Fluorescence measurements of fusion between human erythrocytes induced by poly(ethylene glycol)

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ABSTRACT The kinetics of poly(ethylene glycol) (PEG)–induced fusion between intact human erythrocytes was continuously monitored by a fluorescence lipid mixing method, utilizing the dequenching of the fluorescence probe, 1-oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-*yl*)amino]dodecanoyl] phosphatidylcholine (C_{12}-NBD-PC). The steady-state fluorescence intensity was detected from the surface of cells in a monolayer on an alcian blue–coated glass coverslip. The relief of fluorescence self-quenching after fusion between C_{12}-NBD-PC labeled and unlabeled intact erythrocytes was measured. The extent of fluorescence dequenching was normalized based on the measured concentration of probes in membranes, the projected partial dequenching due both to dilution by intercellular fusion, and the dilution between the inner and outer leaflets of membranes (flip-flop).

There was no significant increase in fluorescence intensity during PEG treatment of 5 min, at 4°C. Intensity increased immediately after the dilution of PEG, and reached saturation in 30 min. The efficiency of fusion increased with the increasing of PEG concentrations. Only 4% enhancement of saturated relative fluorescence intensity was detected in 25 wt% PEG-induced cell fusion; 23% enhancement in 30 wt%; and 66% enhancement in 35 wt%.

The transfer of fluorescent probes between membrane bilayer leaflets (flip-flop) was also monitored during the fusion process. Flip-flop was monitored in confluent monolayers as well as in isolated cells. There was no significant spontaneous flip-flop within 30 min of dilution. The relative fluorescence intensity enhancement contributed by the dilution of probes between fused labeled and unlabeled cells (at a 1:1 ratio) was found to account for only 39% of the observed final dequenching, whereas the contribution by flip-flop associated with cell fusion was found to account for 9%, and flip-flop without fusion contributed ~18%. A portion of the flip-flop is a consequence of hemolysis. Therefore, fluorescence dequenching measurements of fusion of whole cells must be interpreted with caution.

INTRODUCTION

Membrane fusion is involved in a variety of biological events (Poste and Allison, 1973; Blumenthal, 1987). Although many of the physiological functions mediated by fusion events are well known, only recently has the molecular mechanism of membrane fusion received the attention it deserves.

The recent development of fusion assays, using aqueous content mixing and lipid mixing, allows the sensitive and continuous monitoring of fusion kinetics (Wilschut et al., 1980; Hoekstra et al., 1984). The majority of studies using these assays dealt with artificial membrane systems, such as phospholipid vesicles. In some experiments isolated membrane vesicles, as a simplified biological membrane model, were also employed in investigations of membrane fusion (Hoekstra et al., 1984). Erythrocyte ghosts are a popular model for studies of the fusion mechanism of biological membrane, not only for their well-characterized lipid composition and protein distribution, but also for the absence of hemoglobin, which attenuates fluorescence. Although the composition of ghost membranes might be similar to that of the membrane of intact cells, most of the ghosts lose their phospholipid asymmetry during the treatment in a hypotonic buffer (Williamson et al., 1985) and thereby lose their side-specific fusion responses (Tullius et al., 1989). In addition, they may lose or change the composition or structure of their cytoskeleton during hemolysis. Some fusion studies showed that ghosts were easier to fuse than intact erythrocytes by poly(ethylene glycol) (PEG), calcium phosphate and other fusogens (Zakai et al., 1976; Hoekstra et al., 1985; Huang and Hui, 1986).

To obtain a better understanding of the actual membrane fusion mechanism between intact cells, we developed a fluorescence assay for monitoring the fusion kinetics of intact cells. PEG was used to induce fusion between intact erythrocytes. Fluorescence dequenching resulting from the fusion of labeled and unlabeled cells in a monolayer, with fluorescent phospholipid 1-oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]

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phosphatidylcholine (C_{12}-NBD-PC), was monitored during the fusion process. The kinetics of lipid flip-flop during the fusion process was also measured. The normalization of this new assay method is discussed.

EXPERIMENTAL PROCEDURES

Preparation of human erythrocytes

Human whole blood was obtained and used within 2 wk after it had been drawn. Blood was washed three times in Hank's balanced-salt solution (BSS) (Hanks and Wallace, 1949) to remove the plasma and buffy coat.

Incorporation of fluorescent phospholipid (C_{12}-NBD-PC) into erythrocyte membranes

C_{12}-NBD-PC was obtained from Avanti Polar Lipid, Inc. (Birmingham, AL). Insertion of the probes into membranes of erythrocytes was performed as follows. A determined amount of C_{12}-NBD-PC was dried under a stream of nitrogen gas and dissolved in ethanol. This solution was injected into the ambient medium of a cell suspension during vortexing. The ratio of ethanol to medium was limited to 1% (vol/vol) to avoid alcohol mediated effects on the membrane properties (Goldstein, 1984). This suspension was incubated in the dark for 1 h at room temperature, then washed in BSS, and centrifuged three times at 4°C. Nonincorporated C_{12}-NBD-PC in the supernatant was removed and discarded.

To determine the amount of C_{12}-NBD-PC that was incorporated, the membranes were extracted with chloroform/methanol/water by the Bligh-Dyer extraction method (Kates, 1986). An aliquot of the lipid extract (now in chloroform) was taken for fluorescence measurement. The fluorescence intensity curve was calibrated by using known concentrations of C_{12}-NBD-PC in chloroform. The total amount of phospholipid in the extracted lipid samples was determine by phosphate assay. The amount of C_{12}-NBD-PC incorporated into the membrane was expressed as a mole percent ratio of C_{12}-NBD-PC to total phospholipid.

Preparation of cell monolayer in cuvette

Mixed C_{12}-NBD-PC labeled and unlabeled cells in a one to one ratio were allowed to settle on an alcian blue-coated glass coverslip for 5 min. Unattached cells were removed by washing with BSS. The background fluorescence due to NBD-PC absorption on glass is <1% of the maximum intensity of the sample. PEG (mol dt 8,000) was obtained from Fisher Scientific Co. (Pittsburgh, PA) and used without further processing. The coverslip with an attached monolayer of cells, was gently immersed into a desired concentration of PEG in BSS and allowed to stand for 5 min, at 4°C. Immediately after washing out or diluting the PEG with BSS, the coverslip was put into the slot of a holder in the bottom of a cuvette. The slot was at an angle of 38–40° to the direction of the excitation light source. This angle was used to avoid the direct reflection of the excitation beam, which would occur at 45° (Fig. 1).

Fluorescence measurements

C_{12}-NBD-PC fluorescence was measured in an Aminco-Bowman (Urbana, IL) spectrophoto-fluorometer with an attached photon counter.

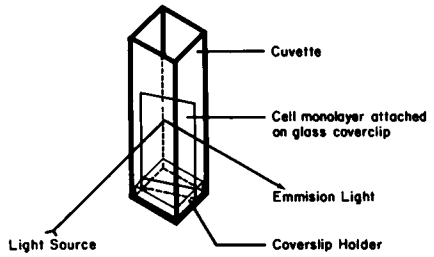

FIGURE 1 Schematic layout of the sample holder. An alcian blue-coated glass coverslip is inserted into a slot at the bottom of the holder. The slot makes an angle of 38–40° to the direction of the excitation light source.

The excitation wavelength for the fusion assay was 465 nm and the emission wavelength was 530 nm. An additional interference filter (model 03FIV028; Melles Griot, St. Irvine, CA) and cut-off filter (model 03FCG067; Melles Griot) were used respectively in the excitation and emission beam path to improve wavelength selectivity. All measurements were carried out at room temperature.

Normalization of the extent of fluorescence during fusion

For fusion kinetic measurements of a 1:1 mixture of C_{12}-NBD-PC labeled and unlabeled erythrocytes, the relative fluorescence intensity was normalized as follows. Within two or three hours after C_{12}-NBD-PC incorporation, cells kept at 4°C can be considered to be labeled only in the outer leaflets of the membrane (Seigneuret and Devaux, 1984). The initial probe concentration on the outer leaflets is represented by X. We assume the entire mixed population participates in fusion. Due to both the diffusion between C_{12}-NBD-PC labeled and non-C_{12}-NBD-PC labeled cells, and the flip-flop of fluorescence probes between bilayer leaflets of each individual cell, the fluorescence labeled areas would increase at the most by fourfold if no probe is lost to the medium from the membrane. The resultant fluorescence dye concentration in membranes would then be X/4. Thus, the maximum possible enhancement of fluorescence intensity I_{max} due to the fusion of the entire population is given by

$$I_{max} = 4I_{X/4} - I_X, \quad (1)$$

where $I_{X/4}$ and I_X are the fluorescence intensities from dye concentrations X/4 and X in the membrane, respectively.

The relative fluorescence intensity $Y(t)\%$ at any time t can be determined by,

$$Y(t)\% = \{[I(t) - I_X]/[4I_{X/4} - I_X]\} \times 100. \quad (2)$$

The concentration X was determined from fluorescence and phosphate assays. The values of I_X and $I_{X/4}$ were determined from a standard curve which was derived separately, using labeled cell monolayers of the same cell density but with different dye concentrations.

From Eq. 2, the fusion kinetics of a m:n mixture of labeled and unlabeled cells can be generalized by,

$$Y(t)\% = \{[I(t) - I_X]/[2(m + n)I_{mX/2(m+n)} - I_X]\} \times 100. \quad (2')$$

Thus, the relative fluorescence intensity $Y(t)\%$ at any ratio of fluorescence labeled and unlabeled cells can be normalized by the same equation.

Correction of fluorescence bleaching

To correct for the effect of bleaching during kinetic measurements, the continuous fluorescence intensity was recorded using samples of the same batch of labeled cells but without PEG treatment. This reduction of fluorescence intensity due to bleaching was used to correct $I(t)$.

Flip-flop measurement by trinitrobenzene sulfonate (TNBS) reaction

Human erythrocytes either treated with 35 wt% PEG or untreated, were diluted with 120 mM $NaHCO_3$-40 mM NaCl buffer, pH 8.6, for 30 min at room temperature, then were reacted with 1.5 mM TNBS. After treatment in ice for 30 min, cells were lysed, and the TNBS reaction was stopped with 1.2% Triton X-100/0.8 N HCl. The 100% value was measured from ghosts lysed with 1.6% Triton X-100 and then reacted with 15 mM TNBS. The control sample contained TNBS unreacted cells. TNP-Amino phospholipids were extracted by chloroform/methanol and determined spectrophotometrically by absorption at 410 nm. The data were corrected for the amount of total phospholipids by phosphate assays in each extraction (Albert and Yeagle, 1982).

RESULTS

Fluorescence characteristics of C_{12}-NBD-PC

Fig. 2 shows the excitation and emission spectra of C_{12}-NBD-PC solubilized in chloroform. The excitation wavelength peak was centered at 470 nm (a), and the emission wavelength peak was centered at 530 nm (b). These peak values were used in the determination of dye concentration in lipid extracts from membranes. The excitation and emission spectra of C_{12}-NBD-PC incorporated into the membranes of intact erythrocytes displayed a similar excitation (a') and emission (b') maxima, with no significant spectral shift.

Incorporation of C_{12}-NBD-PC into the membrane of intact erythrocytes

Upon incubation, the fluorescent amphiphile C_{12}-NBD-PC incorporated spontaneously into erythrocyte membranes. Fig. 3 shows the relationship between the final probe densities in the membranes and varied C_{12}-NBD-PC concentrations in the incubation medium with the same number of erythrocytes (1.2 μmol/ml of lipid). The increase of C_{12}-NBD-PC concentration in the incubation medium eventually led to the saturation of the C_{12}-NDB-PC to lipid ratio. Routinely, in our experiments, erythrocytes (1.2 μmol/ml of lipid) were incubated with 120 μg/ml C_{12}-NBD-PC. After an incubation of 1 h at room temperature, ~13–14% of the probe became membrane associated (Fig. 3). This is equivalent to the final probe density ~1.5 mol% with respect to the total phospholipid of erythrocyte membranes. Negligible flip-flop of lipid across the membrane occurs within relatively short (2 h) incubation periods at 4°C, therefore, the dye seems to remain predominantly localized in the outer leaflet (Seigneuret and Devaux, 1984). Thus, the fluorescent probe concentration in the outer leaflet would actually be 3 mol%.

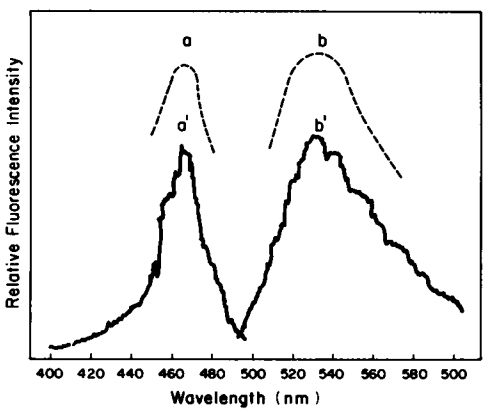

FIGURE 2 C_{12}-NBD-PC excitation spectrum detected at 530 nm (a) and emission spectrum generated by 465 nm excitation (b) respectively, in chloroform. Curves a' and b' show the excitation spectrum and emission spectrum of 1.5 mol% C_{12}-NBD-PC incorporated into the membranes of intact erythrocytes, which formed a monolayer on the alcian blue-coated glass coverslip.

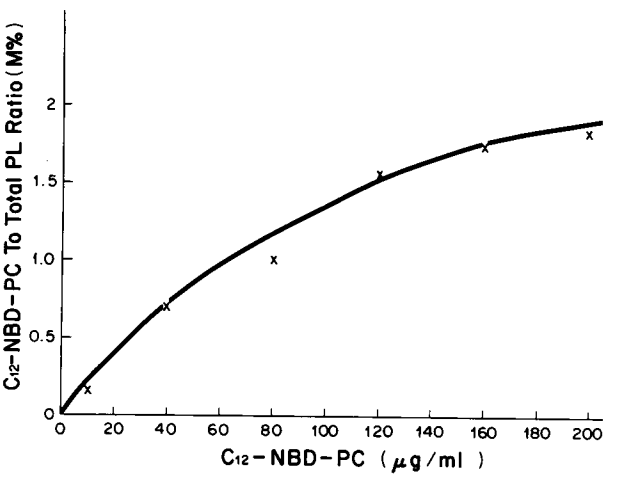

FIGURE 3 The relationship between the C_{12}-NBD-PC probe concentrations in the membranes and its concentrations in the incubation medium with the same quantity of erythrocytes (1.2 μmol/ml of phospholipid).

Self-quenching of C_{12}-NBD-PC fluorescence in the membrane of intact erythrocytes

To normalize the extent of fluorescence during fusion, the values of I_X, $I_{X/4}$ and even $I_{X/2(n+1)}$ must be deduced from the fluorescence quenching curve. It should be noted that, X, used in Eq. (2), should be equal to twice the C_{12}-NBD-PC to total lipid ratio. Relative fluorescence intensities were measured from monolayers of cells labeled with various C_{12}-NBD-PC concentrations. Fig. 4 demonstrates the relative fluorescence intensity as a function of the fluorescent probe concentration of C_{12}-NBD-PC incorporated into erythrocyte membranes. At concentrations of C_{12}-NBD-PC higher than 1 mol% in the membrane, the relative increase of the fluorescence intensity diminished due to the self–quenching effect. Clearly, working in the region of highest dye concentrations vis a vis fluorescence dequenching is desirable in these experiments. However, there are two limitations. First, it is difficult to incorporate high concentrations of NBD-PC probes into the erythrocyte membranes by the incubation method, as shown in Fig. 3. Second, high concentrations of fluorescence probes could change the properties of the membrane. Therefore, a probe concentration ~1.5 mol% was chosen for most subsequent experiments.

Kinetics of fusion induced by PEG

Fig. 5 *a* shows the fluorescence tracings obtained by continuous monitoring of the fluorescence intensity of a cell monolayer of C_{12}-NBD-PC labeled and unlabeled cells (at a 1:1 ratio) after pretreatment with 35 wt% PEG.

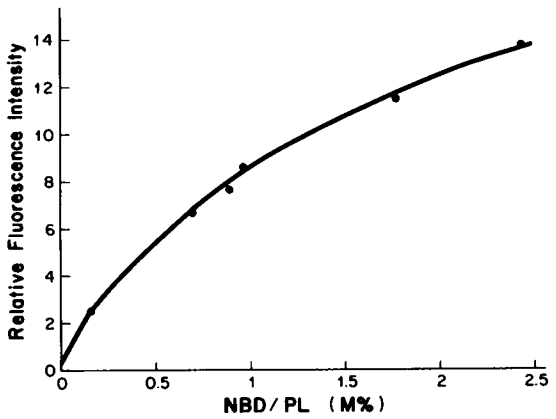

FIGURE 4 Self-quenching of C_{12}-NBD-PC fluorescence in the membrane of intact erythrocytes. Relative fluorescence intensities were measured from monolayers of cells labeled with varied C_{12}-NBD-PC concentrations.

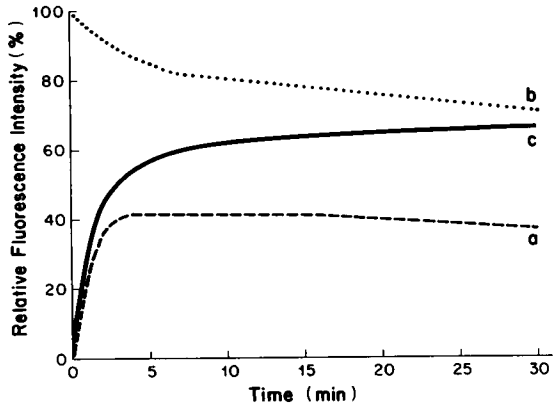

FIGURE 5 Curve (*a*) shows the fluorescence tracing obtained by continuous monitoring of fluorescence intensity of monolayer of mixed C_{12}-NBD-PC labeled and unlabeled erythrocytes (at 1:1 ratio) after dilution from the pretreatment of 35 wt% PEG. The same fluorescence intensities were detected before the treatment with PEG and after dilution (at the initial time 0). This value was defined as the base line of relative fluorescence intensity. Curve (*b*) indicates the loss of fluorescence intensity due to the bleaching effect under the same condition of (*a*) except for the pretreatment with PEG. The net enhancement of fluorescence intensity, after corrected for bleaching, is presented in curve (*c*).

Fig. 5 *b* indicates the loss of fluorescence intensity due to the bleaching effect of a control sample under the same condition of (*a*), except without the PEG pretreatment. Because the cells were labeled at a low C_{12}-NBD-PC density (1.65 mol% in this case), a strong light source was needed to obtain detectable fluorescence emission. Consequently, the bleaching effect could not be neglected. The net enhancement of fluorescence intensity, after correction for bleaching, is presented in Fig. 5 *c*. The same fluorescence intensity was detected before treatment with PEG and immediately after dilution (time 0). This value was defined as the base line of relative fluorescence intensity. The increase of fluorescence intensity started immediately after dilution. This evidence indicates that fusion begins right after dilution. The fluorescence intensity increased sharply, reaching a 55% relative intensity ratio within 5 min. From 5 to 10 min, the increase of fluorescence intensity was relatively slow. Between 10 and 30 min only a 5% increase was recorded.

The release of self-quenching was not a consequence of the C_{12}-NBD-PC probe dissolving in the aqueous medium, with or without PEG, because no fluorescence increase was detected in untreated labeled cells, nor immediately after PEG treatments. Therefore, the dequenching resulted predominantly from dilution of probes in membranes.

To verify the validity of Eqs. 2 and 2′, the fluorescence

tracings of two fusion experiments employing different ratios of C_{12}-NBD-PC labeled and unlabeled cells were compared using the same concentration of PEG (35 wt%). The relative changes in the fluorescence percentage resulting from dilution due to fusion and flip-flop of 1:1 and 1:4 ratios of labeled and unlabeled cells are shown in Fig. 6 *a* and *b*, respectively. These two traces almost overlap.

The fluorescence intensity change was markedly dependent on the PEG concentration used (shown in Fig. 7). The dequenching of fluorescence from samples pretreated with 35 wt% PEG was almost threefold higher than that of the samples pretreated with 30 wt% PEG. The enhancement of relative fluorescence intensity in the final point was only 4% in samples treated with 25 wt% PEG. Apparently, the threshold concentration of PEG required to induce human erythrocyte fusion is >25%. This is consistent with the results in Huang and Hui (1986). Treatments with higher concentration (>40 wt%) of PEG were also tried with this assay, but the continuous monitoring of fluorescence was interfered by hemolysis. The phenomenon of cell lysis in PEG-induced cell fusion from high PEG concentrations was also described by previous studies (Knutton, 1979).

Spontaneous flip-flop

Both the spontaneous flip-flop of fluorescent probes between the outer and inner leaflets of labeled cells, as well as the transfer between adjacent labeled and unlabeled cells provide for the relief of self-quenching. To determine which mechanism was operative in our assay, a monolayer of a 1:1 ratio of labeled and unlabeled cells without PEG pretreatment was intermittently monitored (to avoid fluorescence bleaching), but no significant increase in fluorescence intensity was detected (shown in Fig. 8 [*IV*]). The negative result indicates that there is no significant spontaneous flip-flop or transfer of the fluorescent lipid probe without PEG pretreatment.

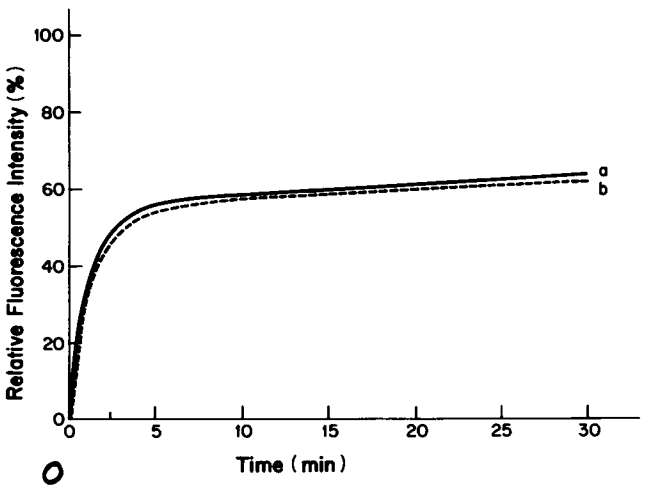

FIGURE 6 The corrected relative fluorescence intensity curves of 1:1 (*a*) and 1:4 (*b*) ratios of C_{12}-NBD-PC labeled and unlabeled human erythrocytes after dilution from 35 wt% PEG.

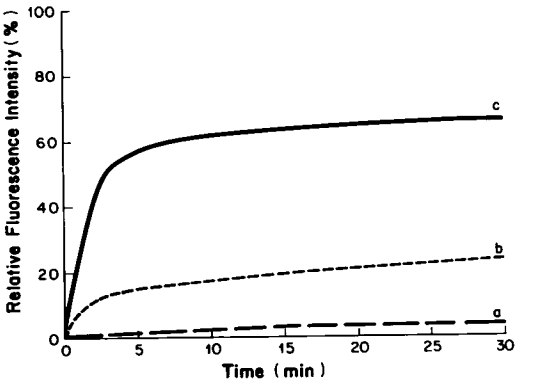

FIGURE 7 The kinetics of bleaching-corrected relative fluorescence dequenching due to PEG-induced fusion of intact erythrocytes with the PEG concentrations of (*a*) 25 wt%, (*b*) 30 wt%, and (*c*) 35 wt% respectively.

PEG-induced flip-flop between the membrane leaflets in isolated cells

A lower density of cells was used to isolate C_{12}-NBD-PC labeled cells in monolayer. <10% of the cells were

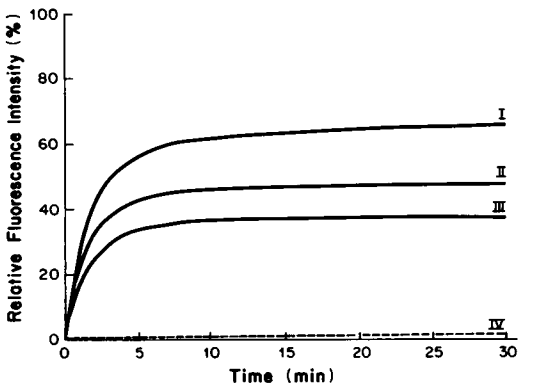

FIGURE 8 (*I*) The kinetics of bleaching-corrected relative fluorescence dequenching in C_{12}-NBD-PC labeled and unlabeled erythrocytes (at 1:1 ratio) following 35 wt% PEG-induced fusion of erythrocytes. (*II*) The relief of fluorescence self-quenching in C_{12}-NBD-PC labeled cells only following PEG pretreatment. (*III*) The relief of fluorescence self-quenching in the isolated C_{12}-NBD-PC labeled cell monolayer following PEG pretreatment. (*IV*) The relief of fluorescence self-quenching recorded from a monolayer of C_{12}-NBD-PC labeled cells without PEG pretreatment.

adjoining other cells as determined by optical microscopy counting. In this way, we restricted the opportunity of cell fusion, while treating the cells in the standard way with PEG (35 wt%). Thus, the relief of fluorescence self-quenching solely through probe transfer between bilayer leaflets of the isolated labeled cells was monitored. The results are shown in Fig. 8 (*III*). The relative fluorescence intensity increased by ~36%. The results revealed that PEG treatment could cause flip-flop between bilayer leaflets of isolated cells without fusion.

Fusion prompted flip-flop

A population consisting of C_{12}-NBD-PC labeled cells only were used to make a confluent cell monolayer in this experiment to determine the rate of fluorescence probe transfer between membrane leaflets following fusion. Fig. 8 (*II*) shows 48% relative fluorescence intensity enhancement by probe dequenching, due to flip-flop after pretreatment with PEG at 35 wt%. The enhancement of fluorescence intensity is greater than the enhancement in a monolayer of isolated cells (Fig. 8 [*III*]). The only difference between the confluent cell monolayer and isolated cell monolayer is that the latter, without cell contact, does not provide the cells any opportunity to fuse. Therefore, the increased fluorescence intensity of the confluent monolayer, relative to isolated cell monolayer, is caused by the phenomenon of cell fusion increasing the rate of flip-flop between the bilayer leaflets.

Flip-flop of amino-phospholipids

To prove that flip-flop is not restricted to fluorescent probes but also to other phospholipids, upon addition and dilution of PEG, we used TNBS to react with membrane amino-phospholipid in the outer monolayer of human erythrocytes in suspension. The percentages of amino-phospholipid in the outer leaflet of membranes, with and without PEG treatment, were 26 ± 2% and 20 ± 2%, respectively. Therefore, it appears that ~6% of the total amino group containing phospholipids (PE and PS) flip-flop from the inner monolayer to outer monolayer 30 min after the dilution of PEG. This is less than what we expected. Because this assay is subject to errors due to membrane leakage, only qualitative assessment can be made. Nevertheless, the result shows that PEG did mediate some degree of phospholipid flip-flop. Moreover, it indicates that the phenomenon of flip-flop happens not only in the cells adhered to the glass slide, but also in cells suspended in solution.

Various mechanisms which contribute to fluorescence intensity enhancement

In comparing the dequenching kinetics of C_{12}-NBD-PC in labeled, and mixed labeled and unlabeled cells (at 1:1 ratio), it is apparent that dilution of C_{12}-NBD-PC due to PEG-induced flip-flop contributed partly to the enhancement of fluorescence intensity shown in Fig. 8 (*I*). We construct a simplified model to explain the various factors contributing to the fluorescence dequenching, using the case of 1:1 mixture of labeled/unlabeled cells as an example. These contributions are calculated from the end (infinite time) points in Fig. 8, and plotted as a pie chart in Fig. 9.

Section I (area shaded by inclined lines) in Fig. 9, which comprises 66% of the pie, represents the end point of curve I in Fig. 8. It is the relative fluorescence intensity enhancement due to the fusion of 1:1 mixture of labeled and unlabeled cells, plus possible flip-flop, induced by 35 wt% PEG. However, the dequenching due to flip-flop could only be detected from a portion of the population, i.e., from labeled (fused or unfused) cells, and unlabeled cells that had fused with labeled cells.

Section III (stippled area) in Fig. 9 represents the end points (36%) of the curve III of Fig. 8. This section of the pie represents the relative fluorescence intensity enhancement of labeled, isolated cells treated with 35 wt% PEG. Thus, flip-flop in the entire population of unfused cells could be detected. One half of this 36% should be detectable in section I because only half of the cells in

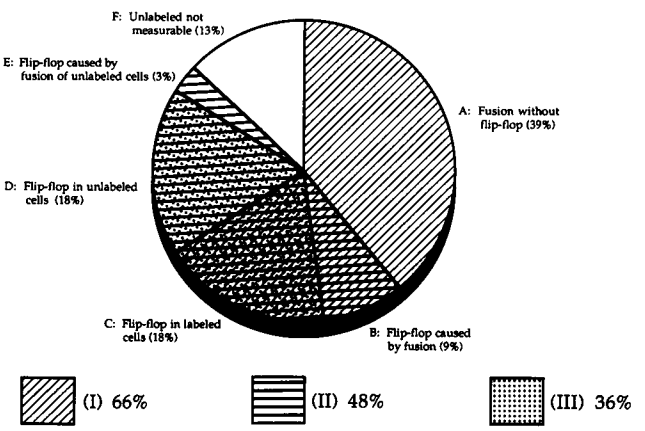

FIGURE 9 Contribution of relative fluorescence intensity at 30 min following PEG pretreatment. *I*, *II*, and *III* are obtained from final values of curves *I*, *II*, and *III* in Fig. 8. Each of the segments is expressed as a percentage of the total dequenching should the entire population of cells undergo fusion and flip/flop. Overlapping segments indicate common subpopulations observable under each experimental condition. Segment *I* is thereby divided into three parts (*A*, *B*, and *C*). The significance of each part is given in the text and in the legends of Fig. 10.

section I are labeled. This portion (18%) is designated section C (flip-flop in labeled cells) whereas the other half is designated section D (flip-flop in unlabeled cells).

Section II (horizontal lines) in Fig. 9 represents the end point of the curve II of Fig. 8. It comprises 48% of the pie representing the relative fluorescence enhancement of adjoining labeled cells treated with 35 wt% PEG. The difference between section II and section III is the inclusion of fusion-induced flip-flop in the former. This accounts for 12%. Out of this 12%, only ¾ (i.e., 9%) is detectable within section I. This is the result of fusion of labeled/labeled and labeled/unlabeled cell pairs at 1:1 ratio, if only pairwise fusion is considered. This section is designated B in Fig. 9. The remaining ¼ (i.e., 3%) of fusion-induced flip-flop is not detectable by the experiment pertaining to section I because it represents fusion of unlabeled/unlabeled cell pairs. It is placed outside sections I and III, and is designated section E in Fig. 9. The area of the pie outside section I, including the unshaded area, represents the fluorescence enhancement that would have been detectable should the entire population in I undergo fusion and flip-flop.

The formulae used to deduce various contributions to fluorescence intensity enhancement are given in the legend of Fig. 10. The curves *A–E* in Fig. 10 are obtained from the algebraic sums of curves I, II and III in Fig. 8. The curves represent the sections of same names in Fig. 9.

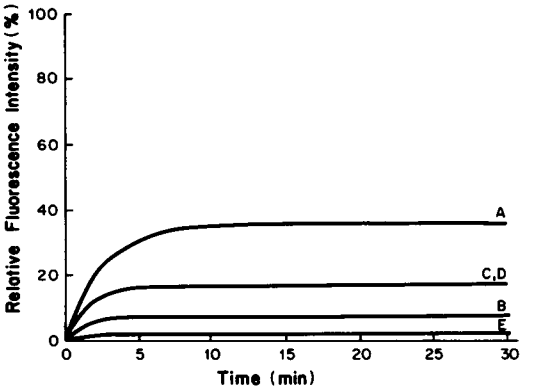

FIGURE 10 (*A*) Relative fluorescence enhancement contributed by PEG-induced fusion of C_{12}-NBD-PC labeled and unlabeled cells (at a 1:1 ratio) without counting probe transfer between bilayer leaflets. (*B*) Relative fluorescence enhancement contributed by probe transfer between bilayer leaflets of fused cells after PEG pretreatment. (*C*) Relative fluorescence enhancement contributed by probe transfer between bilayer leaflets not due to the fusion. (*D*) Relative fluorescence enhancement contributed by probe transfer between bilayer leaflets not due to the fusion, which cannot be detected in Fig. 8. This contribution of relative fluorescence enhancement can be assumed to be equal to the value of (*C*) due to the 1:1 ratio of C_{12}-NBD-PC labeled and unlabeled cells. (*E*) Relative fluorescence enhancement contributed by probe transfer between bilayer leaflets of fused cells after PEG pretreatment, which cannot be detected in Fig. 8. The possibility of fluorescence enhancement contribution from the fusion between these unlabeled cells is ⅓ of the case (*B*). *A–E* are obtained from the experiment curves *I*, *II*, and *III* in Fig. 8 by the following formula: $A = I - \frac{1}{2} \cdot (III) - \frac{3}{4} \cdot (II - III)$; $B = \frac{3}{4} \cdot (II - III)$; $C = D = \frac{1}{2} \cdot (III)$; $E = \frac{1}{4} \cdot (II - III)$.

DISCUSSION

The continuous and quantitative monitoring of the fusion process in intact cells, especially in the initial stages, was crucial to the study of the fusion mechanism in biological systems. The assay described in Experimental Procedures was developed for continuous and sensitive monitoring of membrane fusion. This method is especially applicable in studying intact cells. In previous studies of the PEG-induced fusion of intact erythrocytes, only light microscopic examination was applied to monitor the processes of lipid mixing (Ahkong and Lucy, 1986). The conventional fluorometric measurements of fusion kinetics could not be accurately used in the intact-cell system, due to the fact that fluorescence emission be attenuated by the scattering and inner filtering effects of the cell cytoplasm. In this fluorescence assay, the emission fluorescence was detected from the surface of cell monolayers, at the same side, but at an angle of only 38–40° from the excitation. In this way, not only could most of the direct reflection and scattering of the excitation beam be avoided, but also the unattenuated fluorescence emission from the monolayer surface could be obtained.

By using the present assay method, the fluorescence emission was obtained, unattenuated, from only one side of the cell, vis à vis the upper monolayer surface. However, the fluorescence intensity, thus obtained, is lower than that by the conventional method, in which the fluorescence was obtained from the whole sample suspension. To increase the fluorescence signal, a stronger light source had to be employed. Both C_{12}-NBD-PC (excitation and emission wavelengths of 475 and 530 nm, respectively) and octadecyl rhodamine B chloride (R_{18}) (excitation and emission wavelengths of 560 and 590 nm, respectively) were used as lipid probes in our initial experiments. For R_{18}, the excitation and emission wavelengths differed only by 30 nm. It proved difficult to separate the excitation and emission spectra. Even when interference and cutoff filters were used, the excitation and the emission spectra still overlapped. This probe was later abandoned in favor of C_{12}-NBD-PC, which has well separated excitation and emission spectra (shown in Fig. 2).

Both C_{12}-NBD-PC and *N*-NBD-PE are widely used in lipid-mixing assays, but C_{12}-NBD-PC is preferred over *N*-NBD-PE. One reason is that we would like to keep the

lipid composition asymmetry of erythrocytes as close to nature as possible; another reason is that the fluorescent groups in the C_{12}-NBD-PC locate just below the polar/hydrocarbon interface, whereas those of *N*-NBD-PE are at the polar-hydrocarbon interface (Chattopadhyay and London, 1987). Thus, the fluorophore in C_{12}-NBD-PC is less sensitive than in *N*-NBD-PE to either any chemicals in the medium, or the hemoglobin inside of cells.

PEG was found to interfere with fluorescence of ANTS (Parente and Lentz, 1986). In our PEG-induced C_{12}-NBD-PC mixing experiment, the residual PEG on the cell monolayer after the treatment with PEG was washed away with BSS before the cover glass was inserted into a BSS-filled square curvette. The effect of PEG interference proved to be very limited. This is supported by the fact that the initial fluorescence intensity before PEG treatment was almost the same as the intensity immediately after treatment (at time 0) in the same monolayer sample.

In the calibration of conventional lipid-mixing assays for vesicle fusion, the residual fluorescence of the membranes was taken as the zero level, and the value obtained after the addition of Triton X-100 as 100% (infinite dilution) (Hoekstra et al., 1984) This does not apply to the intact-cell system, especially monolayers. Our calibration is dependent on the fluorescence curve of a given probe's densities in the membranes (Fig. 4) and the assumption of probe transfer between bilayer leaflets (flip-flop) in intact-cell systems. In our experiment, the probe density on the membrane was limited to <2.5 mol%, due to the two limitations previously mentioned in the Results section. Therefore, the dequenching effect was not as pronounced as if a higher probe density had been used. According to the determination of the quenching of C_{12}-NBD-PC in vesicles, if the mole percent of the probe was >10%, the fluorescence intensities would become an inverted function of the probe densities (Nichols and Pagano, 1981), and result in a much more sensitive dequenching assay. Unfortunately, we must be satisfied with a much lower sensitivity range of the dequenching curve.

Different ratios of labeled and unlabeled cells were used in PEG-induced fusion experiments to verify the normalization equation. Although the initial fluorescence intensities at different labeled and unlabeled cell ratios were not the same, the enhancement percentage of the final relatively fluorescence intensity, which is determined by the fusion, flip-flop, and probe diffusion rates alone, and not by the ratio of labeled and unlabeled cells, should be the same. The ratio effect is, thus, normalized with their respective I_{max} (i.e., fluorescence intensity enhancement by the relief of fluorescence self-quenching after complete dilution by lipid mixing and flip-flop). The coincidence of the curves in Fig. 6 indicates the validity of Eqs. 2 and 2'.

The kinetics of PEG-induced fusion (Fig. 5) showed that C_{12}-NBD-PC mixing was initiated at the time of dilution. It has been reported that mere aggregation of the cells might cause lipid transfer (Wojcieszyn et al., 1983). In our experiment, no significant changes of fluorescence intensity could be detected after the cell monolayer was incubated with PEG for 5 min at 4°C, indicating an insignificant probe exchange. This is somewhat surprising, considering that a rapid spontaneous lipid probe exchange was observed between vesicles (Nichols and Pagano, 1981). However, the probe and the concentration we used (1.5–3 mol% of C_{12}-NBD-PC) is considerably lower than theirs (50–100 mol% of C_6-NBD-PC). A recent report (Longmuir and Maliniak, 1989) shows that phospholipid transfer between liposomes and cells is almost entirely collision dependent. Because cells in our experiment are attached to a glass surface, a much lower probe transfer rate is expected. The fact that true fusion rather than probe transfer during cell aggregation is responsible for the transport of probes from labeled to unlabeled cells is supported by microscopy evidence, as described in an accompanying paper.

Various possibilities of fluorescent phospholipid probe transfer between inner and outer leaflets of the cell membrane (flip-flop) during PEG-induced fusion were investigated in parallel experiments. Translocation of highly polar phospholipid headgroup through a hydrophobic membrane interior is energetically unfavorable. However, the phenomenon of lipid flip-flop has been observed in biological membranes. The transmembrane movement, or flip-flop of fluorescence lipid probes has been the subject of considerable experimentation. This phenomenon of lipid flip-flop was observed in our experiments (Fig. 8). This effect may lead to a further dilution of the probes, and, therefore, cause extended fluorescence dequenching, which is not attributable to fusion in lipid mixing assays. Therefore, fluorescence dequenching due to flip-flop must be distinguished from that due to fusion.

The rate of spontaneous phospholipid flip-flop in erythrocyte membranes is both time and temperature dependent (Bloom and Webb, 1983). The rate at room temperature is very slow, and could be in terms of hours or days, depending upon the type of lipid (Seigneuret and Devaux, 1984). As a precaution, before performing an experiment, all fluorescence labeled erythrocytes were kept at 4°C to reduce spontaneous flip-flop. During PEG treatment the monolayers containing labeled cells were kept below 4°C. This not only limited fluorescent lipid probe flip-flop between the membrane leaflets as mentioned previously, but also reduced cell lysis and fluorescent probe transfer between labeled and unlabeled cells. Spontaneous flip-flop without PEG treatment, was also monitored during a control experiment for 30 min at room temperature, but no significant increase of fluorescence intensity was de-

tected. Therefore, within time frames of <2 or 3 h, fluorescent probes can be considered to be incorporated only in the outer leaflets of labeled cells at low temperature.

Having established these conditions and controls, we observed flip-flop promoted by PEG in the experiment with a monolayer of isolated labeled cells. In this case, the relative fluorescence intensity increased by 36% at 30 min after PEG pretreatment. Clearly, PEG induced considerable flip-flop across the bilayer. To ascertain that flip-flop contributes to a major portion of this dequenching, cells were labeled with varying amounts of C_{12}-NBD-PC (1.5–3 mol%). The dequenching results from different experiments are consistent. The pronounced PEG-induced flip-flop may relate to a phenomenon that has been observed during the formation of erythrocyte ghosts. The ghosts lose their phospholipid asymmetry due to hemolysis during treatment with hypotonic solution (Williamson et al., 1985). During the period of dilution after treatment with PEG, some extent of hemolysis occurred (a trace of hemoglobin was found in the supernatant). Even while attempting to prevent leakage by treating cells with a relatively lower concentration of PEG (35 wt%) in 4°C, slight leakage cannot be avoided. This may be related to the extensive flip-flop observed in isolated cells (Fig. 8 [curve *III*]). Fusion related hemolysis may then contribute to the difference curve II and curve III.

The numerical percentages quoted in Fig. 9 are used only as an example. The numerous assumptions used to derive these percentages (i.e., only 1:1 fusion are considered, cells are homogeneously labeled, etc.) render the numerical results to approximations only.

In conclusion, there is considerable flip-flop associated with PEG treatment, and with PEG-induced cell fusion; both flip-flop and fusion begin at the time of PEG dilution. This is the first quantitation of the flip-flop contribution to the fluorescent probe mixing fusion assay. Experiments using lipid mixing assays to quantitate fusion of cells should be interpreted with this in mind.

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