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Interaction of negatively charged liposomes with nuclear membranes: adsorption, lipid mixing and lysis of the vesicles

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Fluorescence energy transfer studies reveal that negatively charged lipid vesicles interact with nuclei from mouse liver cells. This interaction was observed with charged lipid vesicles composed of PA or PS but not with the uncharged PC or PE:PC vesicles. The vesicles were prepared by bath sonication and contained either a fluorescent marker in the lipid bilayer or in the vesicular interior. The negatively charged vesicles showed an adsorption to the nuclear membrane visible by fluorescence microscopy. The results obtained by resonance energy transfer experiments are interpreted in terms of a mixing of the lipids from the vesicles with the nuclear membrane. Encapsulation studies documented a staining of the nuclei only if the dye molecules of high or low molecular weight were encapsulated inside negatively charged vesicles. As consequence of the vesicle-nuclei interaction morphological changes on the nuclear surface became visible.

Introduction

Membrane fusions are widespread phenomena; of the many fusional processes the viral fusion to the host (see, for example, Refs. 1–8) and the fusion of uncoated vesicles to endosomes [9–13] are two important and intensively studied examples. In the light of the potential use of liposomes as carriers for drugs and DNA the fusion of liposomes or vesicles to plasma membranes have gained general attention. Studies on the endocytotic uptake of liposomes and on the intracellular release of the encapsulated material have been reviewed [14]. However, only very recently the intracellular fate of the liposomes has been addressed by model studies [15–19,33]. For an effective liposomal gene transfer the liposomes containing the encapsulated DNA should be directed towards the nuclear membrane and the targeted DNA should be freed into the nucleus. From this laboratory it was recently shown that a targeted (biologically active) DNA can be refound in the coated vesicles of liver cells shortly after the intraveneous administration of the DNA-containing liposomes into mice [20]. Liposomes carrying covalently bound lysozyme fuse with erythrocyte ghosts [21] and the nuclear membrane [22] at low

Abbreviations: PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; PS, phosphatidylserine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; Rh-DOPE, *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine; FITC-dextran, fluorescein isothiocyanate dextran.

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pH. As an extension of our studies on the fusion of negatively charged liposomes to uncoated vesicles which are described in the preceding communication [33] we here report on the interaction of negatively charged lipid vesicles with the nuclear membrane at neutral pH. The comparative studies with neutral and negatively charged lipid vesicles reveal that only negatively charged lipid vesicles are adsorbed on the nuclear membrane. The lipid molecules mix with the nuclear membrane. Encapsulaton studies reveal that low-molecular-weight substances enter the nucleus as consequence of the loss of the vesicle integrity. Encapsulated fluorescent dextrans of high molecular weight also seem to penetrate the nuclear membrane as function of the interactions described.

Experimental Procedures

Materials

Phospholipids were obtained from Lipid Products, Surrey, U.K. (phosphatidylserine from bovine brain, PS), and Sigma, St. Louis, U.S.A. (phosphatidylcholine (PC) from egg yolk, phosphatidic acid (PA) prepared from egg yolk lecithin, L-α-phosphatidylethanolamine (PE) from *Escherichia coli*, L-α-dioleoylphosphatidylcholine (DOPC)). *N*-7-Nitrobenz-2-oxa-1,3-diazol-4-yl) phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine (Rh-DOPE) were from Avanti Polar-Lipids Inc., Birmingham (AL) U.S.A. The lipids were dissolved in chloroform and stored under argon at $-70\,^{\circ}$C.

Fluorescein isothiocyanate dextran of molecular weight 150 kDa (FITC-dextran) and rhodamine-labeled dextran (Rh-dextran) were purchased from Sigma, St. Louis, U.S.A. Inorganic salts and sucrose were from E. Merck, Darmstadt, F.R.G. Sephacryl S-1000, Sepharose 4B, G-25 and LH-20 were from Pharmacia, Uppsala, Sweden. Bisbenzimide H 33258 was purchased from Calbiochem, San Diego, U.S.A.

Methods

The isolation of nuclei from mouse liver followed the protocol of Blobel and Potter [23] as modified by Peters [24]. The nuclei were used within the day of preparation. They were washed four times before use in 0.02 M Tris (0.02% NaN_3, pH 7.20). Examination under the microscope revealed a majority of intact nuclei. Protein concentrations were determined spectroscopically on the basis of $A^{1\%}_{280nm} = 10$ in 6 M guanidine hydrochloride [25].

Small unilamellar vesicles were prepared by a bath sonication of the lipid dispersion under nitrogen. The lipid concentration was usually 0.8 mg in 0.5 ml buffer (0.02 M Tris, 0.02% NaN_3 (pH 7.20) or 0.02 M sodium phosphate, 0.02% NaN_3 (pH 7.33)). For the phosphatidylethanolamine samples a PE:PC ratio of 1 : 1 was used.

The labeling of the liposomes with the (headgroup) fluorescent lipid analogs NBD-PE and Rh-DOPE are described in the preceding communication [33]. Bisbenzimide (BB) and fluorescein isothiocyanate dextran of molecular weight 150 kDa (FITC-dextran) were used as intravesicular markers and encapsulated by cosonication with the lipids. The extravesicular probe molecules were removed by gel chromatography on G-25 or Sepharose 4B-CI.

The mixing of the liposome components with the nuclear membrane was observed by induced changes of the fluorescence energy transfer between a fluorescent donor and acceptor. The lipid analogs NBD-PE and Rh-DOPE served as donor-acceptor pair [26]. The interaction of the lipid vesicles with the nuclei was further assayed by the incorporation of bisbenzimide or fluorescent dextran molecules into the vesicles and subsequent fluorescence microscopy. In all cases the adhesion of the labeled liposomes (if negatively charged) with the nuclei and the spreading of the fluorescence over the nuclei could be followed.

Fluorescent spectra were recorded in thermostated cuvettes in a FICA 55 MK II spectral fluorometer using the ratio mode. Nuclei were observed and photographed using a Leitz-microscope with fluorescence accessory.

Results

Fig. 1 shows the resonance energy transfer results obtained by mixing four kinds of lipid vesicles with nuclei. The nuclei were washed in the same buffer in which the NBD- and Rh-vesicles had been prepared. Similar to the results on uncoated

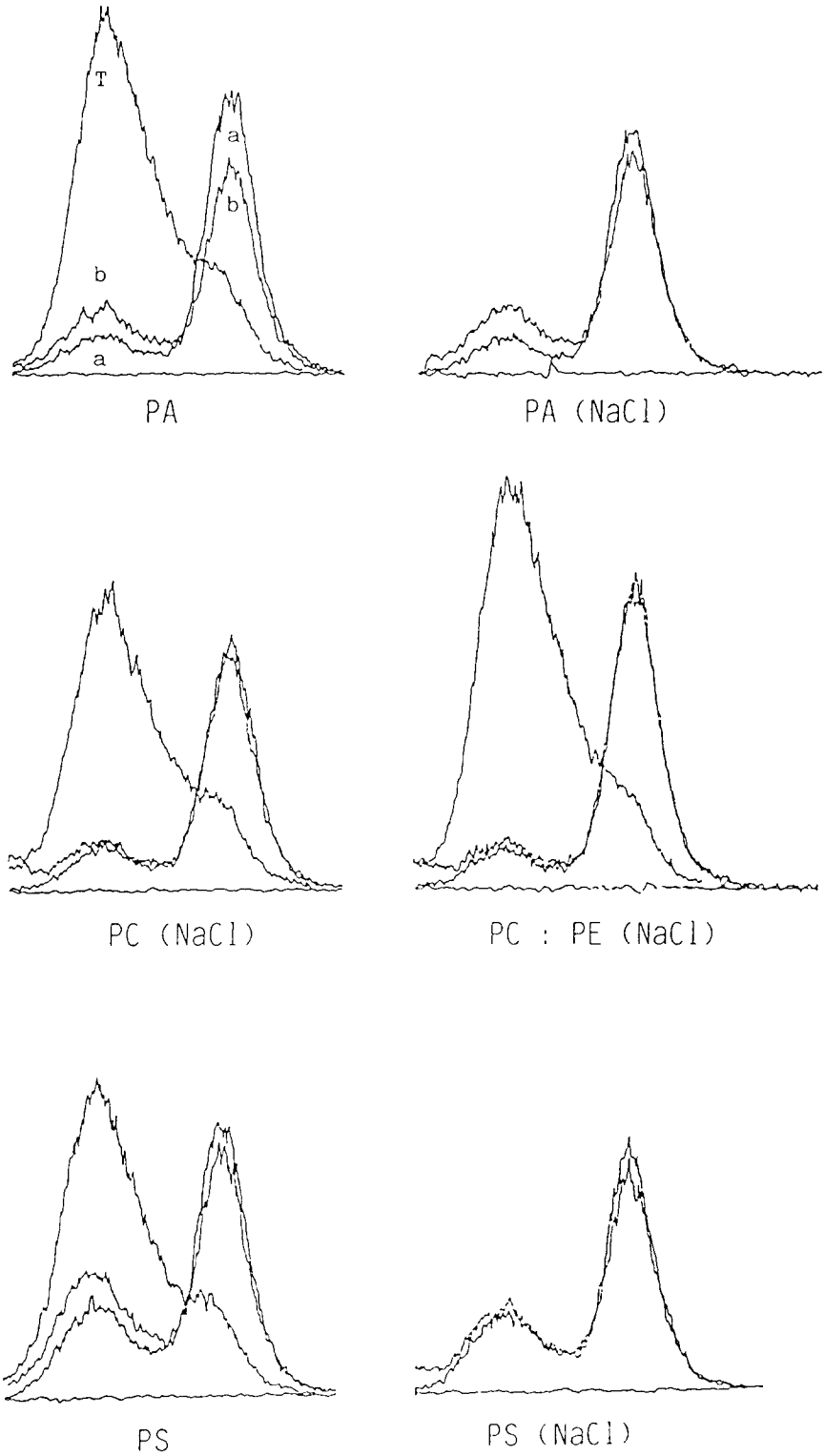


Fig. 1. Resonance energy transfer experiments on the fusion of lipid vesicles to nuclei. NBD-PE/Rh-DOPE labeled vesicles (10 μl + 0.5 ml buffer) were mixed with 10 μl concentrated nuclei in the same buffer. Finally Triton X-100 (final concentration 0.4%) was added. Spectra before (a) and after the addition of the nuclei (b) and in the presence of Triton (T), respectively. Excitation 445 nm, emission from 470 to 650 nm. Buffer either 20 mM Tris or 150 mM NaCl, 10 mM Tris, both at pH 7.20. The PC- and PC : PE spectra at low ionic strength were similar to those shown for high ionic strength. Room temperature.

vesicles described in the preceding paper [33] only vesicles composed of negatively charged lipids showed a loss of resonance energy transfer and therefore a mixing of the lipid bilayer with the membrane of the nuclear envelope. The kinetics of the membrane mixing or insertion is seen in Fig.

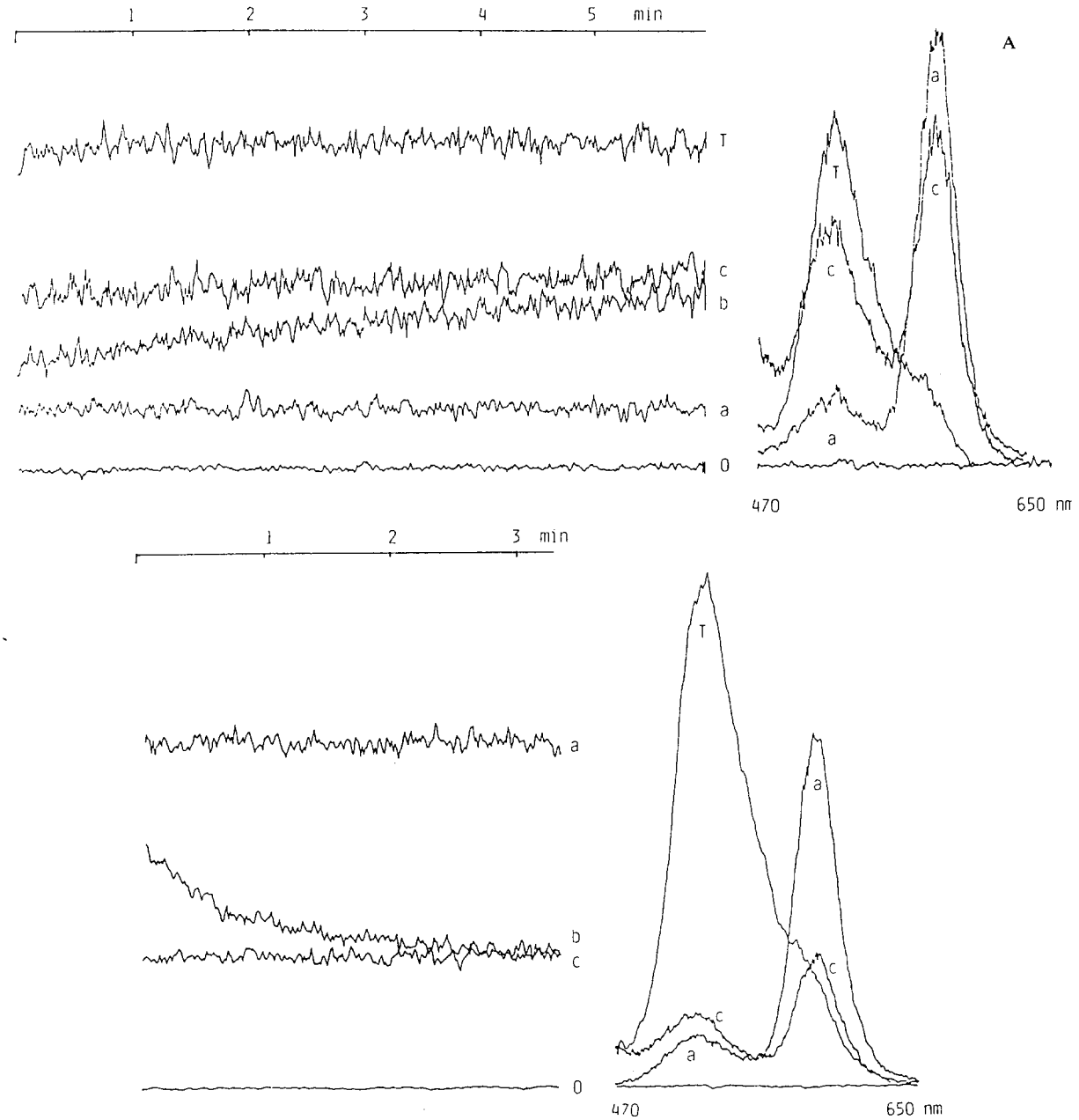


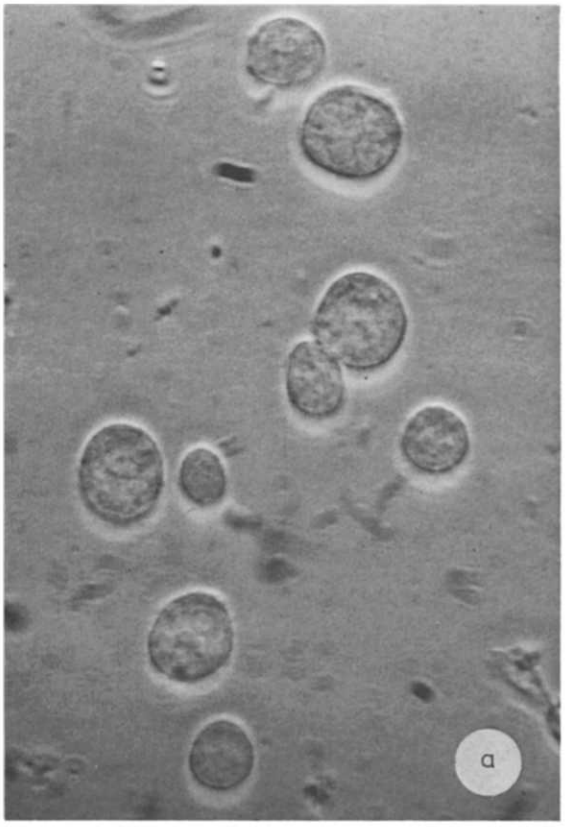
Fig. 2. Resonance energy transfer experiments on the fusion of PA vesicles to nuclei. (A) Excitation 445 nm. Left: time dependence, the increase of the NBD fluorescence at 525 nm is monitored; right: spectra from 470 to 650 nm. (a) 10 μl PA-NBD-PE/Rh-DOPE vesicles +0.5 ml buffer (150 mM NaCl, 10 mM Tris, (pH 7.20)); (b) after adding 10 μl concentrated nuclei; (c) continuation of (b); (T) after adding Triton X-100 (final concentration 0.4%) to (c); (0) zero-line. Room temperature. (B) New sample, same as in (A), except the Rh fluorescence was monitored during the time dependence. Excitation 445 nm, emission 588 nm or emission spectra from 470 to 650 nm.

2. At constant NBD-emission wavelength the time-dependent loss of the resonance energy transfer was monitored by the increase of the NBD-fluorescence signal (Fig. 2A). Though the initial increase could not be observed with conventional fluorescence spectrometers it is obvious that the reaction has come to an end after 3–5 minutes. Complementary experiments on the decrease of the Rh signal show the same trend in the time evolution (Fig. 2B).

The insertion of negatively charged vesicles into the nuclear envelope membrane was further studied by experiments in which the mixing of the intravesicular and the nuclei contents could be monitored. Bisbenzimide was intravesicularly incorporated into four sets of vesicles. The vesicle solutions were adjusted to the same initial fluorescence intensity and subsequently incubated together with the nuclei for 10 min. The nuclei then were well washed and observed under the fluorescence microscope (excitation 350–410 nm/emission 455 cut-off filter, bisbenzimide shows a maximal fluorescence intensity at 450 nm with an excitation at 350 nm in vesicular systems). Only those nuclei fluoresced which were incubated with the negatively charged PS- or PA vesicles. For the PC : PE vesicles the nuclei show a faint but above background fluorescence while the interaction of PC vesicles does not lead to any measurable fluorescent staining of the nuclei. All measurements were performed at pH 7.20 and low ionic strength (0.02 M Tris). Bisbenzimide could have also entered into the nuclei through the pores of the nuclear envelope instead of a direct transfer of the intravesicular content as consequence of fusion. Therefore the experiments were repeated with fluorescent dextran derivatives of molecular wheight 150 kDa which cannot pass the nuclear pores [24].

As in some controls, i.e. the application of free FITC-dextran molecules, a staining of the nuclei at low ionic strength (20 mM Tris (pH 7.20)) occurred, the experiments were then repeated at high ionic strength (150 mM NaCl, 10 mM Tris (pH 7.20)). In both sets of experiments the nuclei appeared fluorescent if the FITC-dextran was intravescularly incapsulated in PS vesicles. No fluorescent labeling was obtained for PC vesicles. The controls at high ionic strength showed also no fluorescence of the nuclei, while in some cases a weak nuclei-fluorescence was visible for controls at low ionic strength. In all experiments the freshly isolated nuclei were incubated in the presence of PS- or PC vesicles containing the entrapped FITC-dextran of molecular weight 150 kDa or in the presence of free FITC-dextran. The amount of FITC-dextran was adjusted so that all samples had the same fluorescence intensity. After various periods of time the nuclei were thoroughly washed and observed in the fluorescence microscope. The PS vesicles seemed to adsorb spontaneously on the nuclei surface. The fluorescence of the nuclei initially appeared as points on the nuclear membrane. With increasing time of incubation the fluorescent points became condensed on the background of a weaker fluorescence spread homogeneously over the nuclei. The fluorescent points disappeared if the nuclei were shortly incubated in 1% Triton X-100. However, the fluorescent background was not totally removed by short periods of Triton treatments. The interaction with PS vesicles also seemed to influence the morphology of the nuclei and induced a nuclei aggregation to larger arrays. These arrays were less pronounced in the controls and for PC vesicles which never led to a staining of the nuclei. Some of the experiments were also performed at pH 4.76 during the incubation periods. The results obtained at neutral and low pH were very similar. PS vesicles always induced a fluorescence of the nuclei in parallel with morphological changes. This was not observed for PC vesicles or the controls.

Three typical sets of experiments are depicted in Fig. 3 where phase contrast and fluorescence micrographs are grouped together. Fig. 3a is the control with free bisbenzimide; all nuclei appear smooth and highly fluorescent because the low molecular weight bisbenzimide could enter into the nuclei through the nuclear pores. Fig. 3b reveals the interaction of bisbenzimide encapsulated within PS vesicles. The amount of nuclei aggregation increased and the nuclei were labed with bisbenzimide forming a fluorescent complex similar as in the control experiment. Bisbenzimide encapsulated within PC bilayers did not lead to any nuclei staining. Fig. 3c shows the nuclei fluorescence following the incubation with PS vesicles containing intravesicular FITC-dextran. Free FITC-dextran and PC vesicles containing FITC-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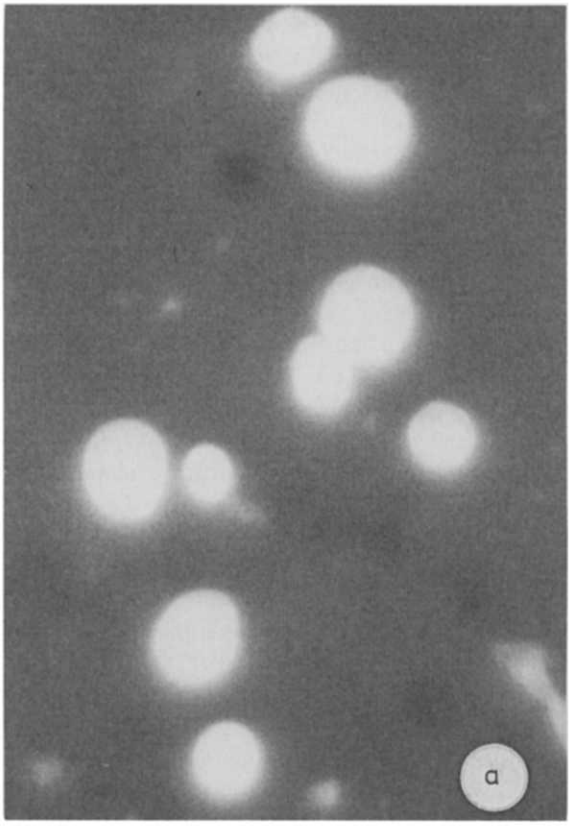
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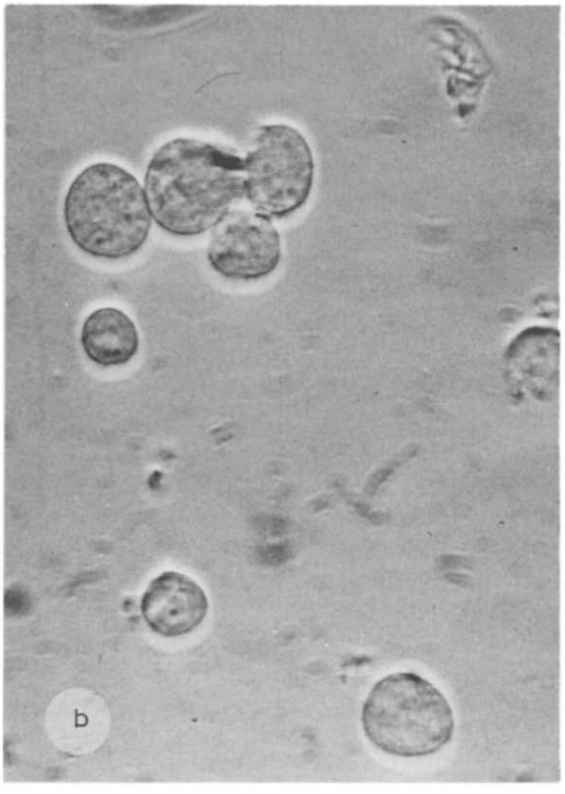
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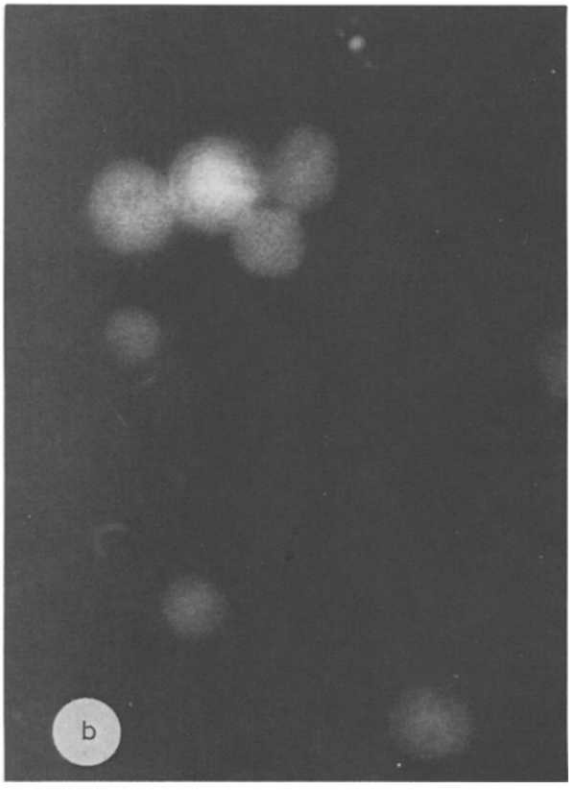
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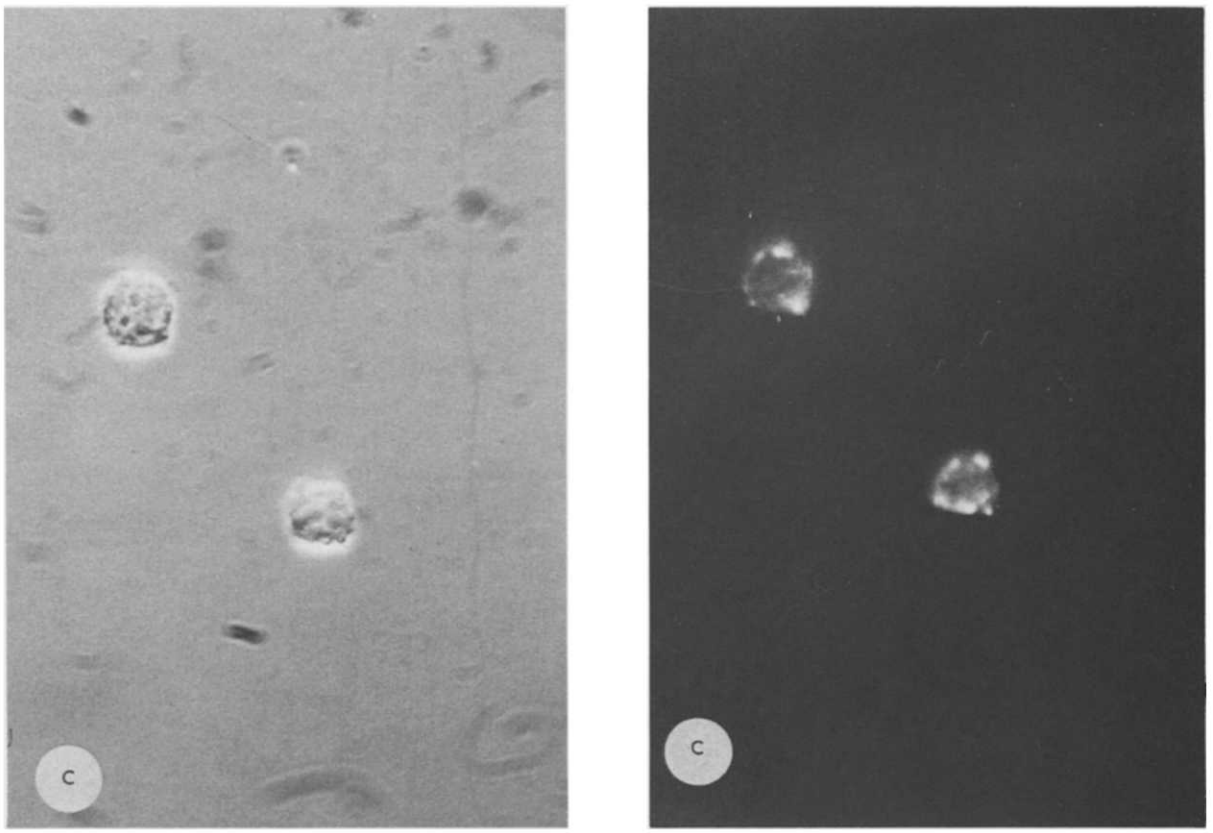

Fig. 3. Phase contrast (left) and fluorescence micrographs (right) of nuclei from liver cells. (a) Nuclei where free bisbenzimide was added. The external (almost nonfluorescent) bisbenzimide was removed by several washing steps. (b) Nuclei where PS vesicles with intravesicularly encapsulated bisbenzimide were incubated for 10 min at 37 °C. The uncomplexed vesicles were removed by several washing steps. In controls it was shown that PC vesicles containing encapsulated bisbenzimide did not lead to a fluorescent staining of the nuclei. (c) Nuclei and PS vesicles containing encapsulated FITC-dextran of 150 kDa molecular weight were incubated (pH 4.76 high ionic strength) for 40 min at 37 °C. The photos were taken after several washing steps against the same buffer at pH 7.20. When either free FITC-dextran or FITC-dextran encapsulated within PC vesicles were incubated together with the nuclei no fluorescent staining of the nuclei was observed. Magnification of the photographs: 2400×.

dextran did not lead to a fluorescent staining of the nuclei.

Discussion

In the preceding sections it was shown that negatively charged vesicles composed of PA or PS interact with and adsorb on the nuclear membrane. The adsorption observed at neutral to acidic pH was followed by lipid mixing of the vesicle components with the nuclear membrane. As a consequence of the lipid mixing a release of the intravesicular content took place possibly due to a lysis of the adsorbed vesicles. The experiments were carried out at room temperature where the lipid bilayers used and the membranes were in the fluid state. In the majority of cases small unilamellar lipid vesicles as prepared by bathsonication were used.

From studies on model and living systems it is known that lipid transfer [27–30] or intercellular diffusion [31] is negligible for the fluorescent lipids used. Thus the results of the resonance energy transfer experiments are an indication of lipid mixing due to insertion into the nuclear membrane. The resonance energy transfer measurements were complemented by fluorescence microscopic studies. The micrographs revealed that

charged PS-vesicles induced morphological changes, in parallel the fluorescence of intravesicular FITC-dextran became more and more spread over the nuclei. This was not observed for PC vesicles or free FITC-dextran. If bisbenzimide was encapsulated, the nuclei fluoresced for the negatively charged PA- and PS vesicles but not for the uncharged PC- and PC : PE vesicles. In contrast to FITC-dextran of molecular weight 150 kDa the small bisbenzimide molecules can enter through the nuclear pores and thus stain the nuclei predominantly by binding to the DNA. The bisbenzimide-DNA complexes are highly fluorescent [32] compared with the free dye. An increase of the fluorescence intensity concomitant with a small shift of the maximum was also observed if bisbenzimide binds to the phospholipid vesicles.

The spectral changes in the resonance energy transfer experiments during the fusion processes critically depend on the initial vesicle preparation (compare Figs. 1 and 2). The transfer efficiency is not only a function of the distance and the angular distribution between the donor and acceptor molecules but depends in vesicular systems also on the asymmetry of the label distribution over both halves of the bilayer. In small unilamellar vesicles the smaller NBD-PE molecules can be symmetrically distributed while the larger Rh-DOPE seems to be predominantly incorporated into the outer leaflet of the bilayer. If the curvature stress is relaxed the two labels become equally distributed over both halves of the bilayer. This behaviour can be demonstrated by comparing resonance energy transfer spectra of small and large vesicles of identical composition.

The fusion of particles of very different sizes should proceed from a stage in which the smaller species are adsorbed on the surface of the larger particles. This case corresponds to the fusion of small vesicles with the nuclear membrane and can show a complex kinetic behaviour as cooperative and fractal phenomena may come into play. The experiments performed at neutral or low (4.76) pH led to similar results in contrast to the pronounced pH dependence observed during the fusion of vesicles carrying lysozyme covalently bound to PE-headgroups with erythrocyte ghost [21] or with nuclei [22]. On the other hand a reduction of the ionic strength from 150 mM NaCl 10 mM Tris (pH 7.20) to 20 mM Tris (pH 7.20) evidently bears an influence on the vesicle-nuclei interaction observed. This different behaviour is possibly due to the higher fragility of the nuclei at lower ionic strengh. The interaction of the negatively charged vesicles with nuclear membranes described above shows the same preference for the negatively charged vesicles as the fusion between lipid vesicles and uncoated vesicles described in the preceding communication [33]. As both the interactions of uncoated vesicles and of nuclei with liposomes showed the same lipid specificity for negatively charged lipid molecules a targeting of the liposomes to the nuclei by lipid molecules seems only conceivable if the liposomes can 'somehow' escape fusion with the endosomes.

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