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The effect of lipid molecular packing stress on cationic liposome-induced rabbit erythrocyte fusion

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Abstract

The effect of curvature stress on the efficiency of cationic liposome-induced fusion between rabbit erythrocytes was studied. Multilamellar cationic liposomes containing 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) and different PEs (1,2-dilinoleoyl-*sn*-glycero-3-phosphoethanolamine (dilin-PE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and lysophosphatidylethanolamine, egg (lyso-PE)) were used to induce cell–cell fusion. It was found that high cell–cell fusion yield (FY) of about 50% could be achieved in sucrose solution by using cationic liposomes containing 50% DOTAP. Cell–cell fusion was assayed by shape criterion and was verified by fluorescence microscopy, using a membrane dye mixing method. The curvature stress, as a result of mixing unsaturated PEs in cationic liposomes, had a significant effect on cell–cell FY which increased with the degree of acyl chain unsaturation, in the order dilin-PE > DOPE > POPE > lyso-PE. Replacement of dilin-PE, DOPE, or POPE by lyso-PE gradually in cationic liposomes lowered the cell–cell FY from 50% to 1%. Furthermore, cationic liposome-induced cell lysis, and fusion between cationic liposomes and cells, as assayed by the *N*-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Rh-PE/NBD-PE) energy transfer method, followed the same order as that for cell–cell fusion. Fusion between the negatively charged PS liposomes and cationic liposomes also followed the same order. The electric double layer screening by electrolytes in NaCl-containing solution and phosphate buffered saline (PBS) was found to reduce the cell–cell FY and cell lysis. These findings suggest that liposome-induced cell–cell fusion was achieved by cationic liposomes serving as fusion-bridges among cells.

Keywords: DOTAP; Cell–cell fusion; Liposome cell fusion; Curvature stress effect; Bending energy; Cationic lipid; Erythrocyte; (Rabbit)

Abbreviations: DOTAP, 1,2-dioleoyl-3-trimethylammoniumpropane; dilin-PE, 1,2-dilinoleoyl-*sn*-glycero-3-phosphoethanolamine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; lyso-PE, lysophosphatidylethanolamine, egg; egg-PC, egg phosphatidylcholine; PS, phosphatidylserine (brain); NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt; FY, fusion yield.

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1. Introduction

Studying the mechanism of artificially induced membrane fusion has contributed to a better understanding of natural membrane fusion, as well as to improve the techniques of artificially induced membrane fusion [1–3]. In different fusion methods, different aspects of fusion mechanisms are involved. Charged lipids, especially cationic lipids, have been used in studying membrane interaction and fusion [4–6] due to their known interaction with cell membranes [7,8]. These lipids have been used recently in gene transfer [9–15].

Unsaturated PEs have been found to cause the destabilization and fusion of lipid bilayer membranes [16–18]. This property of unsaturated PE has been exploited as helper lipids in cationic lipid facilitated fusion [11,19], but how PE can play a role in fusion is still not entirely known. It has been known that the PE headgroup has a relatively low hydration which may favor membrane fusion [18,20]. In addition, the unsaturated PEs have the propensity to form non-lamellar structures due to the curvature stress in monolayer packing [21]. This non-lamellar structures have been associated with membrane fusion [22,23]. However, it remains to be shown whether this packing effect indeed affects cationic liposome-induced membrane fusion.

In this report, cationic liposomes containing different PEs were used to study the effect of the curvature stress or bending energy on cationic liposome-induced cell–cell fusion. It was found that cationic liposomes can induce high cell–cell fusion (about 50%), and the fusion is dependent on the curvature stress of the liposomes. That is, the higher the bending energy on cationic liposome membranes, the higher are the cell–cell, and cell–liposome fusion, the cationic liposome induced-cell lysis, and the liposome–liposome fusion.

2. Materials and methods

DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane), dilin-PE (1,2-dilinoleoyl-*sn*-glycero-3-phosphoethanolamine), DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine), POPE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine), lyso-PE (lysophosphatidylethanolamine, egg), egg-PC (egg phosphatidylcholine), and PS (phosphatidylserine, brain) were purchased from Avanti Polar Lipids (Alabaster, AL); NBD-PE (*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt), Rh-PE (*N*-(lissamine™ rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt) were purchased from Molecular Probes (Eugene, OR).

The preparation of DOTAP containing cationic liposomes was carried out at 4°C with the exception of the case when lyso-PE was involved, in which case lipids were mixed and dried at room temperature in order to allow lyso-PE to dissolve in chloroform with 1.2% methanol and 0.6% deionized distilled water ('water' will be used for simplicity from now on). Because the optimal ratio for cell–cell fusion was found to be 50:50 DOTAP/DOPE in our preliminary experiments, and the popular use of 50% cationic lipid in liposomes for efficient transfection [11–15,19], we used 50% DOTAP in cationic liposomes for cell–cell fusion and cell–liposome fusion. For liposome–liposome fusion experiments, cationic liposomes of 20% DOTAP plus 80% other lipids, and anionic liposomes of 23% PS plus 75% egg-PC, with 1% Rh-PE and 1% NBD-PE were prepared. In general, desired lipids (in mol%) were dried in a glass tube by blowing nitrogen immediately after mixing, and then dispersed into multilamellar liposomes (MLV) by vortexing in 4°C water. Lipid concentration was 0.5 mg/ml for cell–cell fusion and cell–liposome fusion, and 1 mg/ml cationic liposomes and 0.25 mg/ml anionic liposomes for liposome–liposome fusion. Both extruded and MLV could cause cell–cell fusion. However, due to the simplicity in preparation, MLV were used for inducing cell–cell fusion and cell–liposome fusion. For liposome–liposome fusion, cationic liposomes were MLV, whereas the anionic liposomes were sonicated vesicles. The liposomes were kept in an ice bath and used in the same day of preparation.

In the cell–cell fusion assay, the procedures are as following: 10 μl of washed and diluted fresh or stored (no more than 7 days) rabbit erythrocytes (10^8/ml) in sucrose solution (250 mM sucrose + 5 mM Hepes, pH 7.4) was mixed with 50 μl of either sucrose solution, or NaCl solution (150 mM NaCl

+5 mM Hepes, pH 7.4), or PBS (150 mM NaCl +5 mM NaP_i, pH 7.4). Then 10 μl of cationic liposomes (0.5 mg/ml in water) was added during vortexing, and the sample was introduced into a home-made chamber with a 3-parafilm layers (unless specified) thick rectangular trough covered by a piece of cover glass, as described elsewhere [24]. In the chamber, cells could be preserved much longer (as long as 45 min) than those placed between a glass slide and a cover glass, due to less mechanical stress and evaporation. Under the phase contrast microscope (CH-2, Olympus, Japan), cells could be seen to settle down on the glass slide, with a change of shape of the fused cells from a dumbbell shape to a spherical shape by allowing sufficient time to remain undisturbed in the chamber. This shape change served as the criteria for recognizing cell–cell fusion [24] (unless specially mentioned), which was further verified by membrane dye mixing method, as described later. Lysed cells appeared black in phase-contrast microscopy. Unfortunately all cells eventually became lysed, because cationic liposomes could not be removed after initiating fusion. All fused cells, both lysed and unlysed, were counted. According to our experience, fused cells could be distinguished by shape within about 10 min, though fusion areas of dumbbell shape cells were still seemingly separated by membranes, when observed under fluorescent microscope with cells labelled by Rh-PE. Cell–cell FY (fusion yield) was determined by the formula:

$$\text{Cell–cell FY} = (\text{number of fused cells}/\text{total number of cells}) \times 100\%$$

as described elsewhere [24].

There may be some uncertainty in counting fused or unfused cells by the shape criterion alone when cells stay in clumps, but do not involve any shape change. In order to demonstrate the validity of the shape criterion primarily used in this paper in fusion assay, especially in distinguishing unfused cells in cell clumps, we employed the membrane dye mixing method [25]. In this method, 20%–30% erythrocytes were labelled with Rh-PE. The assumption for this method is that fusion occurs only if membrane dyes are mixed. For simultaneous phase contrast and fluorescent photograph recording when the membrane dye mixing method is used, a chamber with only one, instead of three, parafilm layer spacing chamber was used to reduce fluorescence background.

In the determination of cell lysis by cationic liposomes, 40 μl of washed and diluted erythrocytes in sucrose solution (10^8/ml) was suspended in 200 μl sucrose solution. 40 μl of cationic liposomes was added during vortexing. After 10 min in room temperature, 1 ml NaCl solution was added, and the supernatant was collected by centrifuge. The hemoglobin concentration in supernatant was assayed by spectrometry (M4QIII, Carl Zeiss, Germany) at 415 nm. The percentage of lysis caused by liposomes was expressed as the percentage of hemoglobin leaked out to the total hemoglobin lysed by water, as described elsewhere [24].

To measure the fusion between cationic liposomes and cells, the energy transfer method was used [8,26]. Both 2% NBD-PE and 2% Rh-PE were incorporated into the cationic liposomes. Fusion of cationic liposomes to unlabelled cells will result in the reduction of energy transfer between these two dyes. Since hemoglobin and unattached liposomes have to be removed by centrifugation prior to fluorescent measurement, a 10-fold lower lipid to cell ratio, comparing to that used in inducing cell–cell fusion, was used to reduce lipid-induced cell–cell aggregation after centrifugation. Here, 10 μl of liposomes was added to 500 μl sucrose solution previously mixed with 10 μl (10^9/ml) of erythrocytes. After 5 min at room temperature and centrifugation, the supernatant was removed. Under this condition, no cell–cell fusion occurred after centrifugation, and cell lysis was low (about 10%). Then, 5 mM Hepes (pH 7.4) buffer was added to lyse cells, and the ghosts were recovered by centrifugation in an Eppendorf centrifuge (Brinkmann Instruments, Westbury, NY). The ghosts were resuspended in 3 ml of 5 mM Hepes buffer (pH 7.4), and measured by fluorimeter (Ex = 450 nm, Em = 533 nm; SLM-8000, SLM Instruments, Rochester, NY). Triton ×-100 (final concentration 0.03%–0.06%) was used to indicate 100% relief of energy transfer of all liposomes. The FY between cationic liposomes and cells was derived as follows [27]: Assuming the fluorescent intensities of each liposome are i and i_t before and after the addition of Triton, respectively, and I and I_t are the respective intensities of the entire sample. If n liposomes fused

with cells among N liposomes initially attached, and assuming that once liposomes fused with cells, energy transfer is totally relieved, then:

$$I = n \cdot i_t + (N - n) \cdot i \quad (1)$$

$$I_t = N \cdot i_t \quad (2)$$

$$\mathrm{FY} = n/N \quad (3)$$

If Q, the energy transfer efficiency, is defined as

$$Q = i_t/i \quad (4)$$

then from Eqs. (1)–(4), we can derive

$$\mathrm{FY} = \left(Q \cdot (I/I_t) - 1\right)/(Q - 1) \quad (5)$$

Q can be measured by I_t/I from experiment using pure liposomes.

The relief of energy transfer would result in changing of emission color. This phenomena was recorded by color photomicrography and presented as an additional assurance of liposome–cell fusion. A slightly higher cell and liposome concentration, comparing to that used in assaying liposome–cell fusion by fluorimeter, was used to facilitate photographic recording, i.e. 10 μl of liposomes was added to the mixture of 10 μl of erythrocytes (10^9/ml) and 50 μl sucrose solution during vortexing. This liposome and cell concentration still allowed us to centrifuge samples without causing significant cell aggregation.

To measure liposome–liposome fusion, the same energy transfer method was used. 25 μl of 1 mg/ml liposomes containing 20% DOTAP plus other lipids, was mixed with 5 μl of 0.25 mg/ml sonicated PS liposomes containing 23% PS plus 75% egg-PC with 1% Rh-PE and 1% NBD PE. Two minutes later, liposomes were diluted by 5 mM Hepes medium (pH 7.4) for fluorescent intensity measurement. The fusion efficiency was calculated the same way as in cell–liposome fusion, since we assume that once the sonicated anionic liposome fuses with MLV, all energy transfer is relieved.

Using the home-made chamber, which contains 5 or 6 wells on the same glass slide, we could compare cell–cell FY with different parameters at the same time. At least three duplicate experiments were performed for each preparation of liposomes and cells, and the results were reproducible. All experiments were performed at room temperature.

3. Results

3.1. Effect of electrostatic shielding on the efficiency of cationic liposomes-induced cell–cell fusion

Since the interaction between cationic liposomes and cells is known to be initiated by electrostatic interaction [28], it is pertinent to investigate the influence of the ionic strength of the buffering solution prior to fusion experiments. The influence of ionic strength of the suspension media on our experimental end points, namely, cationic liposomes induced fusion yield and cell lysis, were evaluated. The results are given in Fig. 1A and B. All three suspension

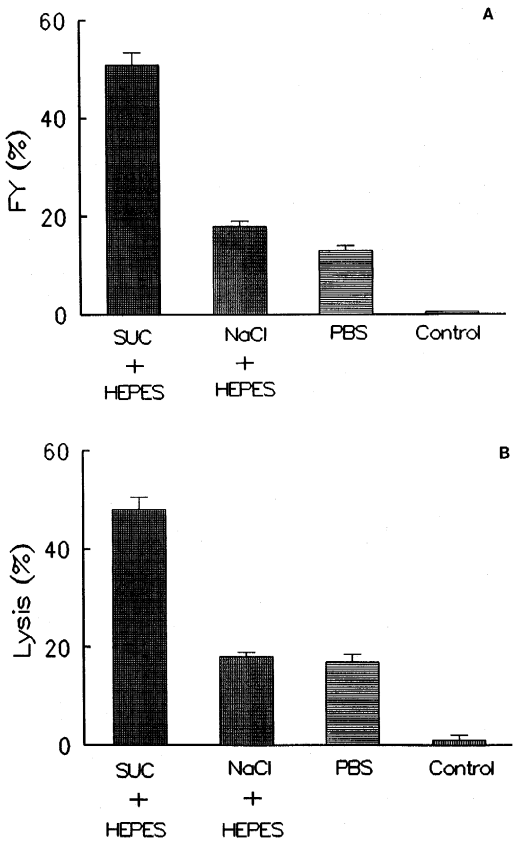

Fig. 1. (A) The effect of various solutions on cationic liposome induced cell–cell FY. 50% DOTAP +50% DOPE MLV were used. Control means no lipid was added. (B) The effect of various solutions on cationic liposome induced cell lysis. MLV of 50% DOTAP +50% DOPE were used. Control means no lipid was added.

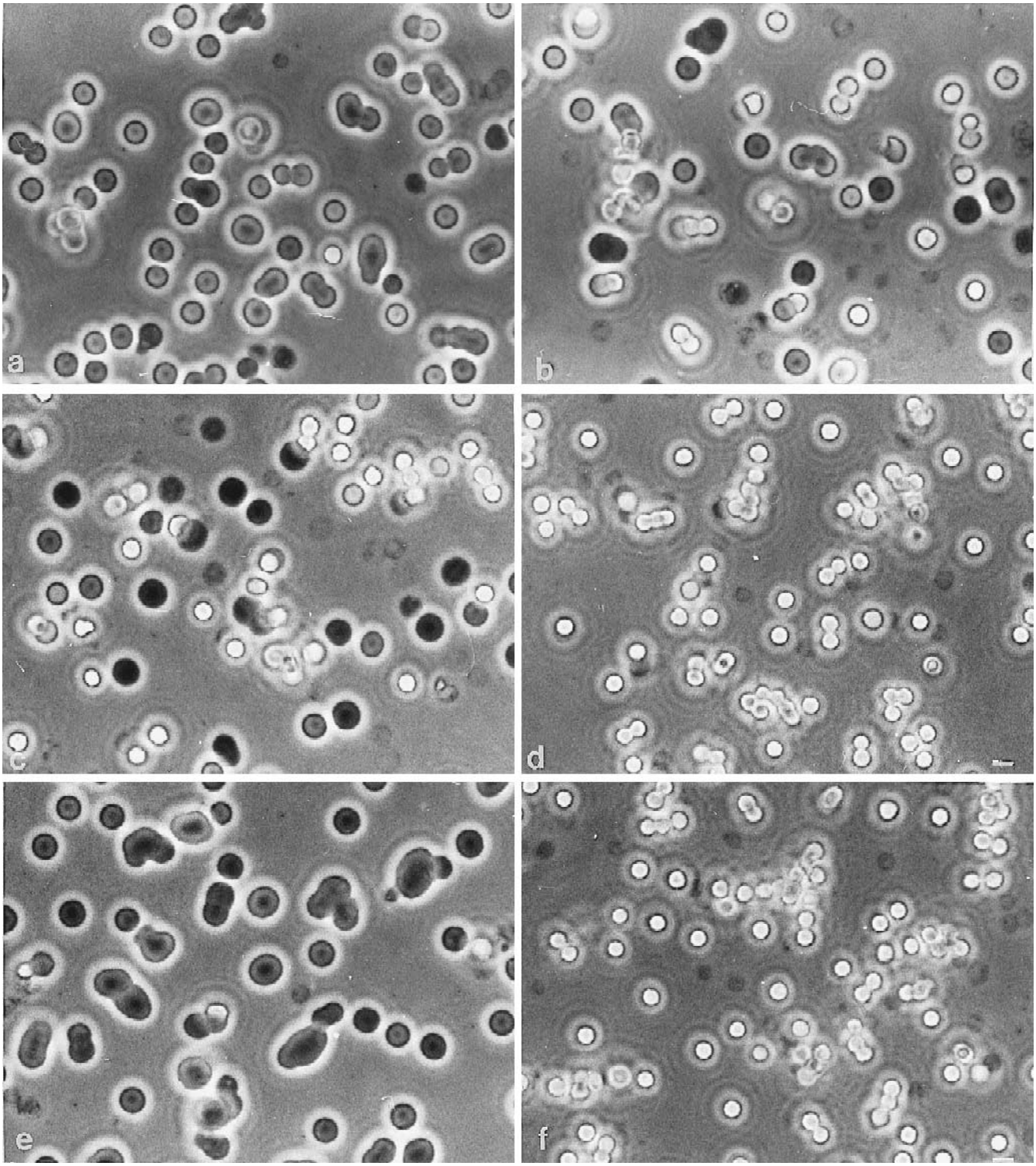

Fig. 2. Phase contrast micrographs of cell–cell fusion by cationic liposomes with 50% DOTAP and 50% (DOPE + lyso-PE). Panels a, b, c and d correspond to 0%, 10%, 20% and 50% lyso-PE, respectively, at 15 min after cells and cationic liposomes were mixed; Panels e and f correspond to 0% (25 min) and 50% lyso-PE (45 min), respectively. The bar is 10 μm.

media are of the same osmolarity. Sucrose solution has the lowest ionic strength, whereas PBS has the highest, containing 5 mM of polyvalent phosphate anions in addition to the 150 mM NaCl ion as in the NaCl/Hepes medium. Both the FY and the lysis results show that the reaction is the strongest in sucrose medium, then decline according to increasing ionic strength in the suspension media. It should be noted that PBS has an additional effect of phosphate polyanion, which tend to react and precipitate cationic liposomes [28]. Hence, sucrose solution was used in all subsequent studies of cationic liposome induced cell–cell fusion and cell lysis.

3.2. Effects of curvature stress caused by helper lipids on cationic liposome induced cell–cell fusion

It has been suggested that the effectiveness of cationic liposome induced gene transfer is partially controlled by the curvature stress caused by helper lipids [29]. The effect is believed to be mediated by curvature stress related membrane fusion between liposome-DNA complexes and cell membranes (plasma membranes or endosome membranes). In order to isolate the effects of headgroup from the curvature stress of the helper lipids, we use a homogeneous series of PEs with different fatty acyl chains,

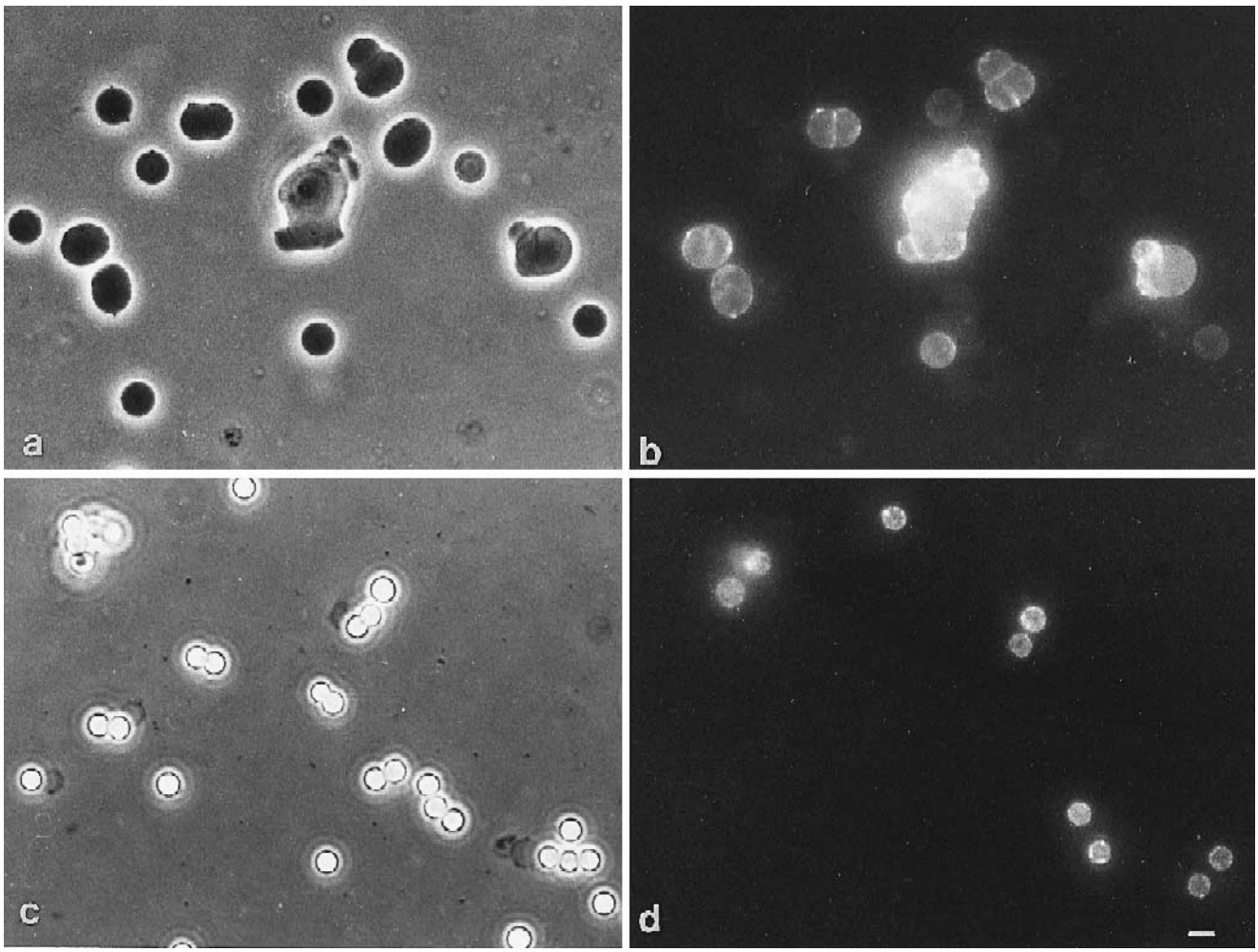

Fig. 3. Fluorescent (b, d) and phase contrast (a, c) micrographs of cell–cell fusion by cationic liposomes with 50% DOPE (a, b), or 50% lyso-PE (c, d). 20%–30% cells were labelled with Rh-PE. The bar is 10 μm

or replace other PEs with the single chain lyso-PE, to manipulate curvature stresses at the same temperature. The various PE used are: dilinoleoyl-(18:2; 18:2), dioleoyl-(18:1; 18:1), palmitoyloleoyl-(16:0; 18:1), lyso-(18:1) PE, and the mixture of lyso-PE and PEs. The decreasing saturation or replacing other PEs by lyso-PE reduce the hydrophobic volume [30,31], thereby lowering the curvature stress while the PE headgroups occupy the same intermolecular spacing [32–36].

The cationic liposome-induced cell–cell fusion was measured by microscopy. A series of phase contrast photographs of cell–cell fusion caused by cationic liposomes with different lyso-PE proportion (DOTAP/(DOPE + lyso-PE) = 1:1) are given in Figs. 2 and 3. Fig. 2 a–d show cell–cell fusion induced by 0, 10, 20, 50% of lyso-PE in DOPE, respectively, at 15 min after liposomes were added to cells. The cell–cell FY was easy to count at this stage even for the 0% lyso-PE sample, because fused cells remained dumbbell shaped. At 25 min after liposome-cell mixing in the chamber, fused cells became more spherical, and it was difficult to identify the number of cells forming giant fused cells, as shown in Fig. 2e. However, for the 50% lyso-PE sample, even after 45 min in the chamber, no cell–cell fusion occurred, by shape criterion, as shown in Fig. 2f. To substantiate the shape measurement, membrane dye mixing method was used to distinguish fused from unfused cells, as shown in Fig. 3. For the 0% lyso-PE sample, dye mixing occurred (Fig. 3b), in agreement with the corresponding phase contrast photograph showing cell–cell fusion (Fig. 3a); whereas for the 50% lyso-PE sample, no dye mixing could be observed (Fig. 3d), even in cell aggregates (Fig. 3c).

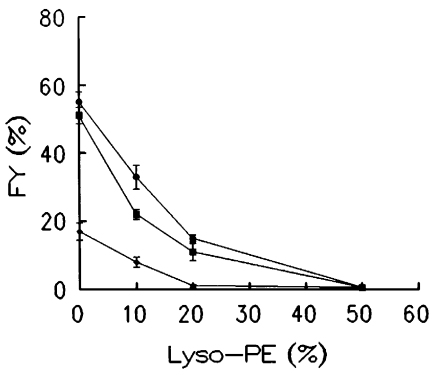

Fig. 4. The effect of different percentage of lyso-PE in cationic liposomes on liposome-induced cell–cell FY in sucrose solution. All liposomes were 50% DOTAP, the other 50% was lyso-PE with either dilin-PE (solid circles), DOPE (solid squares), or POPE (solid diamond).

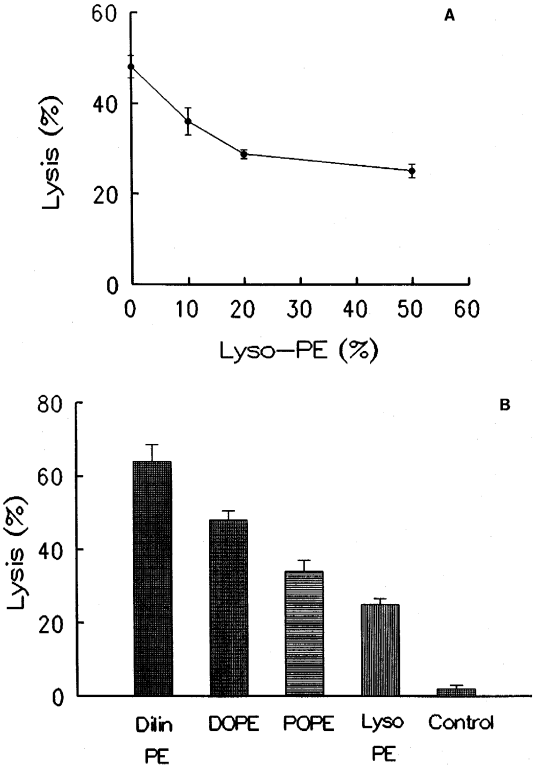

Fig. 5. (A) The effect of different percentage of lyso-PE in cationic liposomes on liposome-induced cell lysis in sucrose solution. All cationic liposomes were 50% DOTAP and 50% (DOPE + lyso-PE). (B) The effect of PEs in cationic liposomes on liposome-induced cell lysis in sucrose solution. All cationic liposomes were 50% DOTAP and 50% either lyso-PE, POPE, DOPE, or dilin-PE. Control means no lipid was added.

The cell–cell fusion was quantitated for each sample. The FY is shown in Fig. 4 when dilin-PE, DOPE, or POPE was stepwise replaced with lyso-PE (from 0% to 50%). The more the dilin-PE (solid circles), DOPE (solid squares), or POPE (solid diamonds) in cationic liposomes was replaced by lyso-PE, the less was the cell–cell FY. When about 30%–50% lyso-PE was in the cationic liposomes, there was almost no cell–cell FY. Also, when the helper lipid of liposomes was changed from dilin-PE to DOPE, POPE and lyso-PE, the cell–cell FY decreased from 50% to about 1%. The cell–cell FY followed the trend: dilin-PE > DOPE > POPE > lyso-PE (Fig. 4).

3.3. Effects of curvature stress caused by helper lipids on cationic liposome induced cell lysis

Since the extent of cell–cell fusion correlates with cell membrane destabilization, it is expected that cell lysis is likewise correlated. By this reasoning, the correlation between the curvature stress caused by helper lipids and cationic liposome induced cell lysis was examined. It was found that, as more DOPE was replaced by lyso-PE (DOTAP/(DOPE + lyso-PE) = 1:1), the extent of cell lysis was reduced (Fig. 5A). When the PE composition of cationic liposomes was changed from dilin-PE to DOPE, POPE, or lyso-PE, the cell lysis also decreased (Fig. 5B).

3.4. Fusion between cell membranes and cationic liposomes

It is not known if cationic liposome induced cell–cell fusion is mediated by cell–cationic liposome fusion. To determine their relationship, we examined the fusion between cells and liposomes in relation to cell–cell FY. First, the curvature stress effect of helper lipids on fusion between liposomes and cells was studied. Liposome–cell fusion was monitored by fluorescence energy transfer. For this purpose, 2% NBD-PE and 2% Rh-PE were mixed with helper PEs in the cationic liposomes. At this concentration, the fluorescence energy of NBD-PE was transferred to Rh-PE. When the cationic liposomes fused with cell membranes, the dyes were diluted, and the energy transfer was relaxed. The fluorescence micrographs of cells treated with cationic liposomes containing different proportion of lyso-PE (DOTAP/NBD-PE/Rh-PE/(DOPE + lysoPE) = 50:2:2:46) were given in Fig. 6. Due to energy transfer from donor NBD-PE to acceptor Rh-PE at high label concentration, the unfused labelled liposomes appeared orange, which was the color of the combination of green and red given by NBD-PE and Rh-PE probe, respectively,

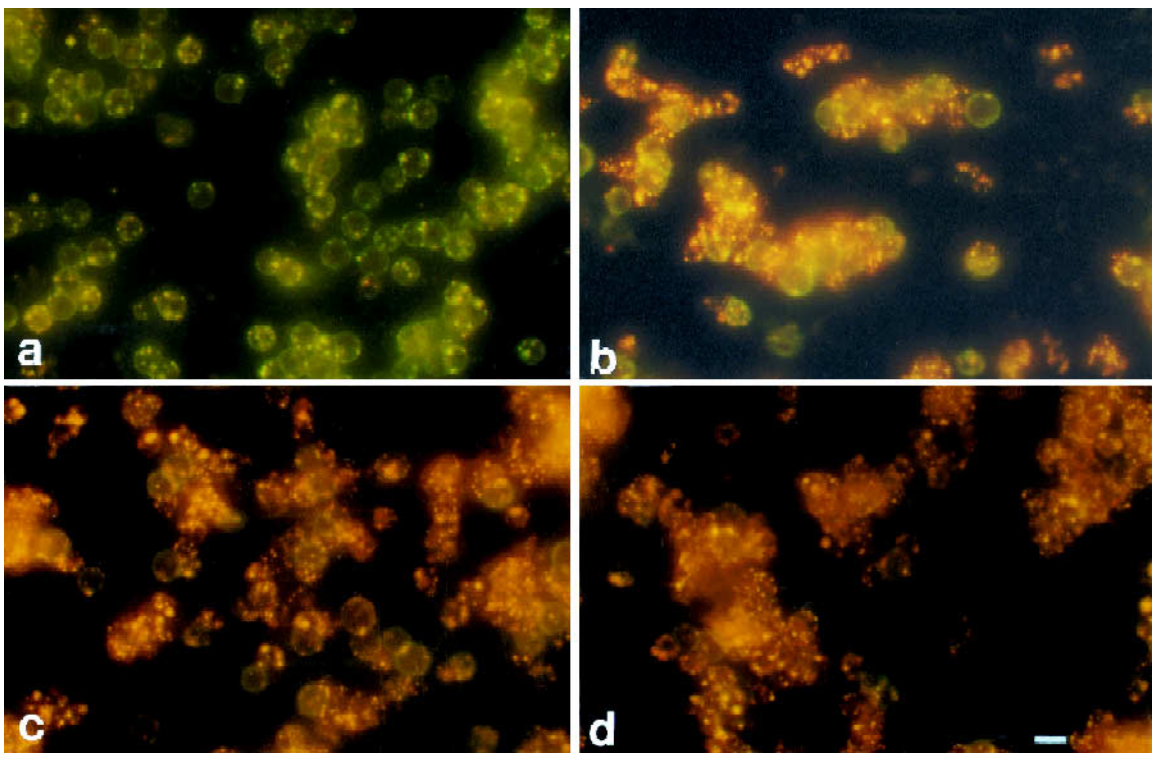

Fig. 6. Fluorescent micrographs of energy transfer occurring during fusion between cells and cationic liposomes with 50% DOTAP +2% Rh-PE +2% NBD-PE plus 46% (DOPE + lyso-PE) taken at about 5–10 min after mixing of cells and liposomes. Panels a, b, c and d correspond to 0%, 20%, 30% and 46% lyso-PE, respectively. The bar is 10 μm.

when the liposomes were excited at about 450 nm. This was the case when only labelled cationic liposomes, irrespective of the ratio of lysoPE:DOPE in liposomes, were in the chamber (data not shown here). However, if fusion occurs, the energy transfer efficiency decreases, and the excitation beam at 450 nm excites the fluorescence of NBD-PE only, with a green emission from this dye. After cationic liposomes were mixed with cells, the number of green cells increased as lyso-PE was increasingly replaced by DOPE, as shown in Fig. 6. For the case of 0% lyso-PE, all cells appeared green (Fig. 6a). The increase of green color indicated the relief of energy transfer from NBD-PE to Rh-PE, which meant the increase of fusion between liposomes and cells, as shown in Fig. 6a,b,c,d (corresponding to 0%, 20%, 30%, 50% lyso-PE). When liposome-cell fusion yield was quantitated by fluorescence spectroscopy, based on the fluorescence energy transfer method, the curvature stress effect had the same trend in Fig. 7A as compared to Fig. 4 (for cell–cell fusion). That is, the more lyso-PE was incorporated into cationic liposomes, the lower was the fusion between liposomes and cells. The effect of different PE acyl chain composition is shown in Fig. 7B. Again, when lipid composition of cationic liposomes was changed from dilin-PE to DOPE, POPE, or lyso-PE, the fusion between liposomes and cells decreased, in analogy to Fig. 4 for cell–cell fusion.

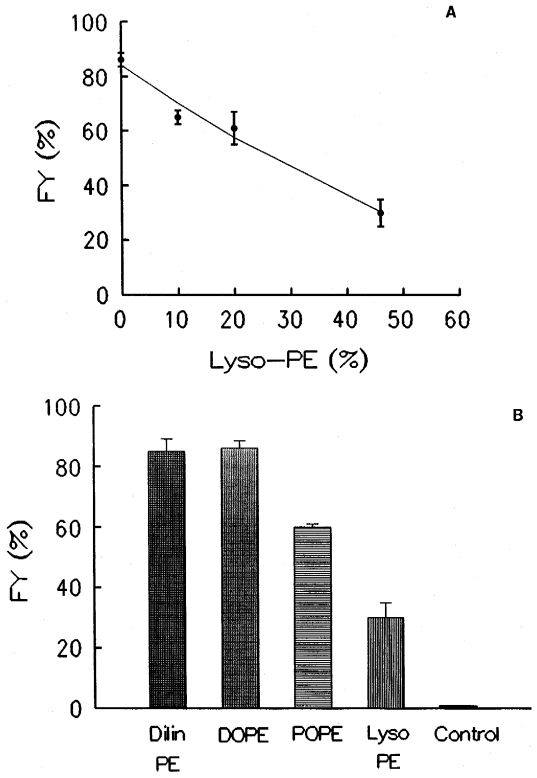

Fig. 7. (A) The effect of different percentages of lyso-PE in cationic liposomes on the fusion between cationic liposomes and cells in sucrose solution. Cationic liposomes composed of 50% DOTAP plus 46% (DOPE + lyso-PE), and 2% Rh-PE + 2% NBD-PE. (B) The effect of PEs in cationic liposomes on the fusion between cationic liposomes and cells in sucrose solution. Cationic liposomes composed of 50% DOTAP, 2% Rh-PE + 2% NBD-PE plus 46% either lyso-PE, POPE, DOPE, or dilin-PE. Control means no lipid was added.

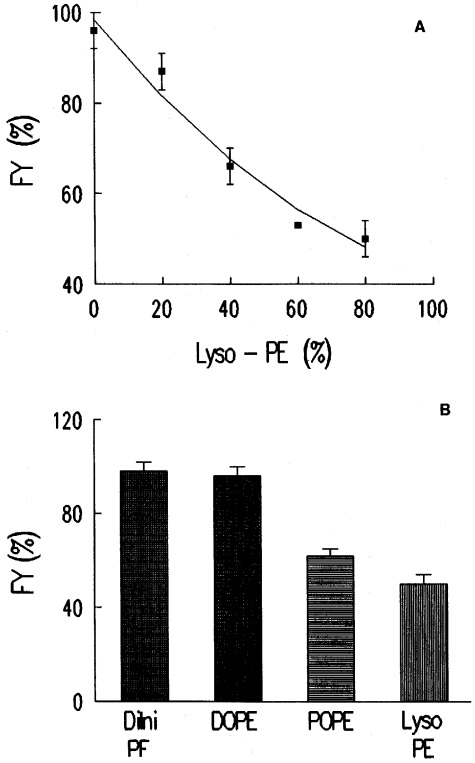

Fig. 8. (A) The effect of different percentages of lyso-PE in cationic liposomes (20% DOTAP + 80% (DOPE + lyso-PE)) on the fusion between sonicated PS liposomes (75% egg-PC + 23% PS + 1% RH-PE + 1% NBD-PE) and cationic liposomes (MLV) in 5 mM Hepes medium (pH 7.4). (B) The effect of PEs in cationic liposome (20% DOTAP + 80% PE) on the fusion between cationic liposomes (MLV) and sonicated PS liposomes (75% egg-PC + 23% PS + 1% Rh-PE + 1% NBD-PE) in 5 mM Hepes medium (pH 7.4).

3.5. Fusion between PS liposomes and cationic liposomes

We further tested the relationship between membrane fusion and membrane curvature in model membranes. Sonicated PS liposomes were fused with cationic liposomes with different curvature stresses. As shown in Fig. 8A and B, clear curvature-dependence of fusion, by energy transfer method, was demonstrated. The FY follows the order: dilin-PE > DOPE > POPE > lyso-PE. Also, the more lyso-PE in the liposome, the lower the FY. These results follow the same trend as that for cell–cell fusion, and cell–liposome fusion, as is likely related to curvature stress.

4. Discussion

Cationic lipids are known to interact and fuse with cell membranes [8,17], PS-containing liposomes [7], and to aggregate and fuse to some extent among themselves in the presence of polyanions [7,8]. This property has been exploited to mediate high efficiency transfection [11]. Although the exact mechanism of cationic liposome mediated gene transfer is not clear, it is believed that, at some stage of the process, the cationic liposomes fuse with either the plasma membranes or the endosome membranes [11,17]. Therefore, identifying the important factors affecting the fusion of cationic liposomes with cell membranes is a crucial step to improve the utilization of cationic liposomes as vehicles for gene and drug delivery [19,37].

Since the specific effects of cationic liposomes is expected to be mediated by electrostatic interaction, we first study the influence of the ionic strength of the suspension media. Indeed, we found that in ion-free sucrose solution, the cell–cell FY and the cell lysis are the highest, whereas in NaCl or PBS media, FY and cell lysis are reduced by about half (Fig. 1A and B). This finding is basically the same as reported [17]. In ionic solutions, the electric double layer of electrolytes screens out the field due to DOTAP, as described [4], thereby reducing the potency of cationic liposomes to bind to cells to induce cell–cell fusion and cell lysis.

Electrostatic interaction alone is not sufficient to cause cell–cell fusion. No cell–cell fusion was observed using pure DOTAP vesicles (data not shown). Additional energy is needed to overcome the hydration/steric barrier [1,38]. It has been reported that stress on lipid molecular packing due to curvature constrain reduces the fusion threshold [18,39–41], and that curvature stress attributed to helper lipids is said to influence the efficiency of cationic liposome mediated transfection [11,15,29]. To focus on the effect caused by acyl chain packing stress, we use a homogeneous series of helper lipids with the same headgroup, PEs, with different acyl chain saturation to vary the negative curvature stress on cationic liposomes [16,21,39]. In addition, lyso-PE, which has only one acyl chain and is supposed to contribute a positive curvature stress because of its small hydrophobic volume [30,35], is used in increasing percentages to reduce the overall negative curvature stress, without altering the headgroup composition.

We found that if different PEs were used as helper lipids, as shown in Fig. 4, the cell–cell FY are: dilin-PE > DOPE > POPE > lyso-PE. The curvature stress induced by these helper lipids follows the hydrophobic volume in the order of: dilin-PE > DOPE > POPE > lyso-PE, so the cell–cell FY follows the same trend as curvature stress. Cationic liposomes with lyso-PE are unable to induce cell–cell fusion. Also, as shown in Fig. 4, when increased amount of any helper PE is replaced by lyso-PE in cationic liposomes (up to 50%), the cell–cell FY decrease. These experiments provide convincing evidence that the cell–cell FY induced by cationic liposomes is controlled by the curvature stress imposed by the helper lipid. The higher is the curvature energy built within cationic liposome membranes, the higher the cell–cell FY.

To show that 50% lyso-PE caused primarily cell aggregation and not cell–cell fusion, we used the membrane dye mixing method [42,43]. We found that labelling 20%–30% of cells was sufficient for the assay. Under this condition, many aggregates had only one labelled cell among many unlabelled ones. Hence, it can be concluded that 50% lyso-PE do not induce cell–cell fusion, as show in Fig. 3.

How can cationic liposomes induce cell–cell fusion? It is possible that cell–cell contact between fusion partners is bridged by cationic liposomes, which in turn fuse with cells in the process. This idea

has been proposed by others [5,6]. If this assumption is correct, the fusion between cationic liposomes and cells should be observable, and the FY is expected to be curvature stress dependent as well. Our data supported this idea microscopically (Fig. 6) and spectroscopically (Fig. 7). Hence, cationic liposomes indeed fuse with fusion cell partners in the process of inducing cell–cell fusion, and the process is curvature stress dependent. In all likelihood, cationic liposomes act as a bridge, and themselves are absorbed into fusing cell membranes in the process.

To further verify the idea of the curvature-dependent membrane fusion, we repeated the experiments on model membranes. As shown in Fig. 8A and B, fusion between model membranes showed the same dependence on curvature stress. It is worth mentioning that charged lipid concentration (PS, DOTAP) had to be reduced from 50% to 20% to distinguish the curvature stress effect from other factors on model membrane fusion.

In this study of cationic liposome induced fusion between rabbit erythrocytes, we established that this cell–cell fusion process is dependent on the curvature stress imposed by helper lipids, and that the cell–cell fusion is mediated through cationic liposome bridges. The finding of curvature stress dependence of membrane fusion also in model membrane makes us believe that this is a general phenomenon in membrane fusion. The factors found to be important in controlling cationic liposome-induced cell–cell fusion and cell–liposome fusion can be used to optimize cationic liposome mediated drug and gene transfer methods.

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