kHz under nitrogen stream, in an ice bath. Sonication was continued until the suspension of vesicles was optically clear.

The sonicated material was then passed through a 0.75- × -50-cm column of Sephadex G-50 coarse (Pharmacia, Uppsala, Sweden) equilibrated and eluted with 50 mM Tris-HCl, pH 8.0 for separating the nonentrapped CF. The elution of the vesicles was monitored spectrophotometrically at 300 nm. The fraction corresponding to the peak value of the turbidity was employed for the incubation of the nuclei and for the assay of the lipid-CF ratio by means of phosphate analysis (1) and fluorimetric determination of the dye content.

Empty vesicles were obtained under the same conditions in the absence of CF. The vesicle preparations were characterized by electron microscopy after negative staining with 1% uranyl acetate and by evaluating the ratio between lipid phosphorus and turbidity at 300 nm as an estimate of their homogeneity (13).

Incubation Conditions

In a typical experiment, 15×10^6 nuclei were incubated at 37°C for the indicated times under the conditions employed for studying the transcriptional and structural response of rat liver nuclei to phospholipids (3,4,11). The mixture contained, in a final volume of 300 μl, 25 mM Tris-HCl pH 8.0, 150 mM KCl, 1.5 mM DTT, 5 mM Mg acetate, 1 mM $MnCl_2$, and the given amounts of PC-entrapped CF. To evaluate the transfer of free CF possibly leaked out from the liposomes during incubation, blank samples consisted of the same composition except for the liposomes and the dye, which were empty vesicles and free CF. The concentration of free fluorophore added ranged from 0.07 to 21.3 μM, corresponding to the input amounts of entrapped dye, based on the assumption that all the vesicle content could be released in the medium. However, since higher local concentration of CF could take place in liposome-nuclei interactions, owing to the limited volume of the reaction mixture, some experiments have been performed with extremely high concentrations of free CF (1 mM).

During some kinetic studies, the standard samples were observed before and after a threefold dilution in Tris buffer. Some cytometric readings were also performed by splitting the sample in two aliquots, and while one was double-washed in medium at 0–4°C as reported, the other was blocked in ice bath and run on the cytometer immediately and after a 10-min incubation at 0°C.

Quantitative Fluorimetry

Quantitative assays of the fluorophore content were done on both freshly prepared CF SUV and on nuclei incubated with free and vesicle-entrapped CF, with a Perkin-Elmer MPF-31 Fluorescence Spectrophotometer (exc = 495 nm, em = 525 nm) operating with 2-mm pathlength cuvettes. The amount of CF encapsulated by the vesicles was determined after lysis with 2% Triton X-100. The amount taken up by the nuclei was estimated before and after treatment with various percentages of Triton X-100 on samples washed twice with 1 ml of reaction mixture. The intranuclear concentration of CF was calculated, taking into account the mean nuclear volume determined on 800× phase contrast micrographs processed with a Zeiss IBAS I semiautomatic image analyzer.

Flow Cytometric Measurements

The flow cytometric analyses were performed at the end of the incubation times on double-washed nuclei with a FACS IV system (Becton Dickinson, Mountain View, CA). The CF was excited by using 300 mW of 488-nm light (argon ion laser 164-05, Spectra Physics, Mountain View, CA). The forward scatter signal was injected in P1 and P4 signal inputs in order to use it for both gating and analysis. Fluorescence and orthogonal scatter emission were collected by focusing at 90° relative to the intersection of the stream with the incident laser light. Ten percent of the orthogonal light was reflected at 90° by an all-wavelength mirror (10:90), passed through a 488-nm bandpass filter into the photomultiplier tube at 500 V (EMI 9924A, EMI Industrial Electronics, U.K.), and the green light was passed through a 520 ± 15-nm bandpass filter into the photomultiplier tube for green fluorescence (EMI 9924A) at 600 V.

This optical combination allowed a multiparameter characterization of each nucleus, on the basis of forward and orthogonal scatter, and green fluorescence. The system was also fitted with a 545-nm longpass filter to analyze the fluorescent signal emitted by CF molecules over the region of absorption of the dye in order to exclude the possible interference between the curve of absorption and the curve of emission of the dye. Since the two systems gave the same results, the fluorescent signal was not affected during the travel from the stream to the photomultiplier tube.

Forward and orthogonal scatter were used for gating the diploid and tetraploid nuclei of parenchymal cells, excluding from the fluorescence analysis the nuclei of endothelial, Kupffer, fat-storing, and pit cells.

The integrity of the nuclei was evaluated according to the similarity of the scatter signal obtained from the CF-treated samples and the controls.

Optical Microscopy

Phase contrast and green fluorescence photographs were obtained with a Zeiss Photomicroscope III equipped with phase optics and epillumination.

Freeze Fracture Electron Microscopy

The nuclear samples, incubated for 20 min with CF-SUV, were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 1 h at 4°C, and pelleted at 700g for 10 min. They were cryoprotected with 25% glycerol in saline for 1 h and then frozen in liquid Freon 22 at −150°C. Fracturing and replication were done with a Balzers 360 M unit.

RESULTS

Both empty and CF-containing vesicles employed in the incubations with nuclei corresponded to the peak fraction of the Sephadex G-50 elution profile, and were eluted shortly after the void volume. They were a homogenous population in terms of the number of layers and size, as indicated by the electron microscopic analysis after negative staining and by the linearity of the absorbance at 300 nm versus the lipid phosphorus content (data not reported) (13).

The integrity of the nuclei, determined on the scatter signal, was higher than 95% throughout the experiments. According to what was reported for cells (9,14), this parameter was estimated by considering that differences in the scatter signal between intact and damaged nuclei are mainly dependent on the physical alteration of the surface and on the change in the internal refractive index.

The intranuclear distribution of CF was not uniform, as shown in Figure 1. In both complete and membrane-deprived nuclei, the dye was mainly located on the nucleoli, while the rest of the nucleus showed a dim level of fluorescence. No extranuclear fluorescence was evident, suggesting that adherent vesicles contained quenching concentrations of CF. The ultrastructural analysis indicated that the nuclei were partly surrounded by the liposomes, some of which showed fusion figures with the nuclear surface (Fig. 2).

The flow cytometric analysis showed three main peaks in the scatter distribution. This reflects the different types of nuclei derived from hepatocytes and from endothelial, Kupffer, fat-storing, and pit cells (Fig. 3). After sorting and analysis of morphology and DNA content, peaks 1a and 1 were shown to correspond to diploid nuclei and peak 2 to tetraploid nuclei. In addition, peak 1a was caused by nuclei showing the same DNA content of peak 1 but smaller size, and was conceivably from nonparenchymal cells (8). The system was then gated to examine only parenchymal nuclei, which fell in the second and third peaks.

This two-peak scatter profile was essentially identical for all the tested conditions and overlapped the fluorescence histogram of the samples incubated with CF-SUV, indicating that all nuclei from each population had taken up the dye in an amount proportional to the area of the nuclear surface (Fig. 4). In contrast, the fluorescence

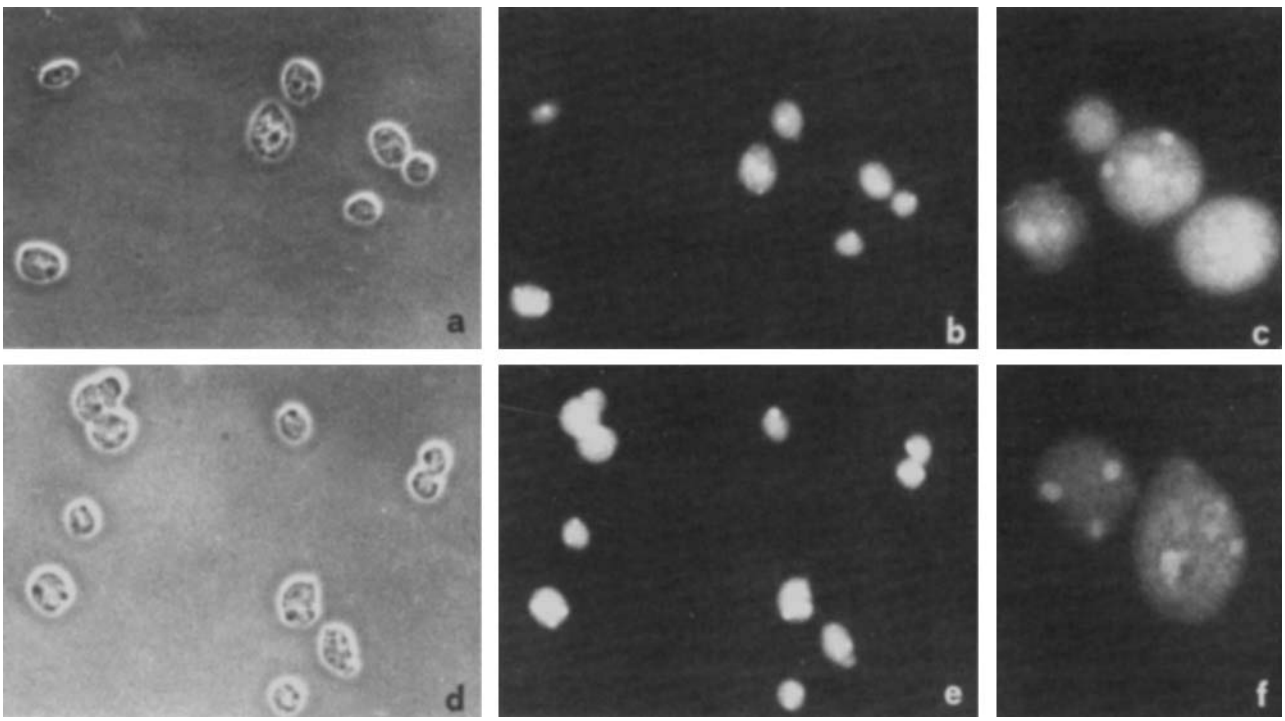

FIG. 1. Phase contrast (a,d) and fluorescence (b,c,e,f) light micrographs of entire (a–c) and membrane denuded (d–f) rat liver nuclei after 20 min of incubation with 6.4 nmol of vesicle-entrapped CF. Magnification: 800×, except c and f: 2,500×.

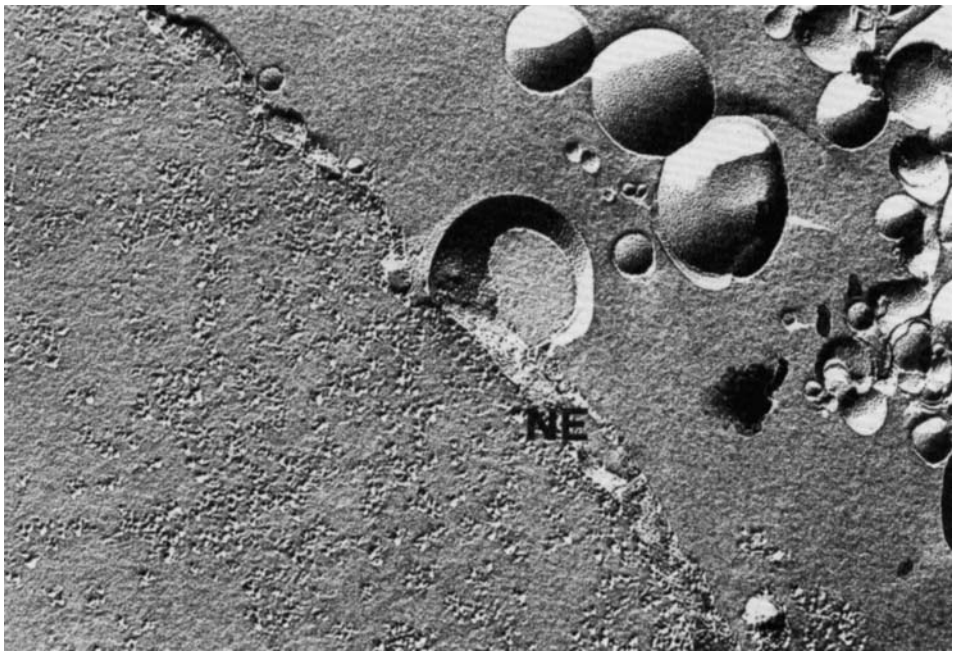

FIG. 2. Freeze fracture replica of CF-SUV-incubated nuclei showing a detail of the interaction between the liposomes and the nuclear surface. A vesicle appears to fuse with the outer layer of the nuclear envelope (NE). Magnification: 60,000×.

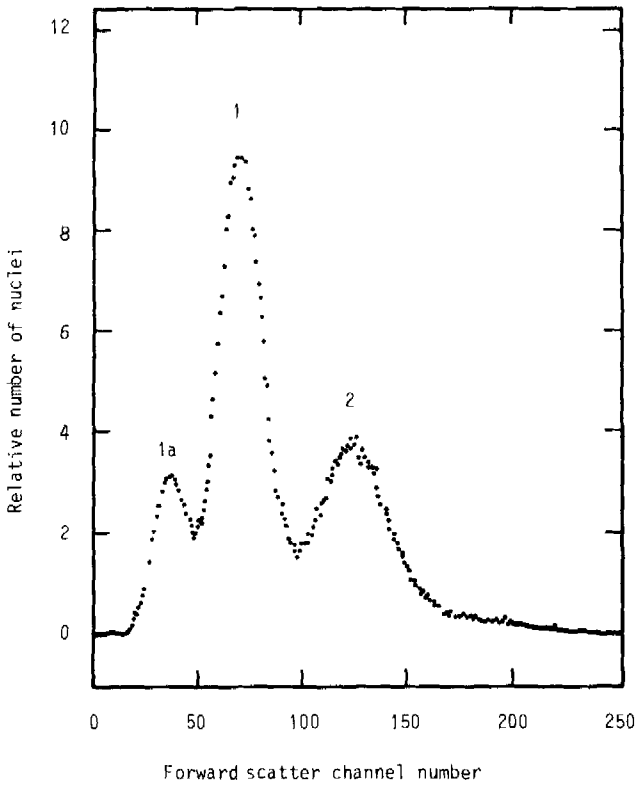

FIG. 3. Light scatter frequency distribution of nuclei isolated from rat liver and incubated for 20 min in the presence of 6.4 nmol of liposome-entrapped CF. Peaks 1 and 2 correspond respectively to diploid and tetraploid parenchymal nuclei.

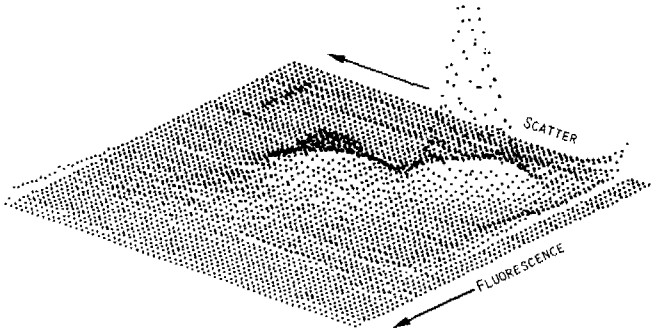

FIG. 4. Scatter and fluorescence dual-parameter histogram of diploid (right peak) and tetraploid (left peak) parenchymal liver nuclei incubated for 20 min in the presence of 6.4 nmol of vesicle-entrapped CF. The analyzing system was gated to exclude nonparenchymal nuclei.

content of blank samples was negligible, showing that the spontaneous transfer of CF, by means of diffusion of the dye dissolved in the medium, was a minor event even for high concentration of free CF (Fig. 5). The addition of an extra volume that diluted the medium did not give any reduction of fluorescence (data not reported). Moreover, no differences were detected between samples not washed and the double-washed ones, even when comparing these samples with those left at 0–4°C for 10 min, showing that PC vesicles interact with nuclei releasing their content only if they are incubated at 37°C, as previously reported for cell-liposome interaction (18).

The fluorescence frequency histograms, which were obtained by gating the diploid population of nuclei

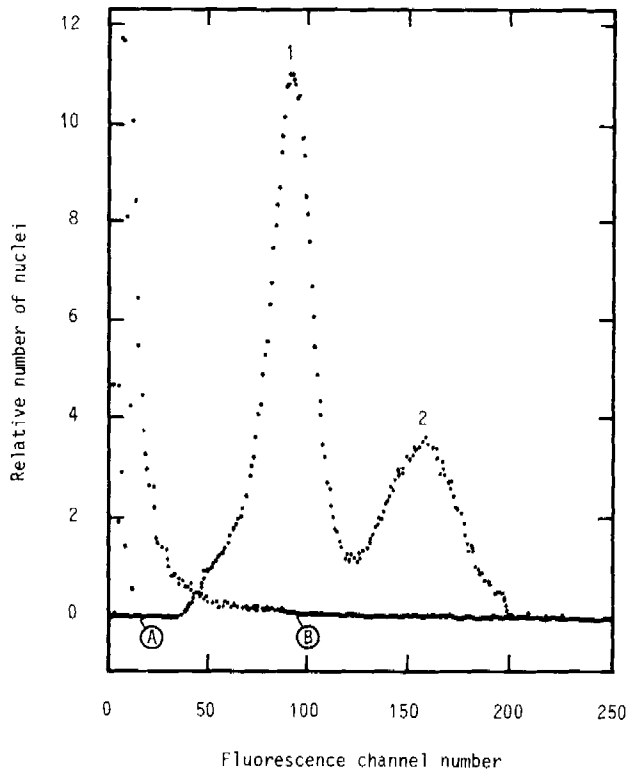

FIG. 5. Fluorescence frequency histograms of nuclei incubated with the same amount of liposome-entrapped (A) and free (B) CF. The nuclei were incubated for 20 min either with 6.4 nmol of vesicle-encapsulated CF or in the presence of 21.3 μM free CF, corresponding to the concentration reached by assuming that all the vesicle content would have been released in the reaction medium. Peaks 1 and 2, respectively, represent diploid and tetraploid nuclei.

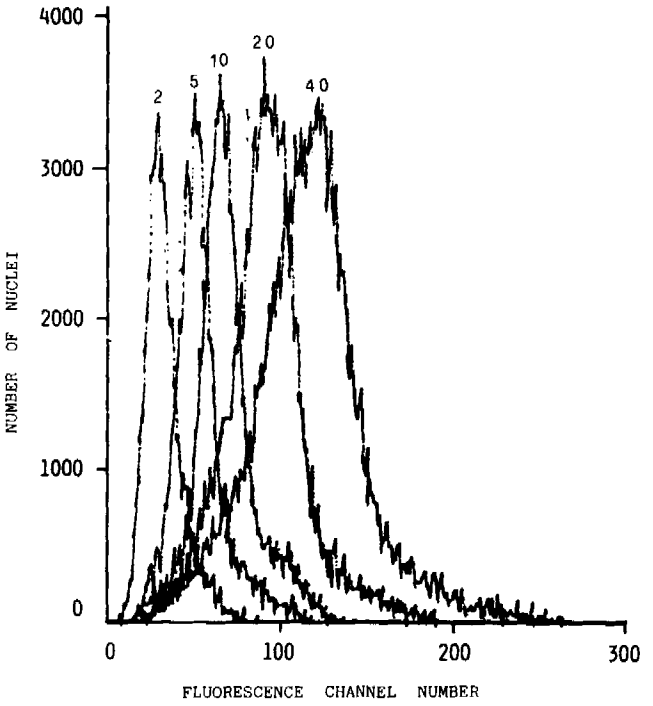

FIG. 6. Fluorescence frequency histograms of rat liver nuclei incubated with 6.4 nmol of liposome-encapsulated CF for times ranging from 2 to 40 min. The system was gated in order to analyze only diploid nuclei.

treated with CF-SUV, indicated that the amount of fluorescence associated with the nuclei rose with the incubation time (Fig. 6). This increase was not directly time-dependent, as shown in the diagram obtained by plotting the fluorescence peak values at different times of incubation, which revealed a time-proportional increase of the uptake of the dye during the first 5 min of incubation and progressive reduction of the uptake after this time (Fig. 7).

The significance of the nuclear membrane in the CF transfer mechanism was estimated by comparing the kinetics of uptake of the dye into complete and membrane-deprived nuclei. The CF incorporation occurred faster in peeled nuclei during the first ten minutes of incubation. Then the slope of the curve decreased drastically after 20 min, and the fluorescence values of both conditions tended to overlap (Fig. 8).

Since the path of the fluorescence was qualitatively similar in both types of nuclei, the factors responsible for the slope reduction and plateau formation were then studied only on complete nuclei.

The presence of saturable sites was primarily approached by analyzing the kinetics of uptake as a function of the different amount of CF-containing vesicles. The CF uptake was a direct function of the vesicle input

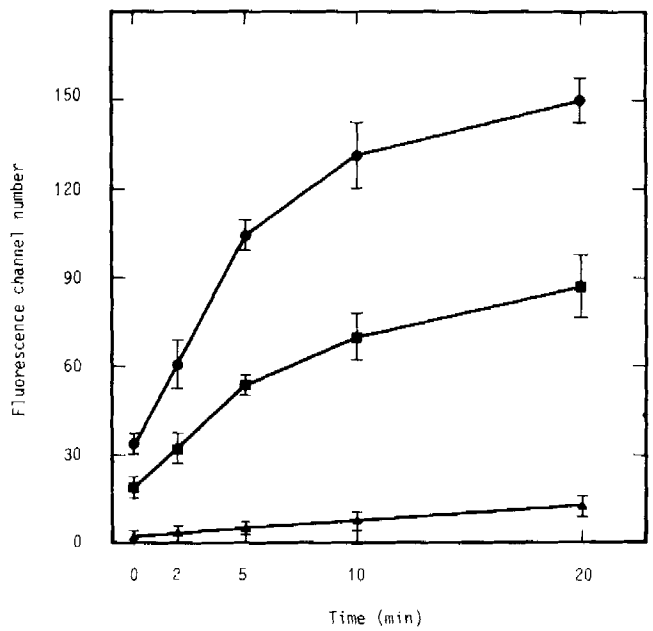

FIG. 7. Kinetic analysis of CF transfer to isolated rat liver nuclei. The data indicate the peak fluorescence channel number of diploid (peak 1, ■—■) and tetraploid (peak 2, ●—●) nuclei incubated with 6.4 nmol of vesicle-entrapped CF. The lower line (▲—▲) refers to the uptake occurring with the same input amount of CF added free to the incubation mixture (21.3 μM). Mean values from three determinations ± SD.

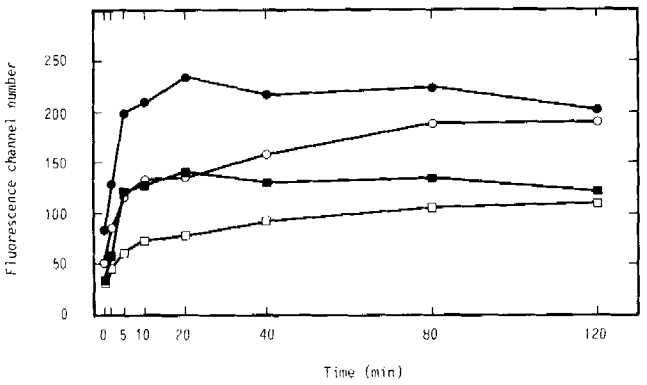

FIG. 8. Time-dependent uptake of CF by entire and membrane-deprived rat liver nuclei. Ordinate values are the peak fluorescence channel number of diploid (□—□, entire; ■—■, peeled) and tetraploid (○—○, entire; ●—●, peeled) nuclei incubated with 6.4 nmol of liposome-entrapped CF.

up to 30 μM lipid, while over this concentration the slope tended to decrease (Fig. 9). The existence of surface saturable sites influencing the uptake over 30 mM lipid was then investigated by preincubating the nuclei with empty vesicles for different lengths of time and then incubating each sample for 20 min with CF-SUV. The amount of dye taken up was essentially independent of preincubation with empty vesicles (Fig. 10).

The occurrence of self-quenching phenomena as the cause of the decrease of fluorescence was assessed by determining the amount of dye contained in the nuclei at the end of the incubation time. The value obtained for the nuclear sample incubated with 600 μM lipid was 12.8 μM CF, largely below the level at which self-quenching starts. This fluorescence represents the total nuclei-associated dye, including also the contribution

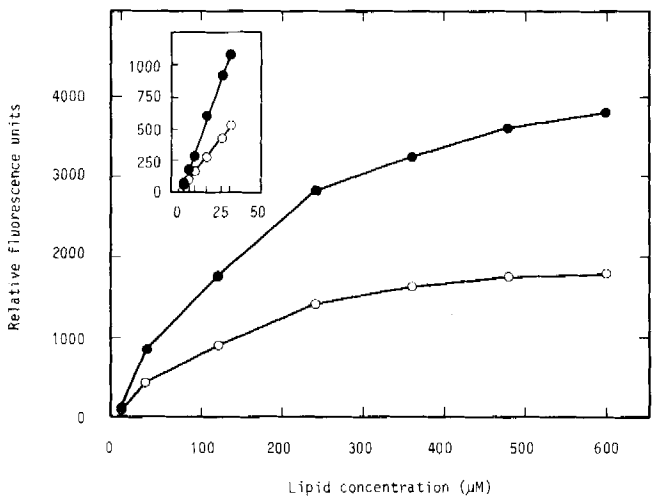

FIG. 9. Dose-dependent uptake of fluorescence by rat liver nuclei incubated with different amounts (2–600 μM lipid, containing 0.02–6.4 nmol of CF) of CF-SUV for 20 min at 37°C. The fluorescence peak values of diploid (○—○) and tetraploid (●—●) nuclei represent the mean of three determinations with a SD below 12%. The insert shows the fluorescence uptake occurring up to 30 μM lipid.

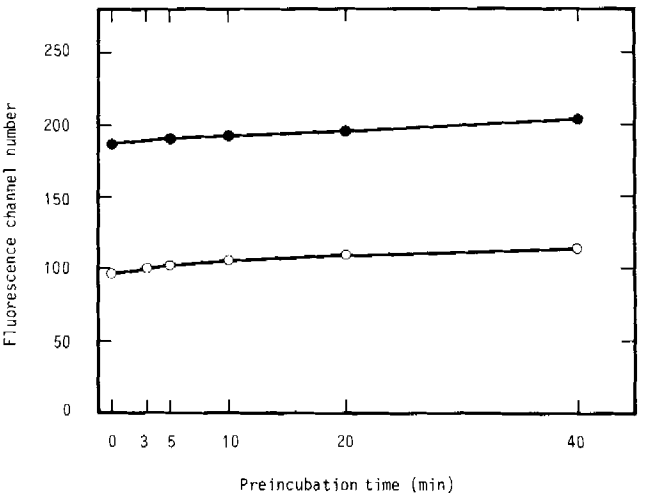

FIG. 10. Influence of preincubation with empty vesicles on the uptake of vesicle-entrapped CF by diploid (○—○) and tetraploid (●—●) rat liver nuclei. The nuclei were preincubated in the presence of 600 μM empty PC vesicles for the indicated times, then 6.4 nmol of liposome-entrapped CF was added and they were allowed to stand at 37°C r an additional 20 min.

due to the tightly bound vesicles, and thus reflects an overestimation of the amount of dye actually contained in the nuclei (2).

An estimate of the amount of CF bound to the inner nuclear components was made by washing the nuclei with 0.1% Triton X-100 after a standard 20-min incubation with CF-SUV. This treatment resulted in the loss of about 80% of the fluorescence with respect to samples washed in the absence of detergents. To ensure the complete solubilization of the nuclear membrane, besides the disruption of the adherent vesicles, some samples were washed with 0.5% Triton. Under this condition, the residual fluorescence was about 10%. Since no further decrease was revealed after additional detergent treatment, this value can be considered as the amount of CF tightly bound to the inner nuclear compartment (Table 1).

DISCUSSION

The CF transfer technique we have employed is a slight modification of that originally described for liposome-cell interactions (2). As indicated by fluorescence microscopy, CF shows a high binding affinity for nucleoli, and also for some other nuclear components. This suggests that the dye, owing to the presence of two carboxyl groups, can presumably bind to nuclear cationic residues, similarly to many polyanions (5).

The uptake of liposome content or components by isolated nuclei might take place with different mechanisms, according to the models currently envisaged for intact cells, i.e., endocytosis, fusion, lipid exchange, and stable absorption (15). Nuclear pore complexes might be also involved, but the transfer of entire vesicles appears to be ruled out by the small functional radius of the pores (6). Among these mechanisms, fusion, or "fusion-like" phenomena for detergent-treated nuclei, might

Table 1
Fluorimetric Determinations of the Carboxyfluorescein Associated to Rat Liver Nuclei Under Different Conditions[a]

Conditions	Relative fluorescence −Triton	+Triton	+Triton:−Triton
CF-SUV nuclei[b]	46	53	1.15
Free CF nuclei[c]	0.6	0.7	1.17
Low detergent nuclei[d]	9.2	9.4	1.03
High detergent nuclei (first wash)[e]	5	5.1	1.02
High detergent nuclei (second wash)[f]	4.9	4.7	0.96

[a]Measurements were done on nuclei either treated or not with 2% Triton X-100, and disrupted by probe sonication for 2 min in an ice bath.
[b]Nuclei incubated with 6.4 nmol of liposome entrapped CF for 20 min as described in "Materials and Methods," and washed twice with the reaction mixture.
[c]Nuclei incubated in the presence of 21.3 μM free CF as in footnote b.
[d]Nuclei as in footnote b, but washed twice with incubation mixture containing 0.1% Triton X-100.
[e,f]Nuclei as in footnote d, further washed once or twice with reaction mixture containing 0.5% Triton X-100.

represent one of the main modes of interaction, on the basis of the fusion capability demonstrated by freeze-fracture electron microscopic observations. The experiments with different concentrations of free CF and unloaded vesicles demonstrate that the transfer into nuclei could occur only for liposome-entrapped and not for free dye. On the other hand, the simple diffusion of CF can be excluded also by the evidence that, upon the dilution of the incubated sample, no reduction of the fluorescence level, which could account for potential gradient across the nuclear membrane, was observed.

The kinetic of the transfer of CF by SUV to nuclei presents a linear pattern within the first 5 min, while a progressive reduction takes place afterward. This behavior is consistent with a two-step mechanism in which, at first, a fraction of all vesicles establish physical collision contacts with nuclei, releasing their content, while the following processes of dye transfer are partly impaired by the presence of adhering vesicles (19). This is further confirmed by the observed linear relations between vesicle concentration and CF uptake by nuclei until 30 μM lipid after standard 10-min incubation time, followed by a progressive reduction of the uptake at higher lipid concentration.

The use of peeled nuclei provides a model in which the number of factors involved is reduced in comparison to complete nuclei. With membrane-deprived nuclei there was a quicker and higher dye incorporation, probably because the liposomes leak upon the interaction with the peripheral lamina proteins, as suggested for liposome cell interactions (19).

The independance of the CF uptake from the preincubation time with empty vesicles suggested that no saturable sites exist in the nuclear surface limiting vesicles-nuclei interactions, as reported for liposome-cell models (11). Self-quenching phenomena were excluded by showing that the intranuclear concentration of CF, even at the maximum lipid input, was largely below the level at which self-quenching occurs. However, it must be considered that the CF distribution is not uniform, so that in some regions, like the nucleoli, the perinuclear cisterna, the proximity of the bound or entrapped dye molecules might affect their fluorescent emission.

In conclusion, the complexity of the system suggests that multiple events take place. As to the intranuclear compartment, the factors likely to be involved are related to the specific binding affinity and to the topological distribution of CF, eventually capable of producing local self-quenching. At the nuclear boundary, it can be assumed that the interactions between liposomes and nuclei are governed by the collision kinetic and by the stable or transient vesicle-nucleus contacts in a highly dynamic fashion, as indicated by the absence of surface saturation. Our data indicate that PC vesicles transfer their content to the inner nuclear compartment of both entire and membrane-deprived nuclei. These results are consistent with previous observations concerning the uptake of labeled PC molecules inserted in the bilayer of liposomes incubated with nuclei (12), and support the possibility that exogenous phospholipids can enter the nuclei and modify the lipid nuclear content. In addition, while confirming the feasibility of the model of nuclei-liposomes as an in vitro system for studying the effects of phospholipids on nuclear structure and function, our results stress the usefulness of flow cytometry as a rapid and dependable technique that can also be easily adapted to isolated nuclei and is capable of giving precise information on the scatter and fluorescence pattern of each particle.

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