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Chemical co-treatments and intramembrane particle patching in the poly(ethylene glycol)-induced fusion of turkey and human erythrocytes

Shi Kun Huang and Sek Wen Hui *

Electron Optics Laboratory, Biophysics Department, Roswell Park Memorial Institute, Buffalo, NY 14263 (U.S.A.)

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Several chemical co-treatments were used to lower the threshold concentrations of poly(ethylene glycol) (PEG) required to induce fusion between turkey erythrocytes and between human erythrocytes. Concanavalin A was used in conjunction with 25% (w/w) PEG to induce turkey erythrocyte fusion. The fusion percentage increased with increasing concentrations of concanavalin A and the duration of concanavalin A treatment. In samples with high percentages of fusion, numerous hemispherical intramembrane particle-free zones (bubbles) in the plasma membrane were revealed by freeze-fracture electron microscopy. However, concanavalin A treatment did not facilitate fusion between human erythrocytes even at 35% PEG, although slight intramembrane particle patching was observed under this condition. Spermidine (0.05% w/v), trichloroacetic acid (100 mM) and ethanol (4% v/v) were found to promote fusion of human erythrocytes in 25% PEG. In all of these cases, intramembrane particle patching was observed by freeze-fracture electron microscopy in the presence of PEG. When applied alone, only ethanol caused a slight intramembrane particle patching. Neither dimethylsulfoxide (2% v/v), lysophosphatidylcholine (lysoPC, 0.15 mM), nor polylysine (mol. wt. 1000–4000, 0.05% w/v) promoted fusion of human erythrocyte in 25% PEG. None of these chemical treatments, alone, or in combination with PEG, caused intramembrane particle patching. We conclude that the positive effect of chemical treatments on PEG-induced cell fusion is closely related to the formation of intramembrane particle-free zones on the plasma membrane.

Introduction

High-molecular-weight polymers of poly(ethylene glycol) (PEG) are commonly used to fuse a wide variety of cells, such as plant protoplasts [1], hen erythrocytes [2], human erythrocytes [3] and other mammalian cells [4]. Usually, aqueous solutions of PEG in excess of 35% are required to induce cell aggregation and fusion; maximum fusion efficiency occurs at a PEG concentration between 40 and 50%. If the concentration of PEG is below 35%, no fusion can be induced [5].

Many factors contribute to the fusogenic efficacy of PEG [6,7]. The creation of intramembrane particle-free zones, and the osmotic swelling of cells after the formation of bilayer contacts during the PEG treatment are considered key steps in PEG-induced fusion of cell membranes [8]. At low concentrations of PEG, intramembrane particle-free zones were not observed [9] and no fusion occurred. However, when treated with high concentrations of PEG, turkey erythrocytes were

* To whom correspondence should be addressed.

haemolysed [3]. Thus, turkey erythrocytes could not be fused with PEG alone [10].

Concanavalin A is an agglutinating factor known to promote attachment of lipid vesicles to eukaryotic cells before PEG-induced fusion [11]. Dimethylsulfoxide (DMSO) and lysoPC are lipid-soluble fusogens. It has been reported that DMSO induces hen erythrocyte fusion [2] and lysoPC induces phospholipid vesicle fusion [12]. The polyamines, spermine and spermidine have been used as modulators to promote aggregation and fusion of certain vesicle types [13,14]. Trichloroacetic acid as a chaotropic ion provides a highly effective means for resolution of membranes and increasing the water solubility of particulate proteins [15]. Ethanol is known to penetrate the core of the bilayer and disorder biomembranes [16]. In this study, we investigate if and how the threshold concentration of PEG necessary to induce fusion of turkey and human erythrocytes can be lowered by a combined treatment with these chemicals. From the concerted actions of one of these chemicals with PEG, we hope to further our understanding of the factors controlling PEG-induced membrane fusion.

Materials and Methods

Chemicals

PEG (mol. wt. 8000) was purchased from Fisher Scientific Company. Concanavalin A (Con A) was purchased from Vector Laboratories. Fluorescein isothiocyanate (FITC)-Con A was purchased from E.Y. Laboratories. Spermidine from Aldrich, poly (L-lysine) (mol.wt. 1000–4000) from Sigma. lysoPC from Avanti Biochemicals, dimethylsulfoxide (DMSO) and trichloroacetic acid from Fisher Scientific Company.

Chemical treatments and fusion of erythrocytes

Turkey and human erythrocytes were washed three times in Hanks' balanced-salt solution [17], then settled on alcian blue-coated glass coverslips. Unattached cells were removed by washing with balanced salt solution. The coverslip, with an attached monolayer of cells, was incubated in a petridish containing the chemical agents at 37°C for 15 to 30 min, followed by washing once with balanced-salt solution. The coverslip was gently immersed into a sub-fusion concentration of 25% (w/w) PEG in balanced-salt solution and let stand for 5 min, then washed once with balanced-salt solution, and incubated at 37°C in balanced-salt solution for 1 h. After that, the percentages of fused cells on the coverslips were counted by phase microscopy [18]. Different protocols were employed to optimize the conditions for turkey and human erythrocytes as observed by light microscopy. For turkey erythrocytes, the samles on the coverslip were fixed with 2% (v/v) glutaraldehyde for 30 min, rinsed and dried. The dried samples were observed in the light microscope, and multinucleated cells were scored. The unnucleated human erythrocytes are too small to be observed in this manner. In order to increase contrast, the wet coverslip was inverted over a supporting microscope slide, bridging over the space between two grease-attached coverslips on the slide. The cells were observed with dark-field light optics [8]. FITC-Con A was used to label concanavalin A receptors in the membrane. Cells were incubated in the 200 μg/ml FITC-Con A at 37°C for 20 min, washed, and observed in a fluorescence microscope.

Freeze-fracture electron microscopy

Samples without PEG were rapidly frozen by the sandwich method [19]. About 0.1 μl of the sample was sandwiched between two thin copper plates and quickly plunged in liquid propane without cryoprotectant. Samples with PEG were concentrated by centrifugation ($1500 \times g$, for 3 min). The pellet of cells with PEG were places on gold cups (Balzer) and frozen in liquid propane. The samples were fractured at -110°C in a Polaron E 7500 unit.

Results

When turkey erythrocytes were treated with 45% or higher concentrations of PEG, more than 75% of the cells lysed. When cells were treated with 40% of lower percentages of PEG, most individual cells remained intact. This confirms the previous observation [10] that turkey erythrocytes cannot be induced to fuse by PEG alone. Conversely, human erythrocytes can be induced to

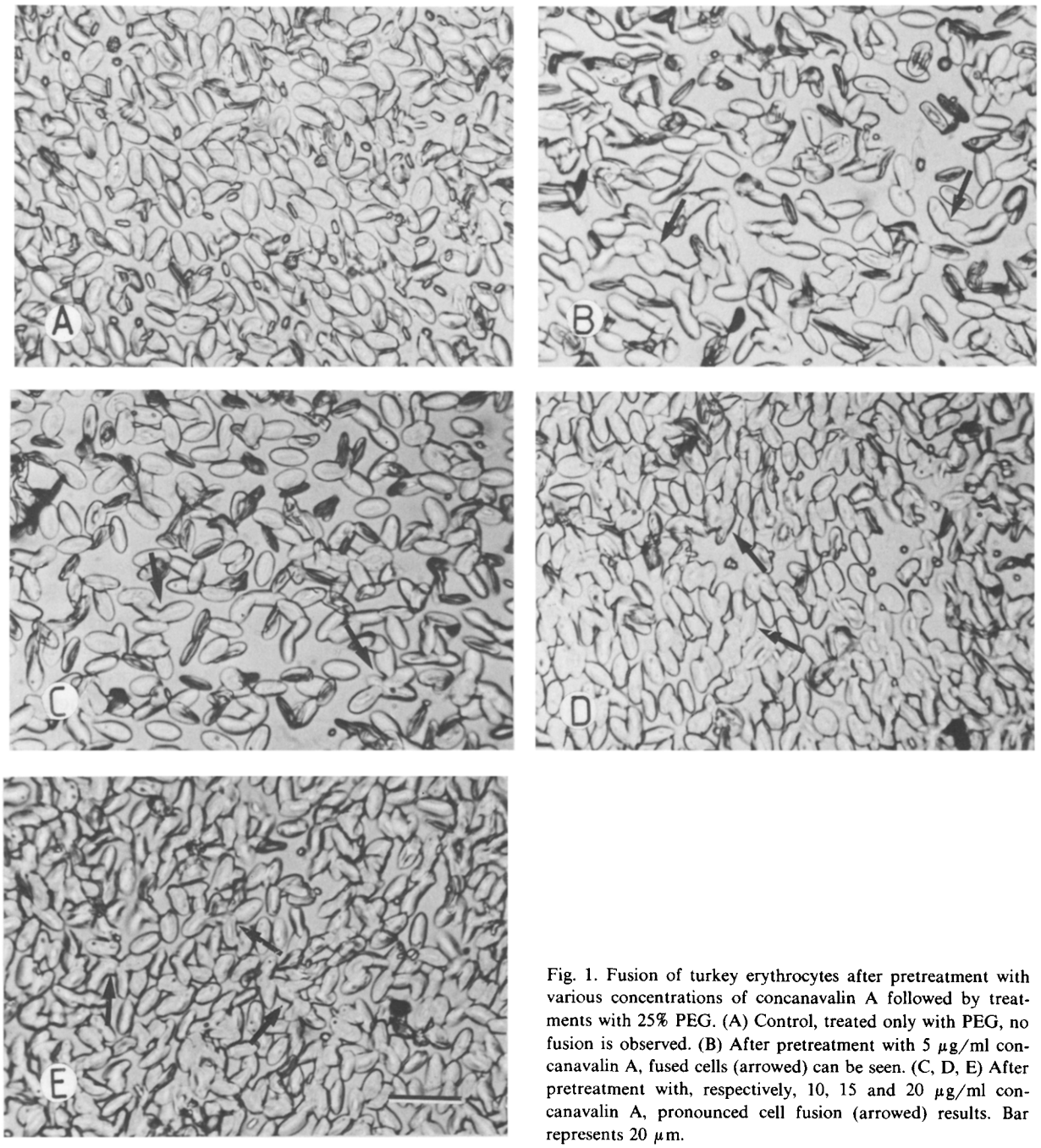

Fig. 1. Fusion of turkey erythrocytes after pretreatment with various concentrations of concanavalin A followed by treatments with 25% PEG. (A) Control, treated only with PEG, no fusion is observed. (B) After pretreatment with 5 μg/ml concanavalin A, fused cells (arrowed) can be seen. (C, D, E) After pretreatment with, respectively, 10, 15 and 20 μg/ml concanavalin A, pronounced cell fusion (arrowed) results. Bar represents 20 μm.

fuse by treatment of 35% and higher concentrations of PEG.

Treatment with concanavalin A only, even at high concentrations (200 μg/ml), does not lead to fusion of either turkey or human erythrocytes, although agglutination was observed in both kinds of cells in suspension. The phenomenon was more pronounced in turkey erythrocytes than in human

TABLE I

CONCANAVALIN A EFFECTS ON PEG-INDUCED FUSION

IMP, intramembrane particle; n.d., none detected.

Sample	Treatment	Agglutination	Fusion	IMP distribution
Turkey erythrocyte	PEG	+ + +	n.d.	No patching
	Con A	+ +	n.d.	IMP-free bubbles
	Con A + PEG	+ + + +	yes [a]	IMP-free bubbles
Human erythrocyte	PEG	+ + +	yes [b]	Small patches [b]
	Con A	+	n.d.	No patching
	Con A + PEG	+ + +	yes [b]	Small patches [b]

[a] Quantitative results are plotted in Figs. 2 and 3.
[b] Only at 35% or higher PEG concentration.

erythrocytes (Table I). Bright fluorescence was observed around the FITC-Con A-labeled turkey erythrocytes, and only a weak periphery could be seen around human erythrocytes. No capping on either type of erythrocyte was observed by fluorescence microscopy.

Different concentrations of concanavalin A were used in conjunction with 25 and 30% PEG to investigate any synergistic effects on fusion. In no case did the concanavalin A treatment result in enhancement of the fusion efficiency to human erythrocytes. In contrast, concanavalin A treatment facilitated turkey erythrocyte fusion. The fused cells showed no boundary between cells, making it easy to discern the fused cells from the unfused cells on alcian blue-coated glass coverslips (Fig. 1). The fusion percentage increased with concanavalin A concentration. However, if cells were treated with 25% PEG only, there was no fusion. When the concentration of concanavalin A was raised from 5 μg/ml to 20 μg/ml, the fusion percentage of the cells rose from 35% to 85% (Fig. 2). The maximum fusion percentage is perhaps limited by the percentage of cells in contact on the cover slip. Obviously, pretreatment with concanavalin A leads to a significant increase in fusion by PEG. Similar results with 30% PEG are also shown in Fig. 2. The fusion percentage rose more rapidly in 30% PEG compared with in 25% PEG, at the same concentrations of con-

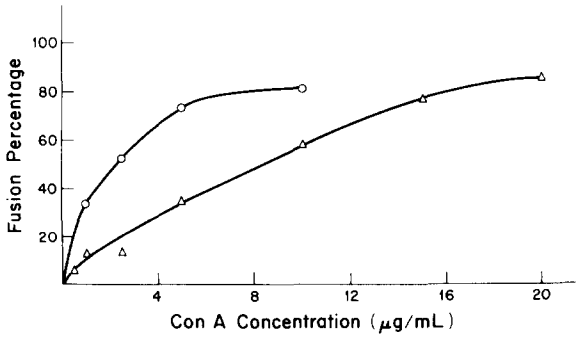

Fig. 2. A plot of the fusion percentage of turkey erythrocytes as a function of the concanavalin A concentration. The erythrocytes were co-treated with concanavalin A and, respectively, 25% PEG (△) or 30% PEG (○). The extent of fusion was quantitated as 'fusion percentage' which is the number of fused cells divided by the total number of cells present, times 100. At least 1000 cells were counted per sample.

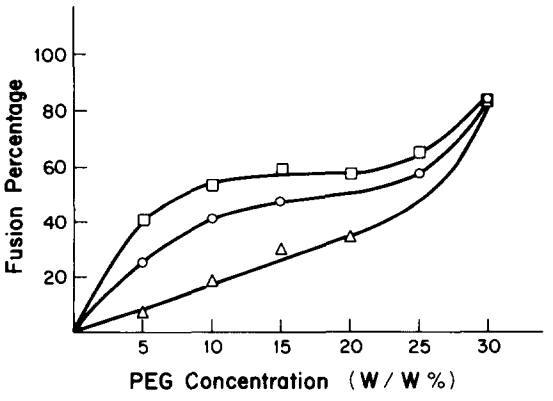

Fig. 3. A plot of fusion percentage of turkey erythrocytes as a function of the PEG concentration, after pretreatment with 7.5 (△), 10 (○), and 15 (□) μg/ml concanavalin A. The fusion percentage was defined as described in Fig. 2.

canavalin A. At 5 μg/ml concanavalin A, the fusion percentage in 30% PEG is almost twice as high as in 25% PEG. The fusion percentage as a function of various concentrations of PEG with the pretreatment in 7.5, 10, and 15 μg/ml concanavalin A is shown in Fig. 3. The fusion percentage declined with the decreasing PEG concentrations, with apparent changes of slope at 10% and 25% PEG. Thus the fusion percentae of turkey erythrocyte depends on the concentrations of both concanavalin A and PEG. Fig. 4 summarizes the data presented in Figs. 2 and 3. This 3-variable plot shows that the combined effect of concanavalin A and PEG is not linear.

The fusion percentage is also a function of the duration of treatment with 20 μg/ml concanavalin A (Fig. 5). After the post-treatment with 25% PEG, the fusion percentage rose with the increasing incubation time in concanavalin A at room temperature.

Freeze-fracture electron microscopy was used to investigate the ultrastructural alteration during concanavalin A and PEG-induced fusion. Human erythrocytes treated with 20 μg/ml concanavalin A or 25% PEG are indistinguishable from control cells. Neither concanavalin A nor low concentrations of PEG cause any observable structure changes of the human erythrocyte membrane. By contrast, small hemispherical zones (bubbles) which were intramembrane particle-free appeared on the turkey erythrocyte membranes treated with concanavalin A alone. The formation and appearance of these bubbles varied with the concentration of concanavalin A. Fig. 6A shows untreated turkey erythrocyte membranes with a seemingly random distribution of intramembrane particles. When the sample was treated with 2.5 μg/ml concanavalin A, relatively small, flat plaques of particle-denuded areas could be seen (Fig. 6B). The small smooth bubbles began to take shape in the treatment with 10 μg/ml of concanavalin A (Fig. 6C). Dominant, hemispherical intramembrane particle-free bubbles were seen over all membrane areas in samples treated with 20 μg/ml of concanavalin A (Fig. 6D). The degree of bubble formation correlates with the increasing fusion percentage, suggesting that these intramembrane particle-free bubbles play an important role in the PEG-induced turkey erythrocyte fusion. These bubbles persisted during the post-treatment in 25% PEG (Fig. 7). The hemispherical bubbles on the P-face are all convex (Fig. 7A), and on the E-face are all concave (Fig. 7B). The concanavalin A-induced formation of intramembrane particle-free bubbles is also time-dependent. The degree of bubble formation increases from 1 to 20 min of concanavalin A treatment, corresponding to the increase of fusion percentage shown in Fig. 5 (results not shown).

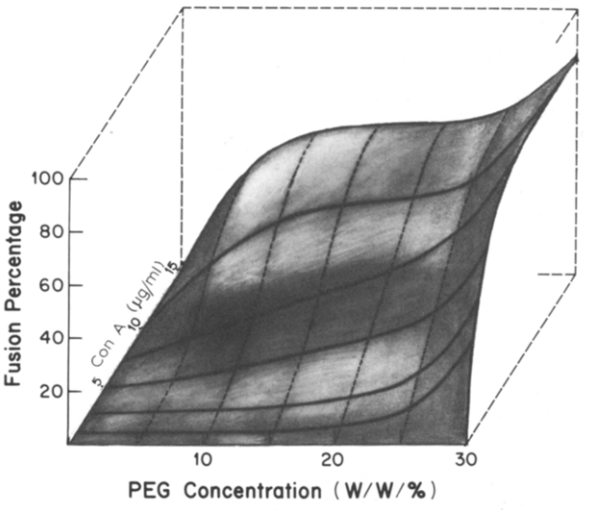

Fig. 4. The 3-variable plot shows that the combined effect of concanavalin A and PEG induced fusion of turkey erythrocytes.

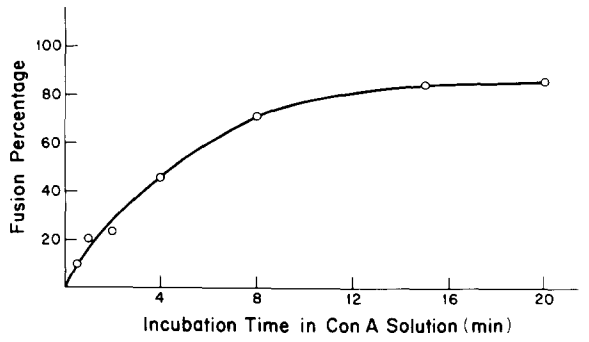

Fig. 5. A plot of the fusion percentage of turkey erythrocytes as a function of the incubation time in 20 μg/ml concanavalin A at room temperature prior to treatment with 25% PEG. The fusion percentage was defined as described in Fig. 2.

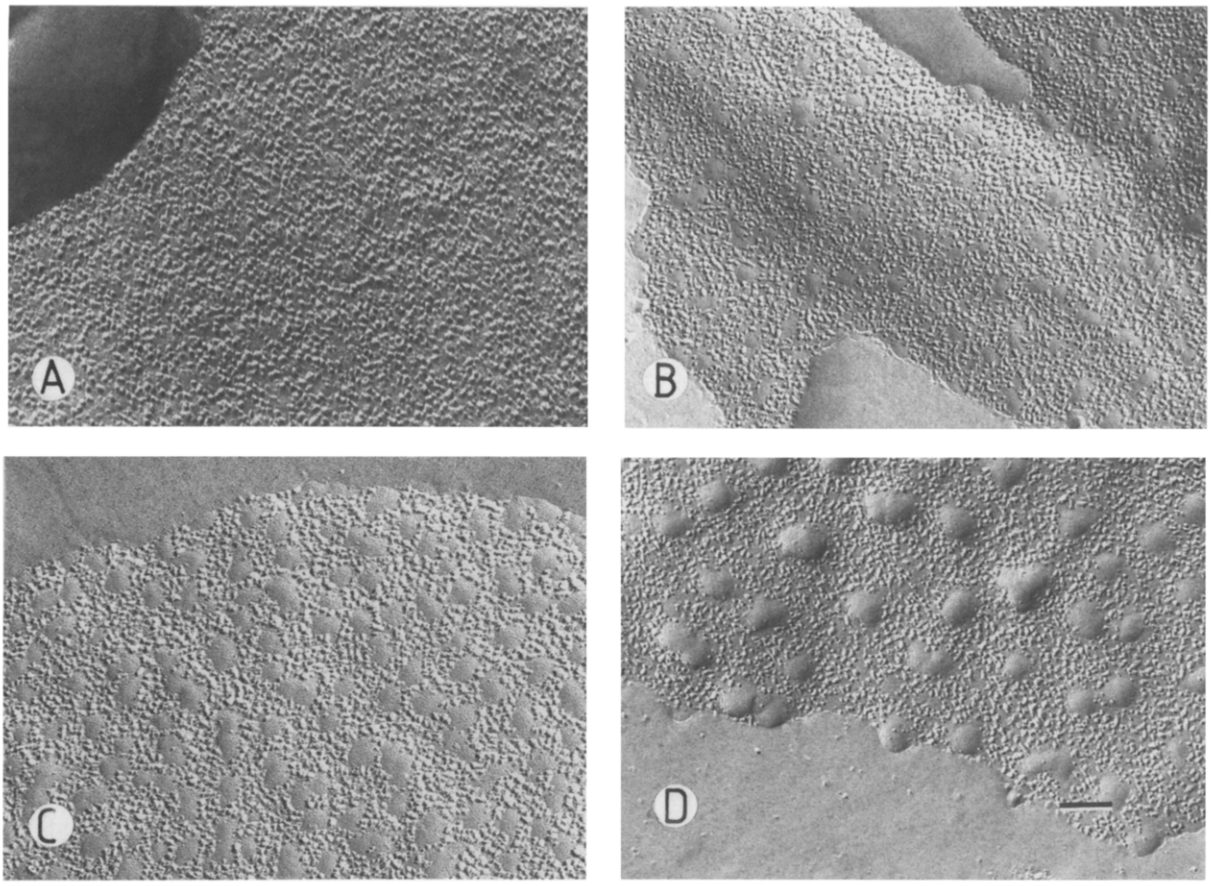

Fig. 6. Freeze-fracture electron micrographs showing the hemispherical intramembrane particle-free bubbles in the membrane of turkey erythrocytes after treatment with various concentration of concanavalin A: (A) control, 0 μg/ml, (B) 2.5 μg/ml, (C) 10 μg/ml and (D) 20 μg/ml. Bar represents 0.2 μm.

Several membrane disrupting agents, such as DMSO, lysoPC, trichloroacetic acid and ethanol; polyamines such as spermidine, and polypeptides such as poly(L-lysine) were also used to investigate if there are any synergistic effects on the PEG-induced cell fusion, and if the enhanced fusion

TABLE II

COMBINED EFFECTS OF PEG (25%) AND OTHER MEMBRANE ACTIVE AGENTS ON HUMAN ERYTHROCYTES

IMP, intramembrane particles; –, none detected.

Chemicals	Concentration		IMP aggregation		Fusion (%)
	Used	Lytic	– PEG	+ PEG	
DMSO	2% (v/v)	> 2%	–	–	–
LysoPC	0.15 mM	> 1 mM	–	–	–
Poly(L-polysine)	0.05% (w/v)	–	–	–	–
Spermidine	0.05% (w/v)	–	–	+ +	30%
Tricholoroacetate	100 mM	–	–	+ +	7%
Ethanol	4% (v/v)	–	+	+ +	12%

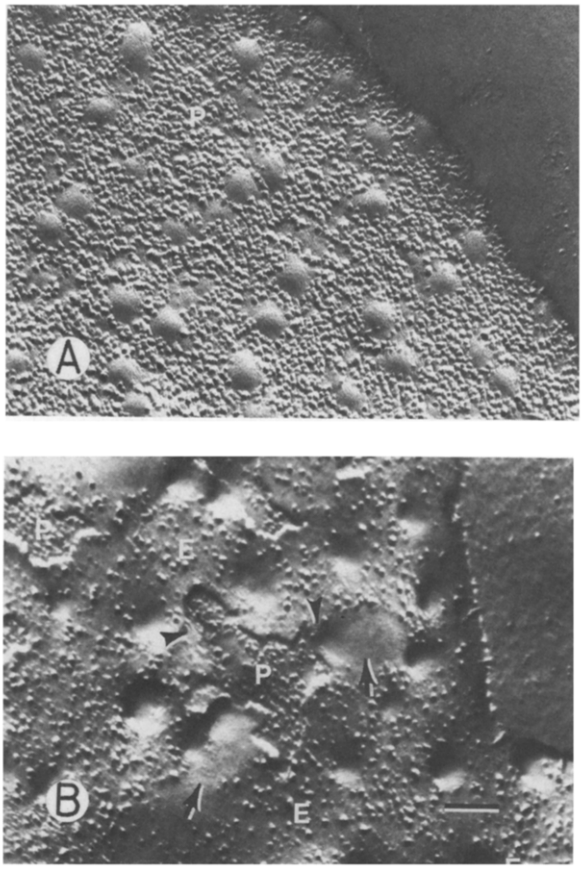

Fig. 7. Freeze-fracture electron micrographs showing turkey erythrocytes pretreated in 20 μg/ml concanavalin A, washed and freeze-fractured in the presence of 25% PEG. All hemispherical bubbles on the P-face are convex (A) and all those on the E-face are concave (B). The arrows indicate expanding intramembrane particle-free bubbles into adjacent membrane areas. The arrowheads indicate the possible fusion sites between two adjacent cells. The E-face of the top cell and the P-face of the bottom cell are marked by letters. Bar represents 0.2 μm.

effects are a result of bilayer disruption, electrostatic interaction, or attachment. Table II summarizes the effects of these chemical agents on human erythrocyte. None of these chemicals cause fusion of turkey erythrocytes.

DMSO [2] and lysoPC [12] are known as lipid-soluble fusogens. In freeze-fracture samples of human erythrocytes treated with sublytic concentrations of DMSO or lysoPC (2% w/w or 0.15 mM, respectively), no aggregation of intramembrane particles was observed prior to PEG (25%) treatment (Fig. 8A), nor in 25% PEG alone (Fig. 8B). Also, fusion was not found in cells treated with DMSO and lysoPC, and post-treatment with 25% PEG (Table II). Similar results were found in samples treated with poly(L-lysine) 0.05% w/v).

Human erythrocytes treated with sublytic concentrations of spermidin (0.05%) and trichloroacetate (100 mM) show no aggregation of intramembrane particles prior to PEG (25%) treatment (Figs. 8C, 8E). However, during the post-treatment with 25% PEG, small plaques of smooth intramembrane particle-denuded membrane were observed (Figs. 8D, 8F). The PEG-induced fusion was 30% and 7%, respectively, in human erythrocytes treated with spermidine and trichloroacetic acid (Table II). Ethanol treatment alone caused minor intramembrane particle patchings, but the degree of patching increased dramatically in 25% PEG. 12% fusion was observed when ethanol was used in conjunction with PEG.

Discussion

We have found that several chemical co-treatments can affect and promote PEG-induced cell fusion, thus lowering the threshold concentration of PEG required for cell fusion. As found by previous studies, PEG plays several roles in mediating cell fusion. First, at high concentrations it creates intramembrane particle-free zones in the membrane [3,8]. If cells are treated with PEG at concentrations below the critical level for fusion, no aggregation of intramembrane particles can be found [9]. Second, it forces close contact by dehydration between the membranes of adjacent cells. The optimum concentration of PEG (in the range of 38–45%) would force the membranes as close together as 5 Å [19,20]. Third, PEG disrupts the bilayer vesicle structure, especially in the subsequent dilution and incubation steps [8]. If another chemical has a positive effect on one or more of these phenomena induced by PEG, it may presumably promote PEG-induced cell fusion upon co-treatment.

Concanavalin A is known to bind to the mannose moiety of glycoproteins of the erythrocyte membrane, and leads to agglutination between cell and vesicles by lectin cross bridging [11]. We observed neither capping nor punctated fluorescence in either turkey or human erythrocytes after

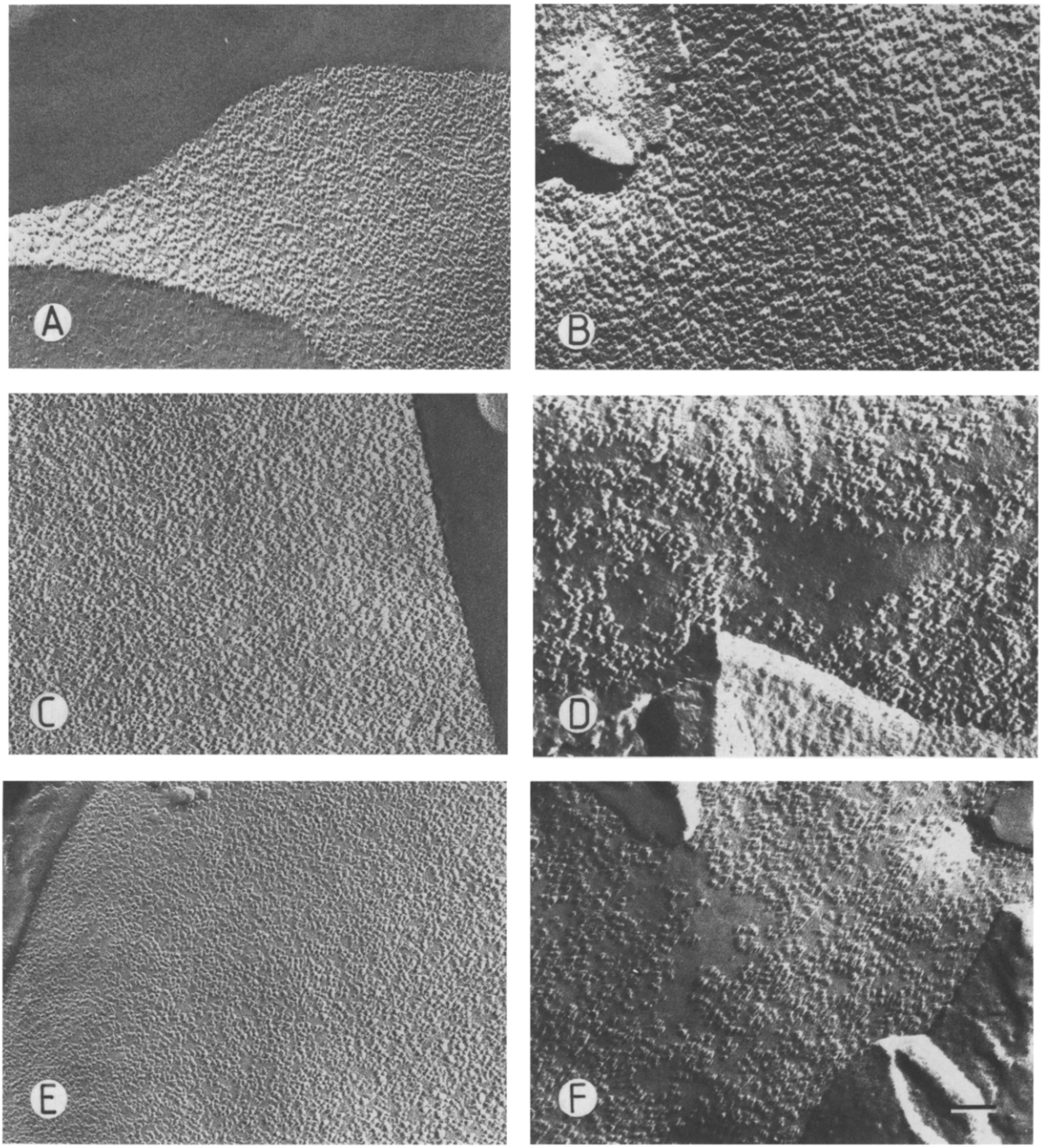

Fig. 8. Freeze-fracture electron micrographs of human erythrocytes showing no aggregation of intramembrane particles in cells treated with 2% (v/v) DMSO alone (A), or after co-treatment with 25% PEG (B). No aggregation of intramembrane particles is seen in cells treated with either 0.05% (w/v) spermidine (C) or 100 mM trichloroacetate (E), but small plaques of smooth intramembrane particle-denuded membrane are observed in cells treated with these respective chemicals and co-treated with 25% PEG (D, F). Bar represents 0.2 μm.

the treatment with FITC-Con A, even at the concentration of concanavalin A that causes bubble formation. Perhaps intramembrane particles and concanavalin A receptors are not equivalent, and the bubble dimension is beyond the resolution of light microscope. The inability to form caps is perhaps due to the characteristic cytoskeleton of the erythrocyte membrane impeding the mobility of concanavalin A receptors. It is interesting to note that the formation of hemispherical intramembrane particle-free 'bubbles' was observed only with turkey erythrocytes and not with human cells. How concanavalin A induces the formation of bubbles is not clear. It may be related to the higher concentration of concanavalin A receptors or the particular structure of the cytoskeleton in turkey erythrocytes. It is of interest to note that the hemispherical bubbles on the P-face are all convex, and on the E-face are concave. It could be that both the concanavalin A-induced agglutination of cells, and the formation of the intramembrane particle-free bubbles promote close contact between opposing lipid bilayers of adjacent turkey erythrocyte membranes, leading to membrane fusion.

The fusion effects of PEG and concanavalin A can be characterized in three concentration ranges (Fig. 4). At PEG concentrations $< 25\%$ and concanavalin A concentrations < 10 μg/ml, fusion increases almost linearly with both concanavalin A and PEG, up to about 50% fusion. In this region, the intramembrane particle-free patch formation by the concanavalin A treatment and the depolarization and dehydration effect of PEG [21,22] work hand in hand to implement fusion. As the concanavalin A concentration reaches and exceeds 10 μg/ml, intramembrane particle-free bubble formation (Fig. 6) greatly facilitates the fusion process, and the fusion efficiency soon reaches a plateau value of 60–70% at a relatively low PEG concentration of 10%. The plateau value probably signifies the maximum percentage of cells with bubble-to-bubble (bilayer/bilayer) contact that fuse at 10% PEG, which is the threshold concentration for fusion of bilayers with structural defects [6]. As the PEG concentration exceeds 25%, the fusion percentage rises sharply at all concanavalin A concentrations. At this PEG concentration, free water diminishes [22]. The dehydration and depolarization effects of PEG cause protein segregation [8] which augments the intramembrane particle patching effect by concanavalin A (Fig. 6). At the same time, it also promotes the fusion of exposed bilayers. 25% PEG is the concentration at which significant fusion of liposomes occurs [6,19]. These additional factors push the fusion efficiency to over 80%. The values for fusion percentages have a $\pm 5\%$ error due to variation of experimental conditions such as cell source, degree of cell attachment to the cover slips, and number of cell-cell contacts.

The results of this study show that a variety of chemicals, e.g. concanavalin A (a lectin), spermidine (a polyamine), trichloroacetate and ethanol (membrane chaotropes) can lower the threshold concentration of PEG required for cell fusion. All of these chemicals are able to cause the formation of intramembrane particle-free zones either in the treatment with the chemical alone, or during the post-treatment with PEG. Treatments with DMSO, lysoPC and poly(L-lysine) did not lead to cell fusion, nor did these treatments cause any intramembrane particle-free zone formation. The excellent correlation supports the hypothesis that the creation of intramembrane particle-free zones is an important step in PEG-induced cell fusion. Those chemical agents which do not create intramembrane particle-free zones, be they bilayer destabilizers or membrane agglutinators, do not promote PEG-induced cell fusion. Once the intramembrane particle-free zones are created in the membrane, even 5% PEG can trigger some fusion events. The fusion percentage increases sharply and becomes less concanavalin A-dependent at $> 25\%$ PEG, which corresponds to the threshold required for the fusion of liposomes in the fluid states [6,19]. The evidence so far points to the scheme that fusion takes place between two intramembrane particle-free zones and the mechanism is similar, if not equivalent to that of fusion between lipid bilayers. The additional PEG concentration (up to 45%) required for most cell fusion work is likely to be associated with the property of PEG, which aggregates and precipitates membrane proteins to expose intramembrane particle-free areas.

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References

1 Kao, K.N. and Kichayluk, M.R. (1974) Planta (Berl.) 115, 355–367
2 Ahkong, Q.F., Fisher, D., Tampion, W. and Lucy, J.A. (1975) Nature (London) 253, 194–195
3 Knutton, S. (1979) J. Cell Sci. 36, 61–72
4 Pontecorvo, G. (1975) Somatic Cell Genet. 1, 397–400
5 Lucy, J.A. (1978) in Membrane Fusion (Poste, G. and Nicolson, G., ed.), pp. 267–304, Elsevier/North-Holland Press, Amsterdam
6 Boni, L.T., Hah, J.S., Hui, S.W., Mukherjee, P., Ho, J. and Jung, C.Y. (1984) Biochim. Biophys. Acta 775, 409–418
7 Herrmann, A., Pratsch, L., Arnold, K. and Lassmann, G. (1983) Biochim. Biophys. Acta 733, 87–94
8 Hui, S.W., Isac, T., Boni, L.T. and Sen, A. (1985) J. Membrane Biol. 84, 137–146
9 Roos D.S., Robinson, J.M. and Davidson, R.L. (1983) J. Cell Biol. 97, 909–917
10 Arnold, W.M. and Lucy, J.A. (1983) in Cell Fusion, Ciba Foundation Symposium 103, p. 78, Pitman, London
11 Szoka, F., Magnusson, K.-E., Wojcieszyn, J., Hou, Y., Derzko, Z. and Jacobson, K. (1981) Proc. Natl. Acad. Sci. USA 78(3), 1685–1689
12 Papahadjopoulos, D., Hui, S.W., Vail, W.J. and Poste, G. (1976) Biochim. Biophys. Acta 448, 245–264
13 Schuber, F., Hong, K., Düzgüneş, N. and Papahadjopoulos, D. (1983) Biochemistry 22, 6134–6140
14 Hong, K. and Vacquier, V.D. (1985) Biophys. J. 47, 109a
15 Hatefi, Y. and Hanstein, W.G. (1969) Biochemistry 62, 1129–1136
16 Goldstein, D.B. (1984) Annu. Rev. Pharmacol. Toxicol. 24, 43–64
17 Hanks, J.H. and Wallace, R.E. (1949) Proc. Soc. Exp. Biol. Med. 71, 196–200
18 Knutton, S. and Bachi, T. (1980) J. Cell Sci. 42, 153–167
19 Boni, L.T., Stewart, T.P., Alderfer, J.L. and Hui, S.W. (1981) J. Membrane Biol. 62, 65–70
20 McCown, J.T., Evans, E., Diehl, S. and Wiles, H.C. (1981) Biochemistry 20, 3134–3138
21 Hartmann, J.X., Galla, J.D. and Emma, D.A. (1976) Can. J. Genet. Cytol. 18, 503–512
22 Baran, A.A., Solomentseva, I.M., Mank, V.V. and Kurilenko, O.D. (1972) Dokl. Akad. Nauk SSSR 207, 363–366