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INTERACTION OF HUMAN ERYTHROCYTE GHOSTS OR LIPOSOMES WITH POLYETHYLENE GLYCOL DETECTED BY FLUORESCENCE POLARIZATION

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Summary: The membrane fluidity of human erythrocyte ghost was temporarily increased by the addition of polyethylene glycol with molecular weight of 7500. On the other hand, the fluidity of dipalmitoylphosphatidyl choline bilayer liposomes was monotonously decreased by the addition of polyethylene glycol. Fusion of liposomes was inhibited by the interaction with polyethylene glycol. The temporary increase in membrane fluidity of erythrocyte ghosts was considered to be the result of the clustering of membrane-bound proteins which is believed to be one of the most important sequences in cell fusion.

INTRODUCTION

The fusion of biological membranes is a widely applicable technique in the fields of biology, immunology, cell engineering and so on. A mechanism of the fusion is not clearly understood in spite of its extensive applications. Recently, many efforts have been made to induce the membrane fusion by chemicals instead of viruses. Polyethylene glycol (PEG) was initially used as a fusogen for plant protoplasts[1], and was also recognized as a potent fusogen for other cell lines such as human, mouse and hen erythrocytes, fibroblasts, lymphocytes, myeloma and so on[2-6]. The change of membrane fluidity in a sequence of the membrane fusion has not been sufficiently investigated yet, although its importance has been suggested in the virus-induced and chemically induced cell fusion [7-9]. In this communication, the effects of PEG on the changes of membrane fluidity have been investigated using plasma membrane and artificial membrane

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mainly by means of the fluorescence polarization technique, and the mechanism of the membrane fusion induced by PEG has been discussed from a new viewpoint of the interpolymer complex formation.

MATERIALS AND METHODS

Human erythrocytes were isolated from whole blood of adult donors. After removing plasma by centrifugation (3 000 rpm for 5 min at 4 °C), erythrocytes were washed and centrifuged three times with 10 volumes of 0.9 % NaCl solution to remove buffy coats. The erythrocytes were lysed with tris buffer (30 mOs, pH 7.4) according to Dodge et al.[10]. Erythrocyte pink ghosts were collected by centrifugation (12 000 rpm for 20 min at 4 °C).

Tris buffer solution of sodium 8-anilino-1-naphthalene sulfonate (ANS)(ANS concentration = 4.0×10^{-6} mol/l, pH 7.4) was added to a suspension of erythrocyte ghosts (2×10^6 cells/ml) by equivolume and then the mixture was incubated at 37 °C for 60 min with gentle shaking. ANS-labeled erythrocyte ghosts were once washed with the same buffer and re-suspended in the same buffer for fluorescence measurements.

A spectroscopic grade ANS was purchased from Tokyo Kasei Co., Ltd. and used without further purification. PEG(#6 000) with average molecular weight of 7 500 was purchased from Wako Pure Chem. Co., Ltd.. PEG was purified by the reprecipitation method as described previously[11]. Dipalmitoylphosphatidyl choline (DPPC) was purchased from Sigma Chem. Co.. The purity of DPPC was checked by thin layer chromatography. Samples which gave only one spot on the TLC plate were used without further purification. Dispersion of DPPC in D_2O was sonicated in water bath (20 °C) for 30 min at 45 W with a sonicator UR-200P (Tomy Seiko Co., Ltd.). Determination of radius of DPPC liposomes was carried out by the same method as previously reported[12].

Fluorescence intensities were measured by a JASCO FP-550 fluorescence spectrophotometer. Fluorescence intensities were corrected for light-scattering back ground using an ANS-free blank solution containing ghosts and PEG. The light at 360 nm was used for excitation of membrane-bound ANS and emission intensities were detected at 476 nm. The slit widths of excitation and emission were 5 nm.

Proton magnetic resonance spectra were measured by JEOL-100 MHz NMR spectrometer with temperature control apparatus. Chemical shifts were measured using an external capillary of 1 % Me_4Si in $CDCl_3$.

Freshly prepared erythrocytes were re-suspended in Eagle's buffer solution at 1 % of hematocrit and 1 ml of this suspension was centrifuged (1 200 rpm, 5 min at 5 °C). The supernatant solution was completely removed and then 40-50 wt% of PEG solution was mixed with the precipitates. After this suspension was incubated at 37 °C for 1-3 min, 10 ml of Eagle's buffer solution prewarmed at 37 °C was added to it and was incubated further at 37 °C for 5 min. Then, fused erythrocytes were collected by centrifugation (1 200 rpm for 5 min at 5 °C) and fixed with 1 % of glutaraldehyde for the optical microscopy. In case of cell fusion, commercial grade PEG was used without any purification because the purified PEG had no ability of cell fusion but a strong ability of cell aggregation[11].

RESULTS AND DISCUSSION

The membrane fluidity of human erythrocyte ghosts was detected as the changes of fluorescence polarization according to Shinitzky et al.[13,14]. The polari-

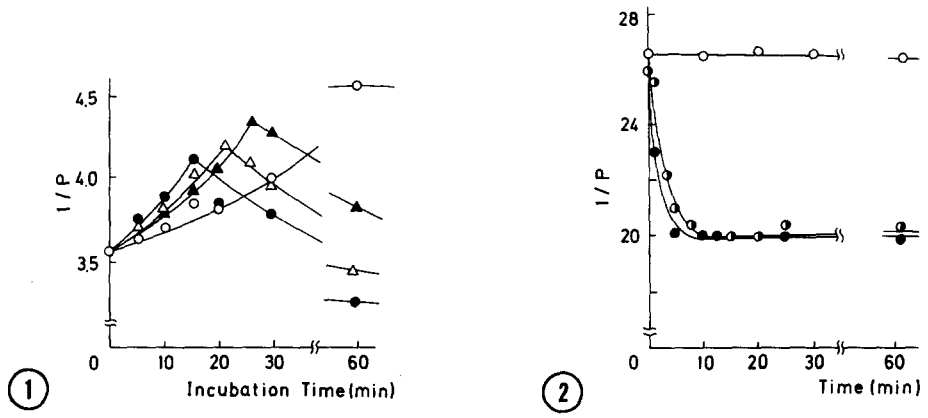


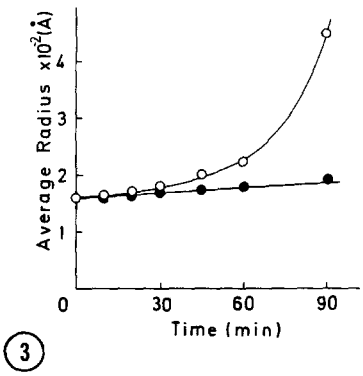
Fig. 1. Effect of PEG with molecular weight of 7 500 on the fluidity of human erythrocyte ghost at 37 °C. ANS-labeled ghost suspensions were mixed with tris buffer solution of PEG and fluorescence intensity was measured immediately. Mixing and measurement were taken at 37 °C. [ANS] = 1.0 x 10^{-6} M, [ghost] = 1 x 10^{6} cells/ml. PEG concentrations were 0.02 (○), 0.09 (▲), 0.9 (△) and 20 (●), respectively in wt%.

Fig. 2. Fluidity change of DPPC liposomes after mixing with PEG solution at 50 °C. Dispersion of ANS-labeled DPPC liposome was added to PEG solution with equivolume mixing and fluorescence intensity was measured. Measurements were taken at 50 °C. Final concentration of DPPC and ANS were 1.0 wt% and 1.0 x 10^{-6} M, respectively.
○ : DPPC only, ◑ : DPPC + PEG (0.1 wt%), ● : DPPC + PEG (1.0 wt%)
Much more concentrated PEG solutions gave the aggregation of DPPC liposomes instantaneously.

zation (P) of fluorescence from ANS was changed by mixing erythrocyte ghosts with PEG as shown in Fig. 1. The inverse of polarization (1/P) temporarily increased and then decreased with incubation time. PEG solution with a concentration of 0.02 wt% made the fluidity of ghost membranes increase and 1/P reached a maximum point 60 min after incubation as shown in Fig. 1 (open circles). On the other hand, a maximum was observed 15 min after incubation with 20 wt% PEG solution (see Fig. 1, closed circles). There appeared an obvious dependence of PEG concentration on the time at which 1/P reached a maximum. In other words, the fluidity of ghost membranes was quickly increased by adding a concentrated PEG solution. The maximum values, $(1/P)_{max}$, by themselves also depended on the concentration of PEG applied. That is, $(1/P)_{max}$ was 4.65 in case of 0.02 wt% PEG solution but it was not so large (4.13) in case of the concentrated PEG solution (20 wt%). The membrane fluidity increased slowly but markedly when relatively diluted PEG solution was added.

The effect of PEG on the fluidity change of liposomes was quite different from that of erythrocyte ghosts. The fluidity parameterm 1/P, monotonously increased and reached a constant value when PEG was added as shown in Fig. 2. The interaction of PEG with DPPC liposomes has been already studied by NMR, fluorescence and DSC measurements[12,15]. It was clarified that the mobility of DPPC molecules in liposome was depressed by the interaction with PEG[12]. The difference in the PEG-induced membrane fluidity change between liposome and ghost membranes should be based on the existence of membrane proteins. It is well known that soluble polymers can interact with each other through Coulombic force, hydrogen bond and/or hydrophobic interaction force to form interpolymer complexes in solution[16-20]. Similar polymer complexes are known to be formed by mixing PEG with proton-donating polymers[19,20]. Recently, we observed the complex formation of PEG with dextran, dextrin or inulin through hydrogen bond in an aqueous medium[21]. The same phenomenon should be observed on biological membranes. As the surface of ghost membranes is surrounded with polysaccharide chains, added water-soluble polymers like PEG or dextran can interact with the polysaccharide chains or hydrophobic parts of membrane-bound proteins and form a kind of interpolymer complexes. PEG is known to cause cell aggregation[22] and the clustering of membrane-bound proteins on biomembranes[23]. Water-soluble polymers also interact with lipid molecules on the membrane surface[12]. Fig. 2 demonstrates that PEG interacts with the lipid molecules of liposomes to decrease the membrane fluidity. The same interaction might be realized on plasma membranes on which intrinsic proteins are clustered and phospholipid molecules are denuded. This must be the reason why the fluidity of ghost membranes was decreased after the temporary increase by adding PEG, as shown in Fig. 1.

The fusion of DPPC liposomes should be inhibited if PEG suppressed the mobility of DPPC molecules. The effect of PEG on the fusion of DPPC liposomes was examined by means of NMR spectroscopy. Fig. 3 shows that the fusion of DPPC liposomes was actually inhibited by the addition of PEG. Liposome fusion is considered to be taken place as a result of the dissipation of surface energy



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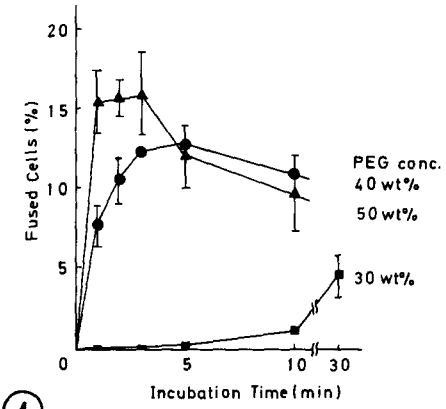

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Fig. 3. Changes of radius of DPPC liposome at 5 °C under the presence (●) and absence (○) of PEG. The radius of liposome was calculated from the ratio of NMR signal intensities of choline methyl groups located on the outward and inward facing membranes.
[PEG] = 0.17 wt%, [DPPC] = 1.0 wt%.

Fig. 4. Effect of PEG concentration and incubation time on the fusion of human erythrocytes at 37 °C.
[erythrocyte] = 1 x 10^7 cells/ml, [PEG] = 50 (○), 40 (●) and 30 (△), respectively in wt%. Standard deviations of each plot of the fusion index indicated are 3 - 5 %.

of liposomes[24,25]. PEG is known to lower the surface energy of liposomes[26]. Fig. 3 suggests that PEG stabilizes the membrane of liposomes so that the membrane fusion of liposome is prevented. On the other hand, in case of erythrocyte membranes, a higher concentration ($\gtrsim$50 wt%) of PEG induced the membrane fusion. However, a longer incubation time ($>$5 min) resulted in the decrease of the degree of fusion (see Fig. 4). The following mechanism of the membrane fusion is considered from the above mentioned results. At the initial stage of fusion, PEG induces the clustering of membrane-bound proteins so that lipids are exposed to water phase. The lipid phase of the denuded membrane is similar to an intact liposome membrane. At the second stage, the denuded membranes are fused. When an excess of PEG further interacts with the lipid layer, the membrane fluidity is decreased and the membrane fusion is suppressed as the result. However, it was found that the fusion was induced again by washing of PEG-treated cells. It was already reported that an excess of PEG should be removed from the surface of bio-

logical membranes in order to perform the fusion of the aggregated cells[23].

It was also observed that the fluidity of PEG-treated cells was recovered by washing

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