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Low pH fusion of mouse liver nuclei with liposomes bearing covalently bound lysozyme

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Lysozyme covalently bound to liposomes induces the fusion of liposomes with isolated mouse liver nuclei. The fusion behavior is very similar to the case of erythrocyte ghosts (Arvinte, T., Hildenbrand, K., Wahl, P. and Nicolau, C. (1986) Proc. Natl. Acad. Sci. USA 83, 962–966). Kinetic studies showed that membrane lipid mixing was completed within 15 min, as indicated from the resonance energy transfer (RET) measurements. For the resonance energy transfer kinetic measurements the liposomes contained L-α-dipalmitoylphosphatidylethanolamine (DPPE), labeled at the free amino group with the energy donor 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) or with the energy acceptor tetramethylrhodamine. The lipid mixing at equilibrium was studied by the fluorescence recovery after photobleaching technique (FRAP). Liposomes (with/without lysozyme) containing Rh-labeled DPPE in their membranes were incubated with nuclei at 37°C, pH 5.2, for 30 min. After washing of nuclei by three centrifugations, 60–70% of the initial amount of labeled DPPE was associated with the nuclei in the case of liposomes bearing lysozyme and only 7–10% in the case of liposomes without lysozyme. For the nuclei incubated with liposomes having lysozyme, about 70% of the total Rh-labeled lipids present in the nuclei diffused in the nuclear membrane(s) (lateral diffusion constant of $D = (1.4 \pm 0.5) \cdot 10^{-9}$ cm^2/s). By encapsulating fluorescein isothiocyanate-labeled dextran of 150 kDa molecular mass into the liposomes and using a microfluorimetric method, it was shown that after the fusion a part of the liposome contents is found in the nuclei interior. In this lysozyme-induced fusion process between liposomes and nuclei or erythrocyte ghosts, the binding of lysozyme to the glycoconjugates contained in the biomembranes at acidic pH seems to be the determining step which explains the high fusogenic property of the liposomes bearing lysozyme.

Abbreviations: DPPE, L-α-dipalmitoylphosphatidylethanolamine; FRAP, fluorescence recovery after photobleaching; FITC-D150, fluorescein isothiocyanate-labeled dextran of 150 kDa molecular mass; GlcNAc, *N*-acetyl-D-glucosamine; G-protein, membrane glycoprotein from vesicular stomatitis virus; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; Rh-labeled, tetramethylrhodamine labeled; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TKM buffer, 50 mM Tris-HCl/25 mM KCl/3.3 mM $CaCl_2$/5 mM $MgCl_2$.

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Introduction

Membrane fusion is a fundamental cell-biological process, but its mechanism is not yet well understood. Many recent studies have focused on the fusogenic properties of biological molecules due to the widely accepted viewpoint that membrane functions are strongly dependent on their composition. It is possible to study the role of biological molecules in the fusion process by investigating liposome–liposome or liposome–bio-

membrane interactions. For example, reconstituted liposomes incorporating purified membrane glycoprotein (G-protein) from vesicular stomatitis virus, fused with small unilamellar vesicles at acidic pH, and the process was G-protein mediated [1]. Vesicles containing influenza virus hemagglutinin glycoproteins were shown to fuse with erythrocyte membranes with an optimum fusion yield at pH 5.2 [2]. Liposomes containing glycophorin fused with erythrocyte membranes in the presence of hemagglutinating virus of Japan, and it was suggested that this fusion is F-protein dependent [3]. We showed recently that liposomes bearing covalently bound lysozyme fused with red blood cell ghosts at acidic pH [4]. At pH 5.2, the optimum of the lysozyme enzymatic activity, the rate of fusion exhibited a maximum while the plot of extent of fusion vs. pH showed a characteristic shoulder. From these observations, as well as from the decrease of fusion rate by the lysozyme inhibitor, N,N',N''-triacetylchitotriose, it was proposed that the enzyme active site of lysozyme was involved in the fusion at acidic pH [4]. In the present study, we tried to determine whether the lysozyme-induced fusion of liposomes is restricted only to erythrocyte membranes or may also occur with intracellular membranes. A combination of experimental approaches, which we present here, yielded evidence that lysozyme, covalently bound to liposomes, induces fusion of these liposomes with isolated mouse liver nuclei.

Materials and Methods

Chemicals. 7-Nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) chloride and tetramethylrhodamine (Rh) isothiocyanate were purchased from Fluka. Fluorescein isothiocyanate Dextran (FITC-D150) (average molecular mass 150 kDa), lysozyme, dimethylsuberimidate and cholesterol were from Sigma.

Lipids. Egg yolk L-α-phosphatidylcholine, Grade 1, was purchased from Lipid Products. Phosphatidylethanolamine (PE) from egg yolk and L-α-dipalmitoylphosphatidylethanolamine (DPPE) were purchased from Sigma. All lipids showed single spots on TLC in the solvent mixture, $CHCl_3/MeOH/H_2O$ (65 : 24 : 4, v/v). *N*-NBD-DPPE and *N*-Rh-DPPE were synthesized by the method of Monti et al. [5], and Vanderwerf and Ullman [6], in the laboratory of Dr. K. Hildenbrand in the Max-Planck-Institut für Strahlenchemie, Mülheim/Ruhr, F.R.G., as described previously [4,7].

Vesicle preparation. For the resonance energy transfer assay the liposomes were prepared by sonication [4]. Thus, stock solutions of egg yolk L-α-PC, PE, cholesterol, *N*-NDB-DPPE, *N*-Rh-DPPE in a molar ratio of 4 : 5 : 1 : 0.05 : 0.1 (10 mg of lipids) were mixed. The solvent was evaporated and 2 ml of 10 mM borate buffer (pH 8.5) containing 0.145 M NaCl, was added to the lipid film. Liposomes were formed by sonication using a Bransonic 12 bath sonicator. The sonication was performed until the solution was clear (i.e., no change in turbidity was observed with further sonication).

Liposomes containing FITC-D150 were made from the same lipid mixture, without *N*-NBD-DPPE. The 2 ml of borate buffer that were added over the dried lipid film, contained 40 mg FITC-D150. After the sonication step, the unincorporated FITC-D150 was removed from the liposomes by filtration through a Sepharose 4B column (30 cm × 1 cm). The column was loaded with 1 ml of the liposome solution. The fraction volume was 1 ml.

Covalent coupling of lysozyme to liposomes. Lysozyme was covalently linked to liposomes using the method of Torchilin et al. [8], as described in Ref. 4. Briefly, the liposome suspension (5 mg lipids/ml) in the borate buffer was mixed with $1.6 \cdot 10^{-4}$ M lysozyme. Dimethylsuberimidate was then introduced in the cold, 10 °C, up to 1.5 mg/ml final concentration. The mixture was incubated at 10 °C for 30 min and then dialysed against the same borate buffer at 4 °C for 2 h. The resulting mixture was then passed over a Sepharose 4B column to separate the liposomes from the unreacted lysozyme and dimethylsuberimidate. The same procedure of lysozyme coupling was applied to liposomes encapsulating FITC-D150.

Phospholipid concentration in the liposome suspensions was determined from the absorbance of the eluted fractions at the rhodamine absorbance wavelength (λ_{abs} = 545 nm). This procedure yielded results similar to those obtained by

the phosphate analysis method [4].

Preparation of nuclei. Nuclei from mouse liver were isolated following the procedure of Blobel and Van Potter [9], with some modifications. The liver was homogenised in an ice-cold buffer containing 50 mM Tris-HCl/25 mM KCl/5 mM $MgCl_2$/3.3 mM $CaCl_2$ (TKM buffer) and 250 mM sucrose (pH 7.2). After filtration through nylon gauze, the homogenate was concentrated by centrifugation at $600 \times g$ for 10 min at 4°C. The volume of the pellet was measured, and then twice this volume of TKM buffer containing 2 M sucrose was added. The suspension was layered over the TKM buffer containing 2 M sucrose and concentrated by centrifugation at $90000 \times g$ for 30 min at 4°C using a Beckman L8-70 ultracentrifuge (SW 50 rotor). The pellet of nuclei was stored at 4°C in TKM buffer containing 25 mM sucrose and was used for experiments within 8 h after preparation. Nuclei concentrations in stock suspensions were determined by the counting method.

Resonance energy transfer measurements. Mixing of lipids from liposomes and nuclei was monitored by the resonance energy transfer method [10,11]. The resonance energy transfer donor-acceptor pair, *N*-NBD-DPPE and *N*-Rh-DPPE, were incorporated into liposomes at 0.5 mol% and 1 mol%, respectively, concentrations at which there was a strong transfer of energy from NBD to rhodamine. Fluorescence measurements were performed on a Fica 55 MK II spectrofluorimeter in a thermostated sample holder at 37°C. NBD was excited at 475 nm, and the lipid redistribution was followed by monitoring the changes in NBD emission intensity at 530 nm [10]. An assay mixture in the cuvette contained 0.665 ml sodium-acetate buffer (0.02 M sodium acetate/0.145 M NaCl), 0.011 ml of liposome suspension (in borate buffer, 0.5 mM final lipid concentration) and 0.025 ml suspension of nuclei (in TKM buffer containing 25 mM sucrose, $6.9 \cdot 10^6$ nuclei). To avoid artifacts due to settling of fluorescent materials, the cuvette medium was continuously mixed during the measurements. The pH values indicated in text were obtained in separate experiments from mixtures of the three buffers.

Normalization of emission intensities. Steady state NBD emission intensities of the incubation mixture of liposomes with nuclei were normalized to the NBD emission intensities of the samples after treatment with Triton X-100 (1% v/v final concentration). The difference between the intensity of the Triton X-100 treated sample (F_T) and the liposome fluorescence at zero time (before addition of the nuclei), F_0, was considered to be 100. Thus, normalized equilibrium emission intensities were calculated as: $[(F_{eq} - F_0)/(F_T - F_0)] \times 100$, where F_{eq} is the fluorescence intensity at equilibrium.

FRAP measurements. Diffusion coefficient (D) and mobile fraction were measured by FRAP [12,13] employing an apparatus described earlier [4,14] and similar to those used by Koppel et al. [12] and Peters [15]. The argon laser beam (488 nm) was focused through the microscope to a gaussian radius of $w = 1.2$ m with an $\times 100$ oil immersion objective. A brief pulse (10 ms, 20 mW) bleached the illuminated area, and the fluorescence recovery was monitored with an attenuated beam ($5 \cdot 10^4$ times), and measured by a 56TVP Radiotechnique photomultiplier. Single photoelectron pulses from the photomultiplier were shaped by a 9302 ORTEC amplifier discriminator and counted by a 2071 Camberra 100 MHz counter. The counter was read through a GPIB bus, by a Minc 11/23 computer. From the fluorescence recovery curves, the diffusion coefficients were calculated using the formula $D = \beta w^2/4t_{1/2}$, where $t_{1/2}$ is the half recovery time and β a factor which depends on the bleached fraction [16].

Assay for the transfer of liposome contents to the nuclei by Triton X-100 treatment and microfluorimetric measurements. 0.050 ml of liposome suspension, with or without lysozyme, containing FITC-D150 (2.5 mM final lipid concentration) was mixed with 1.4 ml sodium acetate buffer and 0.050 ml nuclei suspension ($1.4 \cdot 10^7$ nuclei) and incubated for 30 min at 37°C, pH 5.1. After incubation the sample was concentrated by centrifugation. A Savant Speed-Vac Concentrator was used for 7 min at $1000 \times g$. The pellet after the second centrifugation was suspended in 0.5 ml TKM buffer, and divided in two fractions. Over one fraction Triton X-100 was then added (1% v/v final concentration), mixed, and reacted for about 5 min under gentle shaking. The resulting solubilized materials were removed from the Triton re-

sistant nuclei structures by washing twice with centrifugation. This fraction will be termed envelope denuded nuclei [17]. Microfluorimetric measurements of fluorescent dextrans associated with nuclei for the two fractions were performed on the FRAP apparatus previously described [18]. Briefly, a drop of the suspension of nuclei was spread between a coverslip and a microscope slide and was placed on the stage of the microscope. Observing the sample with the transmitted light of the microscope, the middle plane of the nucleus was focused and placed in the center of the optical field defined by the ocular reticle of a Zeiss Microphotometer attachment (MPM01K). Fluorescence was excited by the attenuated laser beam of the FRAP apparatus ($\lambda = 488$ nm); the beam waist was focused on the nucleus center. The mean value obtained from measuring 20 single nuclei fluorescence intensities was considered as characterising the nuclei fraction.

Results

Study of the lipid mixing by the resonance energy transfer method

The kinetics of the mixing of nuclei and liposome lipids were measured by the resonance energy transfer method [10,11]. Liposomes containing *N*-Rh-DPPE and *N*-NBD-DPPE in their membranes (0.5 and 1 mol%, respectively), with or without covalently bound lysozyme, were incubated with mouse liver nuclei at 37 °C and at different pH values in sodium acetate buffer as described in Materials and Methods. The increase in *N*-NBD-DPPE emission intensity at 535 nm due to lipid redistribution after mixing of liposomes with nuclei was continuously monitored. Fig. 1 presents the equilibrium emission intensities of *N*-NBD-DPPE reached after liposomes containing lysozyme and liposomes without lysozyme had reacted with isolated mouse liver nuclei. These intensities were proportional to the lipid mixing extent. As for the case of erythrocyte ghost membranes [4], there was a strong enhancement of the extent of lipid mixing in the pH 5 region induced by covalently bound lysozyme. A similar effect was observed for the kinetics of lipid mixing. It can be seen in Fig. 2 that around pH 5 the presence of covalently bound lysozyme at the liposome surface leads to a strong enhancement of the ate of mixing of lipid molecules. This is the pH range where lysozyme has its maximum enzymatic activity [4].

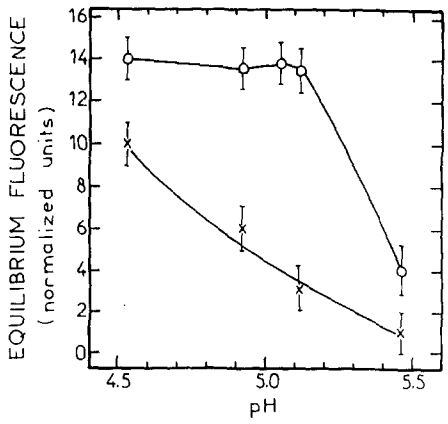

Fig. 1. Resonance energy transfer equilibrium emission intensities of *N*-NBD-DPPE, after incubation of fluorescently labeled liposomes with mouse liver nuclei as a function of pH. The steady-state NBD emission intensities were normalized to the NBD emission intensity of each sample after treatment with Triton X-100. All the reaction mixtures contained the same concentration of liposomes and nuclei (see Materials and Methods). (○) Lysozyme was covalently bound to the liposomes; (×) lysozyme-free liposomes. The lines between the experimental points have no theoretical significance.

Study of the lipid mixing at equilibrium state using the FRAP technique

For FRAP measurements, *N*-Rh-DPPE labeled liposomes with or without covalently bound lysozyme ($3.6 \cdot 10^{-4}$ M lipids), were incubated with a suspension of $2 \cdot 10^6$ nuclei in the sodium-acetate buffer at pH 5.2 for 30 min at 37 °C. The nuclei were subsequently washed by centrifugation four times in a large volume (10 ml of TKM buffer, $1000 \times g$, 4 °C, 5 min). Absorbance measurements of the Rh-chromophore which remained in the pellet after the four washes showed that 60–70% of the initial amount of labeled lipids was pelletable in the case of liposomes bearing lysozyme and 7–10% in the case of liposomes without lysozyme. Under the same conditions of centrifugation the liposomes, with or without lysozyme, could not be pelleted in nuclei-free suspensions. Thus, the centrifugation and absorbance measurements indicate that lysozyme strongly favours the lipo-

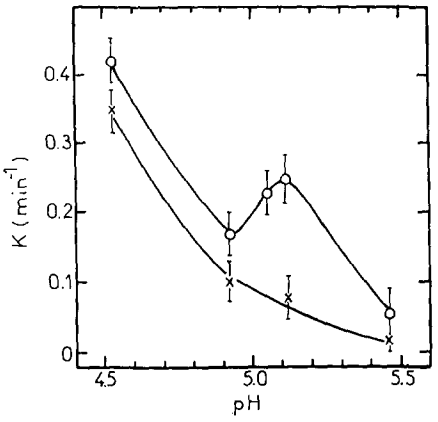

Fig. 2. Rates of mixing of liposome lipids and nuclear membrane lipids as a function of pH. The lipid mixing was monitored using the resonance energy transfer method (see text and Materials and Methods). The mixing rate constant is defined as $k = \ln 2/t_{1/2}$ where $t_{1/2}$ is the half-time measured from the increase of *N*-NBD-DPPE fluorescence intensity in time. (○) Rates of lipid mixing in the case of nuclei incubated with liposomes containing covalently bound lysozyme; (×) the nuclei were incubated with lysozyme-free liposomes. The liposomes and nuclei concentrations were the same in all experiments.

some–nucleus interaction. The recovery of *N*-Rh-DPPE fluorescence intensity after bleaching a small area of nuclear membranes from these pellets is shown in Fig. 3. The fluorescence of the nuclei was strong in the case of lysozyme-bearing

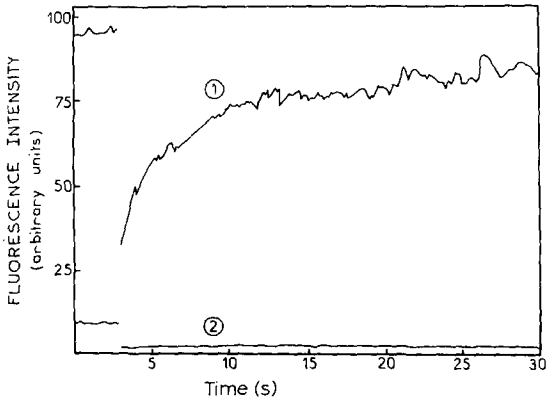

Fig. 3. Typical fluorescence recovery after photobleaching curves measured on mouse liver nuclei incubated with *N*-Rh-DPPE containing liposomes. (1) Lysozyme was covalently bound to the liposomes; (2) lysozyme-free liposomes. The two reaction mixtures contained, before the washing procedure, the same concentrations of liposomes and nuclei (see text). The measurements were performed at 22° C.

liposomes. About 70% of the total *N*-Rh-DPPE lipids present in the nuclei diffused in the nuclear membrane with a lateral diffusion constant of $D = (1.4 \pm 0.5) \cdot 10^{-9}$ cm^2/s. In the case of nuclei incubated with liposomes without lysozyme, the fluorescence intensity was 3 to 7 times weaker than in the previous case, and the mobile fraction of *N*-Rh-DPPE diffused with a diffusion coefficient of $(2.5 \pm 1) \cdot 10^{-9}$ cm^2/s in the nuclear membrane. This presented only about 10% of the total *N*-Rh-DPPE (Fig. 3, curve 2). These *D* values are of the same order of magnitude as the value, $D = (3.8 \pm 1.3) \cdot 10^{-9}$ cm^2/s, reported by Schindler et al. [19], for *N*-NBD-DPPE incorporated in nuclear membranes by ethanol injection. This result suggested that the mobile fraction of the *N*-Rh-DPPE lipids bound to the nuclear membrane was mixed with the nuclear membrane lipids.

Microfluorimetric measurements of the content mixing

To study the transfer of liposome contents to the nuclei interior, dextran of 150 kDa molecular weight labeled with fluorescein isothiocyanate (FITC-D150) was encapsulated in liposomes as described in Materials and Methods. After incubation of these liposomes at 37° C with nuclei (in 0.02 M sodium-acetate buffer/0.145 M NaCl (pH 5.2)), the nuclei were washed by centrifugation three times and divided into two fractions. One of the fractions was reacted for 5 min with

TABLE I

FITC-D150 FLUORESCENT INTENSITY OF NUCLEI INCUBATED WITH LIPOSOMES WITH AND WITHOUT LYSOZYME

Incubation of nuclei with liposomes, the treatment with Triton X-100, and the microfluorimetric measurements of FITC-D150 fluorescence intensities are described in Materials and Methods. Results presented are means ± S.D. for measurements of 20 nuclei.

Liposomes	Fluorescence intensity (arbitrary units)	
	intact nuclei	nuclei treated with Triton X-100
With covalently bound lysozyme	11 ± 3	3 ± 1
Without covalently bound lysozyme	4 ± 2	2 ± 1

Triton X-100 (1% v/v). The dextran fluorescence of nuclei from the nuclei fractions was measured under the microscope of the FRAP apparatus (see Materials and Methods). As a result of detergent treatment the liposomes, the nuclear membrane lipids, and 10% of the nuclei proteins, were solubilized and could be removed by centrifugation [17]. Consequently this last fluorescence was proportional to the dextran which had penetrated in the nucleus interior. The nuclei incubated with liposomes bearing lysozyme were about three times more fluorescent that those incubated with liposomes without lysozyme (see Table I). After the treatment with Triton X-100, the envelope denuded nuclei still contained FITC-D150 as shown in Table I, this being an indication that some FITC-D150 molecules passed the two nuclear membranes and entered in the nucleus interior.

Discussion

In a previous work, we incubated liposomes composed of egg yolk L-α-PC, PE and cholesterol (4 : 5 : 1, molar ratio) with erythrocyte ghosts at 37 °C and acidic pH [4]. Resonance energy transfer and FRAP measurements showed that the lipids of the liposome membrane mixed with the erythrocyte ghost membrane. When lysozyme was covalently linked to these liposomes, the extent and rate of mixing was increased. The maximum enhancement was observed at pH 5.2, where lysozyme has its maximum activity. The enzymatic activity of lysozyme covalently bound to liposomes is less than the activity of the equivalent amount of free lysozyme. It generally represents 15–20% of the free, unbound enzyme [4]. An increase of this value up to 60% was observed after addition of Triton X-100 to the lysozyme-liposome suspension, which suggests that the linking of lysozyme to PE shields about 40% of the active sites of the enzyme from the aqueous environment, [4].

In the present study, we have extended our previous observations by showing that lysozyme, covalently bound to liposomes, significantly increases liposome mixing with mouse liver nuclei at acidic pH. Fusion was assayed by techniques that showed membrane lipid mixing and transport of the liposome contents to the nuclei interior. By resonance energy transfer measurements, we found an enhanced mixing of liposome and nuclear membrane lipids when lysozyme was covalently bound at the liposome surface. By FRAP measurements we found that the fluorescence intensity before bleaching was considerably higher for the liposome with lysosome than liposome without. This meant that adherence of liposomes was increased by the bound lysozyme. In addition the mobile fraction of the fluorescent lipids was also much higher for the lysozyme liposome, which meant that the fraction of liposomes which mixed their lipids with the nuclear membrane was also increased by the presence of the bound lysozyme. Notice that the immobile fraction represented the fraction of liposome which adhered to the nuclear membrane but which did not mix with it. This enhancement was maximal in the pH 5 region. It is generally accepted that the mixing of lipids is a necessary step in the membrane fusion reaction. Thus, membrane fusion assays based on lipid intermixing have been described [10,11]. However, lipid mixing can also occur as a result of lipid transfer through the aqueous phase [7,20,23], or by exchange at the area of surface contact between membranes [22]. It has been shown in a number of studies that phospholipids having long chains and labelled head groups are not exchanged between lipid vesicles either through aqueous phase or vesicle aggregates [7,10,20]. Long exchange time has been found with the labelled and non labelled lipids of vesicles interacting with cells [10,20,22, 27–31].

The relatively rapid mixing we observed is difficult to conciliate with the exchange time reported in the literature. Therefore the most probable explanation of our resonance energy transfer and FRAP experiments is that lipid mixing is due to fusion of the liposomes with the nucleus membranes. In order to further demonstrate that fusion occurred we studied the interaction of the nuclei with fluorescent dextran encapsulating liposomes. We compared the fluorescence of intact nuclei with the Triton X-100 treated nuclei. It has been shown, that this Triton X-100 treatment removed completely the nuclear membranes but that the pore complex, the peripheral lamina and the interior structure of the nuclei was retained [32,33]. Therefore we concluded that the fluorescence by

the Triton X-100 treated nuclei was emitted by the dextran fraction transported in the nuclei interior during the fusion process. We already used this technique in a previous work in which we studied the interaction of liposomes with hepatocyte nuclei at pH 4.5. The result obtained by this technique was confirmed by studying the variation of the fluorescence intensity of intact nuclei, as a function of their radius [18]. As already noted in this previous work the quantitative interpretation of these data was complicated by the fact that the nuclear envelope comprises a double lipid bilayer membrane. This implied that the transport of dextrans could only occur with liposomes comprising at least two lipidic bilayers in order to perform successively two fusions with the two nuclear membranes.

In the present case lysozyme was covalently bound to the outside bilayers of the liposomes, so that the inside bilayers which fused with the nuclear membranes were without lysozyme. In this context the relatively small difference between the transport dextran into the nuclei by liposomes with and without lysosome might be explained if the fusion rate of these liposomes with the internal nuclear membrane was the limiting rate of the whole transport process. The possibility that lysozyme accelerates lipid transfer or exchange processes seems unlikely, because when liposomes bearing lysozyme were mixed at pH 5.2 with egg yolk L-α-PC liposomes, no mixing of lipids occurred, as resulted from resonance energy transfer measurements (data not shown). For these reasons we can consider that the resonance energy transfer and FRAP experiments provide a strong indication that covalently bound lysozyme has a high fusogenic effect. In addition, we used a microfluorimetric technique showing that a fraction of a high molecular weight fluorescent dextran encapsulated in the liposomes was transferred to the nucleus interior. Since the dextran molecules were too big to penetrate through the nuclear pore [24] we can conclude that transfer occurred by fusion of liposomes with the nuclear membrane.

Lysozyme is an enzyme which catalyses the hydrolysis of linear (1 → 4) polymers of *N*-acetyl-D-glucosamine (GlcNAc) if the number of monomer units is larger than four. Smaller oligomers are not hydrolysed but bind specifically to the lysozyme molecule with maximum affinity around pH 5 [25].

Recently it was shown that O-linked GlcNAc residues are found on different proteins in virtually every subcellular compartment, except mitochondria, of rat liver cells [25]. This supports our hypothesis that one of the steps in the fusion process of lysozyme-liposomes with biological membranes is the binding of the lysozyme molecules to glycoconjugates of these membranes.

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