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VIRUS-INDUCED FUSION OF HUMAN ERYTHROCYTE GHOSTS

I. EFFECTS OF MACROMOLECULES ON THE FINAL STAGES OF THE FUSION REACTION

KIYOTOSHI SEKIGUCHI [a,*], KAZUMICHI KURODA [b], SHUN-ICHI OHNISHI [b] and AKIRA ASANO [a,**]

[a] *Institute for Protein Research, Osaka University, Osaka 565, and* [b] *Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606 (Japan)*

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Human erythrocyte ghosts prepared by hypotonic hemolysis can be fused by Sendai virus, provided that certain macromolecules (bovine serum albumin, dextran and others) are sequestered in the ghosts. Since fusion of the ghosts is dependent on intactness of the F(fusion)-glycoprotein of the virion, and since the other requirements for this reaction are also similar to those for the Sendai virus-induced fusion of intact erythrocytes, this system can be used as a model for the Sendai virus-induced cell fusion reaction. Sequestered macromolecules seem to be required for rounding of locally fused ghosts. Under low osmotic swelling conditions, such as use of ghosts sealed without macromolecules or using bovine serum albumin-loaded ghosts sealed in the presence of external macromolecules, no apparently complete cell fusion (large spherical polyghost formation) could be observed. Even under these conditions, however, occurrence of local cell fusion could be demonstrated either by transfer of fluorescent-labeled albumin from one ghost to an other, or by observation of polyghost formation after osmotic swelling in the cold. Thus, final stages of the fusion reaction can be divided into local cell-cell fusion which could not be observed by phase-contrast microscopy, and rounding (i.e. formation of spherical polyghosts). For the observation of fusion of ghosts, the last step seems to be important.

Introduction

Virus-induced cell fusion has served as a good experimental model for studies of membrane fusion, because in this system rapid and extensive membrane fusion can be attained under easily controllable condition [1]. To elucidate the mechanisms of membrane fusion, especially the structural changes of the cell membrane responsible for the cell-cell fusion, the use of human erythrocytes seems to have several advantages over the use of nucleated cells because erythrocytes are devoid of intracellular organelles and their plasma membrane can be prepared easily by simple hypotonic hemolysis. Furthermore, the erythrocyte membrane is one of the most extensively characterized biomembranes. There have been a lot of studies on the fusion of both human and chicken erythrocytes induced by virus and chemical fusogens [2–17]. Through these investigations, it has been shown that mobilization and subsequent rearrangement of membrane glycoproteins, which can be visualized as intramembrane particles with freeze-fracture electron microscopy [18–20], play a critical role in fusion of adjacent cell membranes [3,8–15]. The

* Present address: Biochemical Oncology, Fred Huchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104, U.S.A.
** To whom correspondence should be addressed.

Abbreviations: Buffer A, 40 mM Tricine-NaOH containing 135 mM NaCl and 5.4 mM KCl (pH 7.6); Buffer B, 5 mM sodium phosphate containing 0.9% NaCl (pH 7.6); HAU, hemagglutinating units.

mobility of the erythrocyte membrane glycoproteins seems to be restricted by spectrin which forms a meshwork structure with erythrocyte actin and band 4.1 protein at the inner surface of the membrane [18,21]. It is, therefore, of importance to examine whether spectrin and other cytoskeletal proteins participate in the fusion of red blood cells. For this purpose, it seems to be advantageous to utilize erythrocyte ghosts because the internal environment of such ghosts can be easily controlled, if necessary, by loading various substances within them.

Several attempts to demonstrate the fusion of erythrocyte ghosts prepared by hypotonic hemolysis have been unsuccessful. Thus, such erythrocyte ghosts, when mixed with Sendai virus *, became agglutinated but did not produce polyghosts [4,9,12]. Recently, we found that the ability of such hypotonically prepared ghosts to fuse could be restored by sequestering bovine serum albumin within them [12]. In the present investigation, we have studied the detailed conditions for the observation of fusion reaction of the erythrocyte ghosts. The results indicate that overall cell-cell fusion consists of two distinct steps, local cell-cell fusion and rounding of the locally fused ghosts. The loading of macromolecules inside ghosts is not required for the local cell-cell fusion but is essential for subsequent colloidal osmotic swelling of the locally fused ghosts. Quantitative or semi-quantitative measurement of the degree of cell fusion reported in most of the previous papers [2,4,5,9–14,22] utilized the last step, i.e. rounding, as the criterion of cell fusion. Thus, methods for the measurement of the local cell-cell fusion developed in this study will be of some help for further studies on the mechanism of membrane fusion reactions.

Most of these results have been reported at the Annual Meeting of the Japanese Biochemical Society held at Tokyo in 1979 [23].

Materials and Methods

Chemicals. Bovine serum albumin (fraction V), fatty acid-free bovine serum albumin, ovalbumin, trypsin, soybean trypsin inhibitor, polyvinyl pyrrolidone (PVP-40, $M_{r_{av}}$ 40 000) and Tricine (*N*-tri(hydroxymethyl)methylglycine were obtained from Sigma Chemical Co., dextran T70 ($M_{r_{av}}$ 40 300) was from Pharmacia Fine Chemicals, fluorescein isothiocyanate was from Merck, adenosine 5′-triphosphate disodium salt was from Kohjin Co., glutaraldehyde was from Ladd Research Industries Inc.. Spin-labelled phosphatidylcholine (PC*) was prepared by reacting egg lysophosphatidylcholine with 12-nitroxide stearic acid anhydride by the method of Hubbell and McConnell [24]. Other inorganic chemicals were of reagent grade of the highest grade available. A buffer consisting of 135 mM NaCl, 5.4 mM KCl and 40 mM Tricine-NaOH, (pH 7.6) was used as the medium throughout and in referred to as buffer A.

Cells and virus. Human blood, stored in acid-citrate-dextrose solution, (2.0 g trisodium citrate, 0.5 g citric acid, 2.2 g dextrose in 100 ml H_2O, pH 5.0), from donors of different blood types, was a generous gift from Dr. Katsuhiko Akashi of Osaka Red Cross Research Center and was used within two weeks. For the fusion experiments of intact erythrocytes, cells were washed four times with buffer B, twice with buffer A and finally suspended in buffer A to about 2% (v/v). HVJ (Sendai virus), Z strain, was propagated in embryonated eggs. The virus was purified from the infected chorioallantoic fluid by two cycles of differential centrifugation [22] and suspended in buffer A. The dose of the virus is expressed in terms of its hemagglutination units (HAU), which was determined by the pattern method of Salk [25].

Trypsin digestion of Sendai virus was carried out according to Shimizu and Ishida [26]. Sendai virus washed once with 10 mM sodium phosphate (pH 7.2), and resuspended in the same buffer, was incubated with 25 μg/ml trypsin at 37°C for 20 min. The digestion was stopped by adding 50 μg/ml soybean trypsin inhibitor. The digested virus was washed twice with buffer A, and suspended in buffer A.

Preparation of ghosts and loading of albumin and other macromolecules. Erythrocyte ghosts were prepared according to the method of Steck [27]. Washed erythrocytes were hemolysed by adding 20 vol. 5 mM sodium phosphate buffer (pH 8.0). The lysed cells were immediately sedimented by centrifugation at 15 000 rev./min for 20 min in a Sorvall SS-34 rotor at 4°C and washed once with the same buffer. One volume of packed ghosts was mixed with 4 vol. of

* Sendai virus is synonymous with hemagglutinating virus of Japan (HVJ).

buffer A containing bovine serum albumin or other macromolecules (loading medium) and allowed to stand for 15 min at 0°C, followed by resealing by incubating at 37°C for 60 min. Ghosts loaded with albumin or other macromolecules were washed twice with buffer A and suspended in the same buffer to give a concentration of about 2% (v/v) for usual fusion experiments or about 4% (v/v) for fluorescent-labeling experiments.

Fusion experiments. Unless otherwise stated, 0.19 ml of intact cells or ghosts was mixed with 0.01 ml of Sendai virus and kept on ice for 15 min to allow agglutination. The resultant aggregates were incubated at 37°C for 30 or 60 min and then chilled on ice to terminate the fusion reaction. Fused cells or ghosts were examined by phase contrast microscopy.

Preparation of fluorescein isothiocyanate-labeled bovine serum albumin. Fluorescent-labeled albumin was prepared as follows. 4 g of bovine serum albumin was first dissolved in 100 ml of 0.1 M $NaHCO_3$ (pH 9.0) to which 40 mg of fluorescein isothiocyanate was added and gently agitated overnight at 4°C. The reaction was stopped by the addition of 1 ml of 1 M ethanolamine (pH 8.0) and then extensively dialyzed against buffer A. Free fluorescent dye was further removed from fluorescent-labeled albumin by passing through a column of Sepharose LH20 equilibrated with buffer A. Fluorescent-labeled albumin thus obtained contained 0.96 mol of fluorescein per mol of albumin.

Observation and quantitation of the spreading of included fluorescent-labeled albumin. To a mixture of 0.185 ml of nonfluorescent albumin-loaded ghosts and 0.030 ml of fluorescent-albumin-loaded ghosts, 0.01 ml of Sendai virus was added and kept in ice for 15 min. To the resulting aggregates were added 0.025 ml of buffer A, to which various macromolecules were dissolved when effect of external macromolecules was examined, and further incubated in ice for 15 min. The fusion reaction was started by incubating the aggregates at 37°C, and after 30 min the mixture was chilled on ice to stop the reaction. Samples were observed by phase contrast and fluorescence microscopy using a Nikon fluorescence microscope FL. Photographs were taken with Kodak Tri-X film; The time of exposure was 2 s and 30 s for phase contrast and fluorescence microscopy, respectively.

For quantitation of the increase of fluorescence-bearing ghosts during cell fusion reaction, nonfluorescent and fluorescent ghosts were fused as described above except that the viral dose was considerably reduced (to 284 HAU/ml). The fusion reaction was performed in duplicate and for each sample at least four fields were photographed. To make the counting of the number of ghosts easier, each field of negative was projected on a screen by an enlarger. The number of the fluorescence-bearing ghosts was counted, and the number of original single ghosts before the fusion reaction was estimated for the same field. Since the extent of the apparent fusion reaction was considerably lowered by applying only a small amount of virus, most of locally-fused ghosts could not undergo rounding. It is therefore possible to estimate the number of the ghosts of the original single ghosts for each aggregate of ghosts. The number of the ghosts estimated after the fusion reaction was almost coincident with that measured before the fusion reaction, with a counting error of less than 10%, even when viral dose was maximal (284 HAU/ml).

Preparation of spin-labeled erythrocyte ghosts. Spin-labeled erythrocyte ghosts were prepared as described by Maeda et al. [6]. Spin-labeled phosphatidylcholine suspended in buffer A (2 mg/ml) was sonicated under a stream of nitrogen gas in an ice bath for 10 min and the sonicated mixture was centrifuged at 100 000 × *g* for 60 min at 4°C. The supernatant was mixed with an equal volume of human erythrocytes and incubated at 40°C for 1 h, followed by removal of the unicorporated spin-label by centrifugation. Ghosts were prepared from the labeled-erythrocytes as described above.

Assay of phospholipid intermixing between the erythrocyte membranes. Unlabeled albumin-loaded ghosts were mixed with spin-labeled albumin-loaded ghosts to a ratio of 2 to 1, then the virus was added at 4°C. After incubation on an ice bath for 15 min, the agglutinated ghosts were collected by centrifugation at 650 × *g* for 5 min. The packed aggregates were immediately taken-into a quartz capillary tube and ESR spectra were recorded at 37°C on a JELCO model ME-X spectrometer. Increase in the central peak hight was measured and plotted against incubation time.

Assay. Protein was determined by the method of Lowry et al. [28] using bovine serum albumin as a standard.

Results

Restoration of polyghost-forming ability of hypotonically prepared erythrocyte ghosts by loading with bovine serum albumin

Human erythrocytes have been shown to apparently lose their capability to undergo virus-induced cell fusion after they were lysed in hypotonic solutions (Refs. 5, 13, 20; also see Fig. 1A). The erythrocyte ghosts prepared by different procedures other than hypotonic hemolysis, such as those lysed by prolonged incubation at 44°C in 0.17 M Tris-HCl [29] and by osmotic shock with ethylene glycol under isotonic conditions [30], were also incapable of producing 'large, spherical polyghosts' upon incubation with the virus at 37°C (data not shown). However, the hypotonically prepared erythrocyte ghosts, which apparently lacked the fusion capability per se, could have the capability restored by loading bovine serum albumin within them. The polyghost-forming ability of the albumin-loaded ghosts was dependent on the albumin concentration of the loading medium. The erythrocyte ghosts resealed in the presence of less than 1% albumin did not produce fused polyghosts, instead, they remained agglutinated even after prolonged incubation at 37°C (Fig. 1A and B). The ghosts loaded with 5–7% albumin exhibited highest fusion capability and produced large, almost spherical polyghosts (Fig. 1D and E). Loading with more then 9% albumin resulted in partial destruction of the fused cell membrane after virus treatment and the apparent fusion ability of these ghosts was rather decreased (Fig. 1F). Thus the albumin concentration in the loading medium was fixed at 5% throughout the following experiments unless otherwise stated. Although the erythrocyte ghosts could restore their polyghost-forming ability by loading with albumin, they required more than 3 000 HAU/ml of Sendai

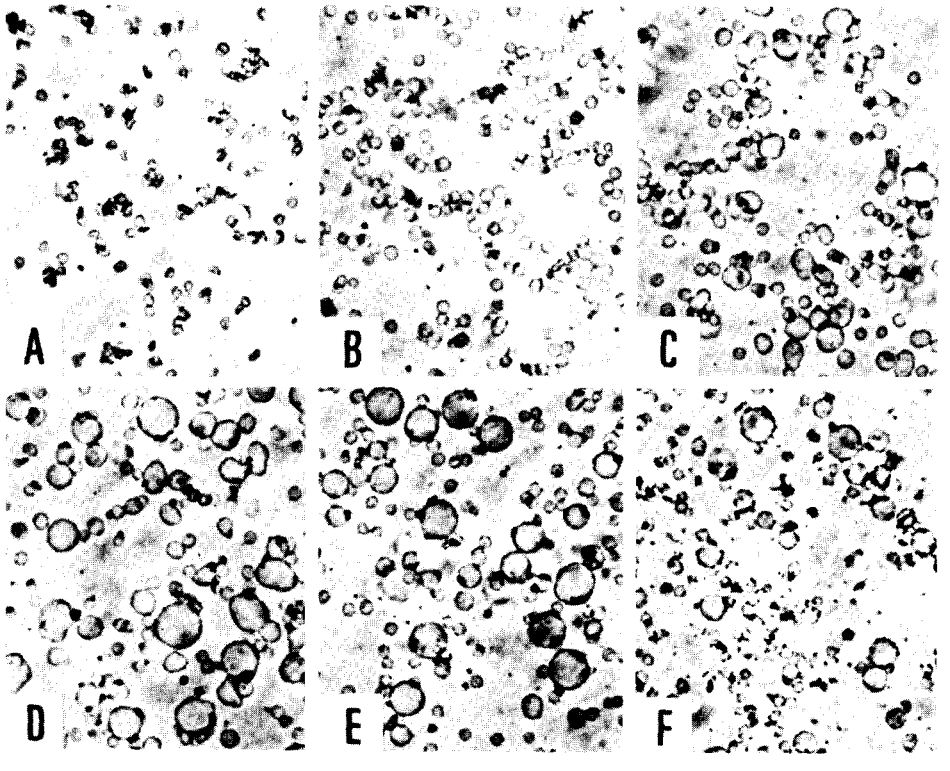

Fig. 1. Dependency of the restored polyghost-forming ability on the concentration of bovine serum albumin. The ghosts loaded with albumin to different concentrations were prepared as described in Materials and Methods. These albumin-loaded ghosts were agglutinated by Sendai virus (10 000 HAU/ml) for 15 min in an ice bath and subsequently incubated for 30 min at 37°C. A, 0%; B, 1%; C, 3%; D, 5%; E, 7%; F, 9%. Phase contrast microscopy. Magnification: ×350.

virus to obtain the maximal fusion, which almost 10-times as high as that required for intact erythrocytes (data not shown).

Participation of F glycoprotein in the fusion reaction of albumin-loaded ghosts

The biological activities of Sendai virus are associated with two glycoproteins which form distinct spikes on the viral envelope [31]. Hemagglutination and neuraminidase activities are associated with one glycoprotein termed HANA, whereas hemolytic and cell fusion-inducing activities are associated with the other glycoprotein, F. Selective cleavage of F glycoprotein by mild digestion with trypsin [26] results in the loss of the hemolytic and cell fusion-inducing activities of the virus, although it can still agglutinate the erythrocytes. The trypsin-inactivated virus could no longer induce the fusion of intact erythrocytes nor the albumin-loaded ghosts (data not shown), confirming that the fusion of the albumin-loaded ghosts is dependent of F glycoprotein and therefore they can be a prototype of other virus-induced cell fusion reaction.

Restoration of polyghost-forming ability by loading ghosts with other macromolecules

Various macromolecules other than bovine serum albumin were also tested for their capability to restore the polyghost formation when sequestered within the ghosts. Both defatted bovine serum albumin and ovalbumin could support the polyghost-forming ability, although the polyghost-forming efficiency of the ghosts loaded with these proteins was slightly lower than that of control bovine serum albumin-loaded ghosts (Fig. 2A–C). Gammaglobulin prepared from rabbit serum could also restore the fusion ability of the ghosts, but is was less effective than bovine serum albumin (data not shown). Interestingly, hemoglobin was almost incapable of restoring polyghost formation at any concentrations up to 10% (Fig. 2D). Not only proteins but also some other macromolecules were effective for the formation of

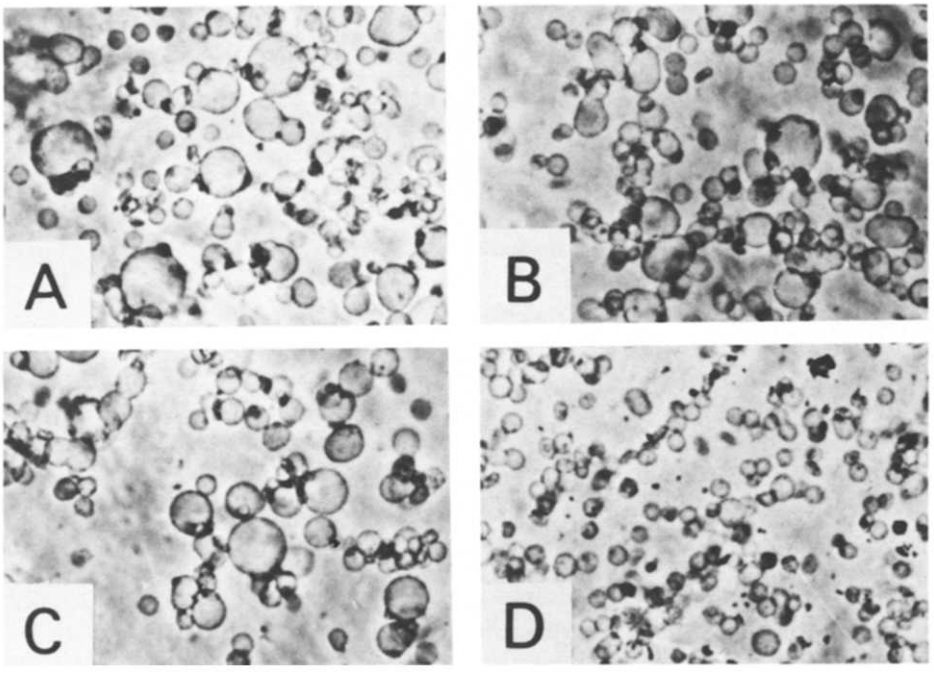

Fig. 2. Restoration of fusion ability of the erythrocyte ghosts by loading of other macromolecules within the ghosts. Ghosts loaded with the following proteins were prepared as described in Materials and Methods. A, 5% bovine serum albumin; B, 6% fatty acid-free bovine serum albumin; C, 5% ovalbumin; D, 5% human hemoglobin. These ghosts were agglutinated by Sendai virus (7 000 HAU/ml for A, B, D and 4 000 HAU/ml for C) for 15 min in an ice bath and then incubated at 37°C for 30 min. Phase contrast microscopy. Magnification: ×300.

polyghosts. Dextran was capable of restoring the polyghost-forming ability of the ghosts, although higher concentrations of dextran T70 (20–30%) were required to obtain a comparable extent of fusion to that of albumin-loaded ghosts (data not shown). Polyvinyl pyrrolidone, a synthetic polymer, could also restore the polyghost-forming ability to a lesser extent, whereas another synthetic polymer, polyethylene glycol was ineffective (data not shown). Bovine serum albumin was most effective among these macromolecules so far tested.

Inhibition of spherical polyghost formation by externally added macromolecules

To elucidate the role of included albumin and some other macromolecules for restoration of polyghost formation, we examined the effect of externally added albumin and other macromolecules which could reduce concentration gradient of macromolecules across the cell membrane. Fig. 3 shows that apparent cell fusion (spherical polyghost formation) was significantly inhibited by increasing concentrations of external albumin. When external albumin concentration was 2%, apparent cell fusion was almost completely inhibited. Rounding of unfused single-ghosts was also suppressed under the conditions emplyed (Fig. 3E). However, it is notable that the ghosts remained agglutinated even after prolonged incubation at 37°C. Similarly, other macromolecules such as dextran and polyvinyl pyrrolidone, when added to the reaction medium at almost the same concentration as albumin, could also suppress the virus-induced polyghost formation of albumin-loaded ghosts (see Fig. 4D and E).

Reversibility of the effect of externally added macromolecules

Since the ghosts remained agglutinated even after prolonged incubation under conditions where the apparent fusion reaction was inhibited by externally added macromolecules, it seems possible that such agglutinated ghosts were actually interconnected by

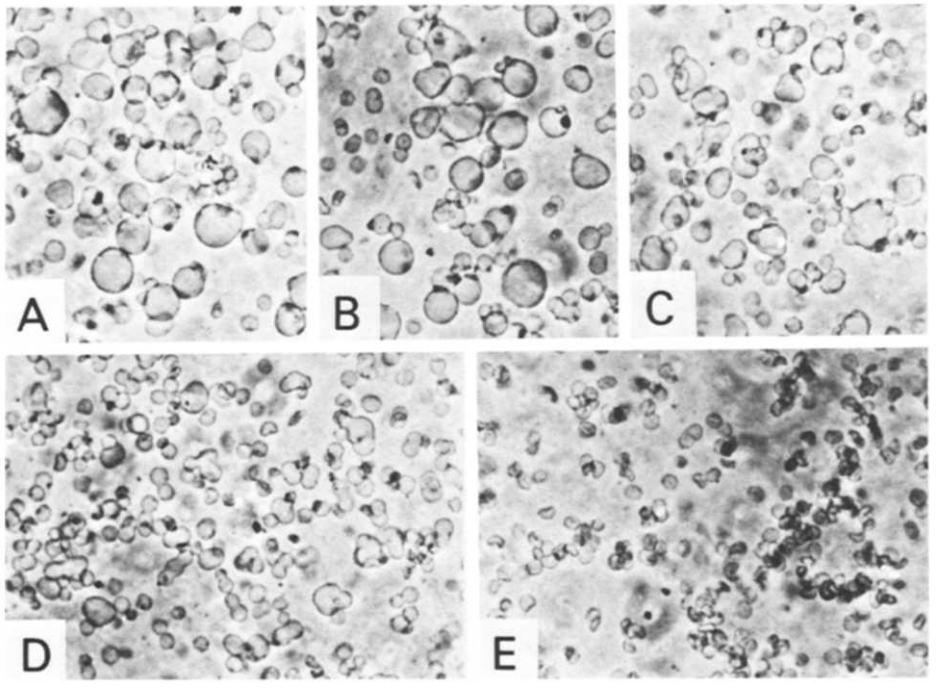

Fig. 3. Effect of externally added albumin on the fusion reaction of albumin-loaded ghosts. The albumin-loaded ghosts were agglutinated by Sendai virus (3 000 HAU/ml) for 15 min in an ice bath, then albumin was added to different concentrations and the ghosts were immediately incubated at 37°C for 60 min. A, 0%; B, 0.1%; C, 0.5%; D, 1.0%; E, 2.0%. Phase contrast microscopy. Magnification: ×350.

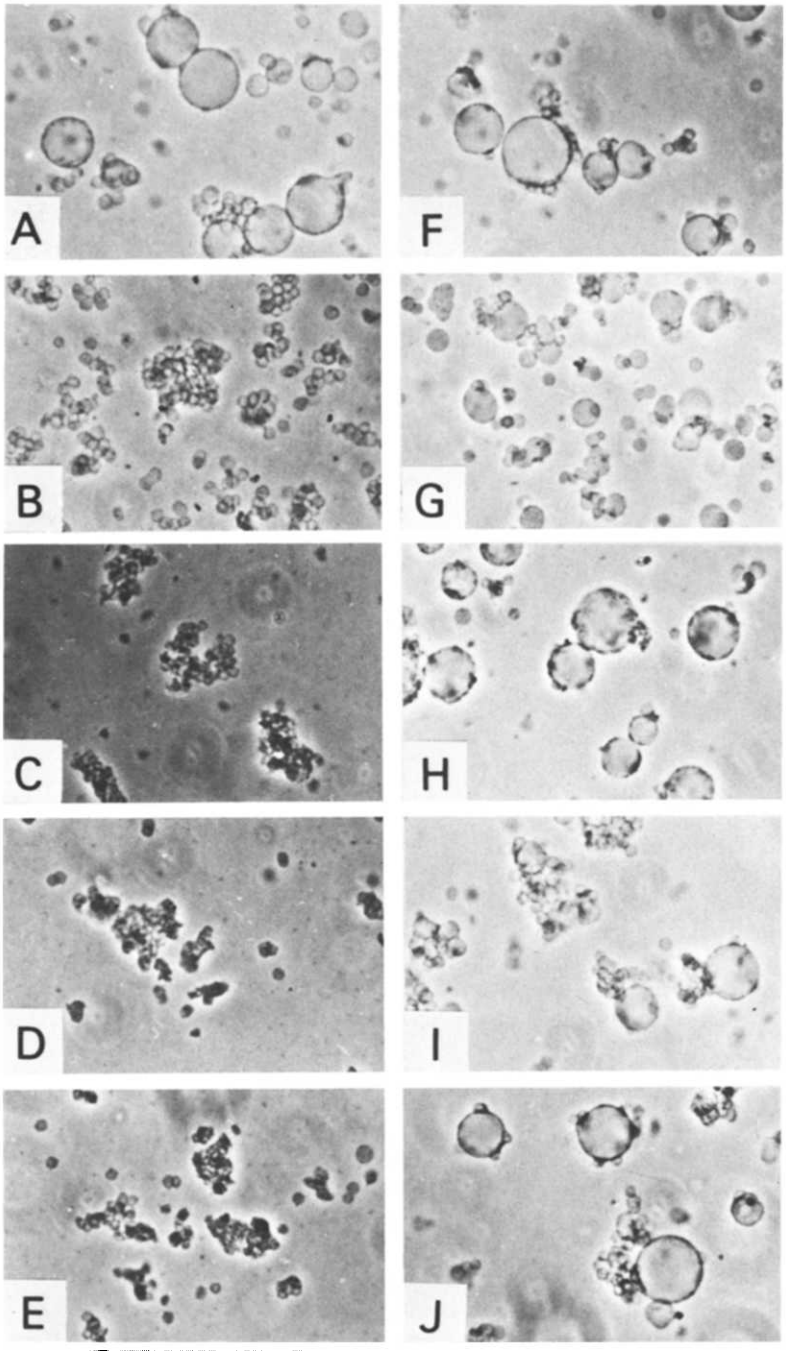

Fig. 4. Reversibility of the effect of externally added macromolecules on the fusion of albumin-loaded ghosts. The albumin-loaded ghosts were agglutinated by Sendai virus (3 200 HAU/ml) for 15 min in an ice bath, then the macromolecules specified below were added and the ghosts were further preincubated for 15 min at 0°C. The agglutinated ghosts were incubated at 37°C for 60 min and the fusion reaction was terminated by cooling the samples in an ice bath (A–E). To remove external macromolecules, the ghosts were then washed once with fresh isotonic buffer (buffer A) containing no macromolecules (centrifuged at 800 × *g* for 20 min) and resuspended in cold buffer A (F–J). Macromolecules added were: (A) and (F), no addition; (B) and (G), 2% bovine serum albumin; (C) and (H), 5% bovine serum albumin; (D) and (I), 2% dextran T 70; (E) and (J), 2% polyvinyl pyrrolidone. Phase contrast microscopy. Magnification: ×350.

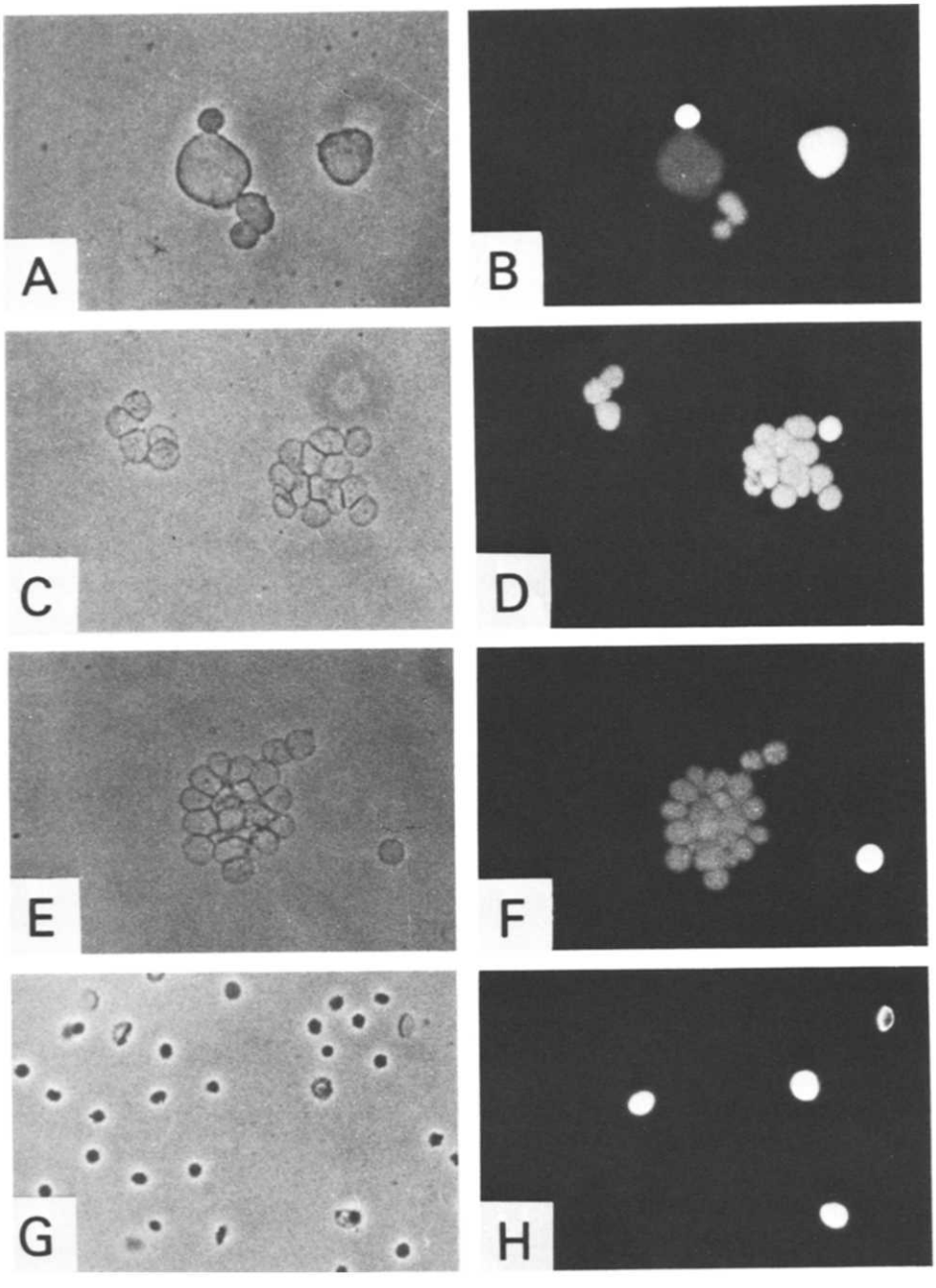

Fig. 5. Detection of local cell-cell fusion by observing the spreading of included fluorescent-labeled albumin. A mixture of fluorescent (0.030 ml) and nonfluorescent (0.185 ml) albumin-loaded ghosts was agglutinated by Sendai virus (5 700 HAU/ml) in an ice bath for 15 min, then 25% albumin (0.025 ml, final concentration 2.5%) was added and the mixture was further preincubated at 0°C for 15 min. Agglutinated ghosts were incubated at 37°C for 30 min. Samples were first examined by phase contrast microscopy (A, C, E, G) and then by fluorescence microscopy (B, D, F, H). (A and B), ghosts fused in the absence of external albumin; (C–F), ghosts fused in the presence of external albumin; (G and H), ghosts incubated at 37°C for 30 min without Sendai virus nor external albumin. Magnification: A–F, × 550; G and H, × 400.

local, thus not visible by phase-contrast microscopy, fusion of adjacent cell membrane. If such local membrane fusion did exist and only the rounding process was inhibited by external macromolecules, it could be expected that the removal of external macromolecules allowed these locally-fused aggregates to swell and thereby resulted in the appearance of large fused polyghosts.

When the agglutinated ghosts, obtained after the incubation at 37°C in the presence of external albumin (Figs. 4B and C), were pelleted and then resuspended in fresh isotonic buffer A at 4°C, they gradually swelled and transformed into large fused polyghosts with almost spherical shape (Fig. 4G and H). Since a prerequisit of the virus-induced cell-cell fusion, i.e. viral envelope-cell membrane fusion, can not proceed at 4°C [6], the results support the hypothesis described above that local membrane fusion can occur in the presence of external macromolecules. In a similar manner, the aggregates of the ghosts formed in the presence of other macromolecules, dextran T70 and polyvinyl pyrrolidone (Fig. 4D and E), could also yield large fused polyghosts when these macromolecules were removed, although most of the ghosts thus swollen were apt to remain aggregates (Fig. 4I and J). These results suggest that external macromolecules did certainly inhibit the apparent cell-cell fusion when observed with a phase-contrast microscope, but they did not actually inhibit fusion between adjacent cell membranes which occurred only locally (see below). This local cell-cell fusion does not seem to affect most of the cell membranes juxtaposed between two adjacent cells as observed at the initial stage of the virus-induced cell fusion by electron microscopy [32], thus the occurrence of this step is not visible by light microscopy.

Effect of external albumin on local cell-cell fusion

The presence of the local cell-cell fusion under such condition that rounding of the locally fused ghosts was inhibited by external macromolecules was more directly demonstrated by the transfer of fluorescent-labeled albumin among the aggregated ghosts. We prepared the ghosts loaded with either fluorescent-labeled or non-labeled albumin and the mixture of these ghosts were fused by Sendai virus. When the fusion reaction was performed in the absence of external albumin, large fused ghosts were obtained and most of them appeared as bright spheres under a fluorescence microscope (Fig. 5A and B). On the other hand, when fusion reaction was performed in the presence of external albumin, the ghosts did not undergo apparent fusion; they remained aggregated as described above (Fig. 5C and E). Under a fluorescence microscope, however, strong fluorescence was observed to cover the aggregates (Fig. 5D and F). Virus-mediated agglutination made at 4°C, however, did not cause such spreading of included fluorescent albumin (data not shown). Thus, these results confirm that local membrane fusion among the aggregated ghosts did occur at 37°C even under the presence of external albumin. The evaluate the effect of external albumin on the local cell-cell fusion more quantitatively, relative increase of the fluorescence-bearing ghosts in the whole ghost populations was measured under the conditions where apparent cell fusion was lowered by applying small amounts of Sendai virus. The reason viral dose was

TABLE I

EFFECT OF EXTERNAL ALBUMIN ON THE LOCAL CELL FUSION REACTION OF ALBUMIN-LOADED GHOSTS

A mixture of fluorescent-labeled and non-labeled bovine serum albumin-loaded ghosts was agglutinated with increasing concentrations of Sendai virus in an ice bath and then incubated at 37°C for 30 min in the presence or absence of external albumin (2.5%). The relative increase in the number of fluorescent-bearing ghosts after virus-treatment was determined as described in Materials and Methods.

Sendai virus concentration (HAU/ml)	Fluorescent ghosts (%)			
	without external albumin		with external albumin	
	Mean ± S.D.	Δ (−control)	Mean ± S.D.	Δ (−control)
0	16.0 ± 4.5	–	–	–
36	19.4 ± 2.3	3.4	20.6 ± 6.4	4.6
71	22.0 ± 7.0	6.0	20.5 ± 4.8	4.5
142	28.9 ± 7.2	12.9	33.8 ± 6.2	17.8
284	39.5 ± 9.7	23.5	41.4 ± 12.4	25.4

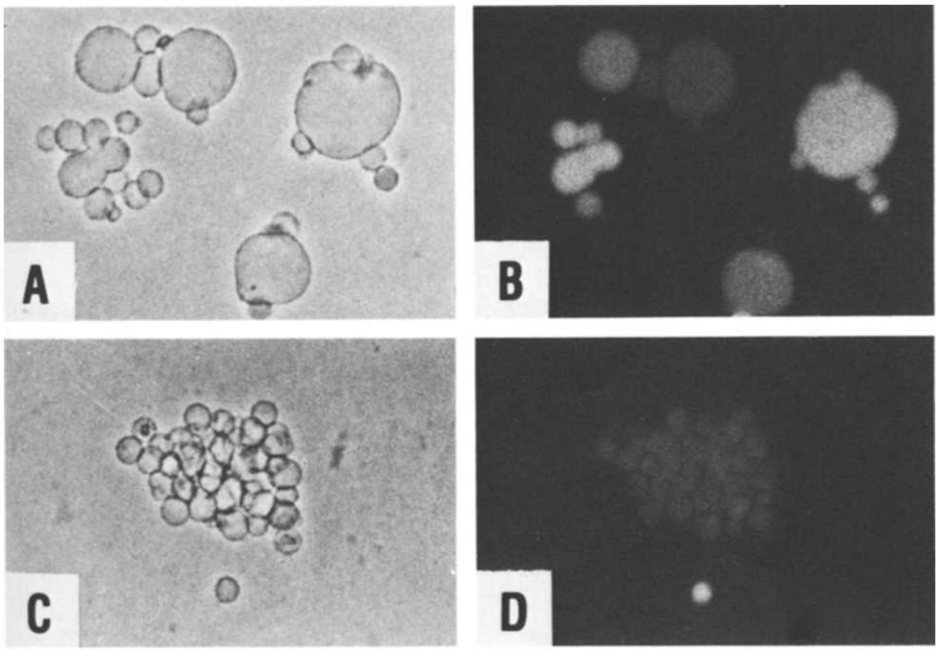

Fig. 6. Detection of local cell-cell fusion among ghosts loaded with a trace amount (0.5%) of albumin. A mixture of fluorescent and nonfluorescent ghosts loaded with either 5% (A and B) or 0.5% (C and D) albumin was incubated with Sendai virus (3 000 HAU/ml) as described in Materials and Methods. It can be seen that albumin included inside the ghosts, which were loaded with 0.5% albumin, diffused into neighboring ghosts although these ghosts did not undergo apparent cell fusion. Magnification: X 550.

suppressed to a very low level was to diminish the errors which might be introduced when we estimate the total ghost number after the end of the cell fusion reaction. High viral dose results in the appearance of large spherical polyghosts from which it is quite difficult to estimate the initial number of the ghosts participating in the formation of the polyghosts.

Table I summarizes the effect of external albumin on the extent of local cell-cell fusion determined by measuring the number of the fluorescence-bearing ghosts. The number of fluorescent ghosts was increased by increasing the viral concentration, but there was no significant difference between those with and without external albumin, suggesting that external albumin does not affect the local cell-cell fusion stage.

Do the ghosts resealed in the absence of albumin not undergo local membrane fusion?

Because the ghosts resealed in the absence of albumin do not undergo apparent cell fusion but remained agglutinated, it is important to examine whether unloaded ghosts could undergo the local membrane fusion or not. For the measurement of the local cell fusion of unloaded ghosts, we included a trace amount of a fluorescent probe within the ghosts. When the ghosts, loaded with either nonlabeled or fluorescent-labeled albumin (0.5%), were mixed and then incubated with Sendai virus, diffusion of included fluorescent albumin was observed as shown in Fig. 6, although the intensity of the fluorescence was weak. Further quantitative examination indicates that there was a slight but not significant decrease of the efficiency of local fusion of the ghosts loaded with 0.5% albumin when compared with the ghosts loaded with 5% albumin (data not shown). These observations indicate that the resealed ghosts containing a trace amount of albumin inside them can undergo the local cell-cell fusion but do not proceed to the next stage, i.e. rounding process, probably because of lack of colloidal osmotic gradient necessary to swell.

We also examined whether unloaded ghosts did undergo the local membrane fusion by the spin-label-

ing technique developed by Maeda et al. [7,12]. We prepared the albumin-loaded ghosts whose cell membrane were densely labeled with spin-labeled phosphatidylcholine (PC*) with 12-nitroxide stearic acid attached at the 2-position [37], and measured the virus-dependent transfer of phospholipid molecules between spin-labeled and non-labeled ghosts. This transfer of PC* incorporated into the cell membrane is a sensitive indication of cell-cell fusion reaction, because this represents the extent of the intermixing of phospholipid between non-labeled and spin-labeled cell membranes. Thus, we can estimate the extent of local membrane fusion by measurement of virus-dependent intermixing of phospholipids [6,8]. The extent of phospholipid intermixing can be estimated through the relative increase of central peak of the ESR spectrum of PC* [6]. As shown in Fig. 7, phospholipid intermixing can be observed during the incubation of unloaded ghosts with the virus. There seemed to be no significant difference between unloaded and albumin-loaded ghosts. When the virus was pretreated with trypsin to inactivate F glycoprotein, the virus-dependent phospholipid intermixing could not be observed.

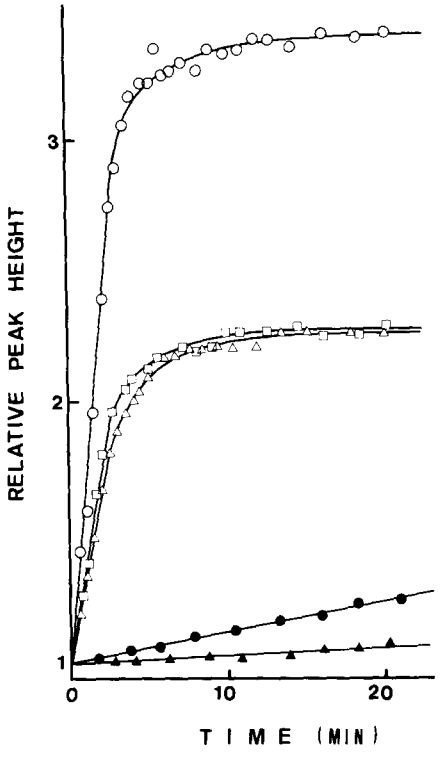

Fig. 7. Virus-dependent phospholipid intermixing between the erythrocyte membranes. Phospholipid intermixing between the erythrocyte membrane was measured from the changes of ESR spectrum of spin-labeled phosphatidylcholine (PC*) incorporated into the erythrocyte membrane as described in Materials and Methods. Briefly, a mixture of one volume of spin-labeled erythrocyte ghosts (or intact erythrocytes) and two volumes of unloaded ghosts (or intact cells) was agglutinated by Sendai virus (8 300 HAU/ml) at 0°C and then incubated at 37°C. The extent of phospholipid intermixing between ghosts (or intact cells) was expressed as relative increase of central peak height of ESR spectrum of PC*. (○), intact cells treated with native Sendai virus; (●), intact cells treated with trypsinized Sandai virus; (△), albumin-loaded ghosts treated with native Sendai virus; (▲), albumin-loaded ghosts treated with trypsinized Sendai virus; (□), unloaded resealed ghosts treated with native Sendai virus.

Discussion

To elucidate the mechanism of cell fusion reaction at a molecular level, the virus-induced fusion of human erythrocyte ghosts seems to be most convenient model because we can perform the cell fusion reaction using almost pure cell membrane preparations under easily controllable conditions. Ghosts prepared by hypotonic hemolysis did not undergo apparent cell fusion, i.e. formation of large spherical polyghosts, although they remain agglutinated after prolonged incubation with the virus at 37°C. Several efforts have been made to fuse erythrocyte ghosts. Perez et al. [4] reported that ghosts prepared by gradual hemolysis, i.e. dialysis of intact cells against 40 mM NaCl/10 mM Tricine-NaOH (pH 7.4) containing 3% bovine serum albumin could be fused by Sendai virus. They assumed that rapid hemolysis led to the leakage of a membrane consituent(s) required for the fusion process, and suggested that albumin added in the dialysis medium prevented this leakage [5]. On the other hand, we succeeded in restoring the polyghost-forming ability of the ghosts prepared by hypotonic hemolysis, washed once with hemolysing buffer and thus almost free from endogenous soluble compounds, by loading bovine serum albumin of other macromolecules inside the ghosts. Therefore, the apparent loss of the fusion ability by hemolysis may not be due to the leakage of an essential membrane constituent(s). According to this method, restoration of the polyghost-forming ability can be

obtained by a two-step reaction, that is, almost complete lysis and then loading of albumin, therefore easily allowing to include other impermeable compounds such as immunoglobulin inside the ghosts. In fact, by loading specific antibodies against spectrin we succeeded in inhibiting both virus-induced cell fusion and redistribution of intramembrane particles [12].

There are several lines of evidence indicating that the function of albumin-loaded ghosts still holds almost the same characteristics as the fusion of intact erythrocytes. Thus, the fusion reaction of the ghosts is dependent on the activity of viral F glycoprotein. Specific splitting of this glycoprotein by mild tryptic digestion completely inhibits the cell fusion reaction. Further, the fusion of the albumin-loaded ghosts does not require external Ca^{2+} nor metabolic energy in the form of ATP (data not shown). These results are in good agreement with those obtained for intact cells [4].

One of the possible explanations for the albumin-dependent restoration of the polyghost-forming ability is that sequestered albumin partially restores the colloidal osmotic gradient across the cell membrane which is lost upon hypotonic hemolysis. The osmotic gradient is necessary for the ghosts to swell and produce round fused ghosts. As discussed in detail below, we could not detect, at least with a phase contrast microscope, the cell-cell fusion unless the rounding of the locally-fused ghosts takes place, even if such local cell-cell fusion occurs. In support of this idea, inclusion of some other macromolecules such as ovalbumin and dextran within the ghosts, which can also induce the colloidal osmotic gradient, is effective in reactivating the fusion ability of the ghosts. Another line of evidence was obtained when the effect of externally-added albumin on the apparent cell fusion reaction was examined. The external albumin reduces the colloidal osmotic gradient across the cell membrane, and therefore, is considered to supress the rounding process. As expected, the external albumin almost completely inhibited apparent cell fusion. Furthermore, not only albumin but also other macromolecules, were effective in inhibiting apparent cell fusion. These results also support the idea that the osmotic gradient across the cell membrane is one of the prerequisites for observing the cell fusion under a phase contrast microscope.

The role of included albumin in restoring the polyghost-forming ability of the erythrocyte ghosts is not, however, simply attributable to the resulting colloidal osmotic gradient, because the capability of various macromolecules to restore the polyghost-forming ability is variable; serum albumin was most effective but hemoglobin was almost ineffective and ovalbumin had intermediate capability. Since included hemoglobin can also induce a colloidal osmotic gradient across the cell membrane, the failure of included hemoglobin to promote the fusion of the erythrocyte ghosts might be due to the lack of another requirement(s) which serum albumin and other effective macromolecules could satisfy. However, the basis for this requirement is not yet clear. It is unlikely that hemoglobin itself has an inhibitory effect on the virus-induced fusion of erythrocytes or their ghosts, because intact erythrocytes which are filled with high concentration of hemoglobin can be efficiently fused by the virus.

Although externally added macromolecules inhibited the overall cell fusion reaction, several lines of evidence indicate that the external macromolecules do not inhibit the local cell-cell fusion but do inhibit the subsequent swelling process. Thus, removal of external macromolecules allowed the locally fused ghosts to swell into spherical polyghosts. Moreover, such local cell-cell fusion could be visualized by the spreading of sequestered fluorescent-labeled albumin into the adjacent nonfluorescent ghosts. Further quantitative studies revealed that the external albumin did not affect the local cell-cell fusion.

Similarly, the ghosts loaded with a trace amount of albumin (0.5%) could undergo local cell-cell fusion although they could not proceed to the subsequent stage. Thus, the ghosts loaded with 0.5% albumin did not give rise to any apparent polyghosts by the virus treatment, instead, they remained agglutinated even after prolonged incubation at 37°C. However, extensive spreading of included fluorescent albumin all over the agglutinated ghosts was observed. Although these ghosts are not identical with unloaded ghosts, these results, together with the fact that the unloaded ghosts also remained agglutinated after prolonged incubation at 37°C, indicate that the unloaded ghosts also underwent local cell-cell fusion but they could not proceed to the subsequent rounding stage. Measurement of the rate of the phospholipid intermixing

between adjacent cell membranes further indicates that there is no difference in the rate of phospholipid intermixing between unloaded and albumin-loaded ghosts, indicating that ghosts can undergo the local membrane fusion with almost the same efficiency as albumin-loaded ghosts.

Fig. 8 is a schematic representation of the stages of the virus-induced fusion of erythrocyte ghosts. Stage I is an agglutination step. Ghosts are agglutinated by the virus but neither viral envelope-cell fusion (referred hereafter as 'envelope fusion') nor subsequent cell-cell fusion takes places at 4°C. Stage II is 'envelope fusion'. When cell aggregates formed at 4°C are warmed to 37°C, viral envelopes immediately begin to fuse with the cell membrane. This step is strictly dependent on the activity of viral F glycoprotein, since specific splitting of F glycoprotein by mild tryptic digestion resulted in the complete inhibition of envelope fusion [26]. Insertion of the viral envelope into the cell membrane causes a transient increase in permeability which is followed by the leakage of internal macromolecules [32,33]. The inserted viral envelope components may affect the integrity of spectrin-actin skeleton and loosen it up to allow the subsequent steps.

Stage III is the local cell-cell fusion. The local cell-cell fusion is defined as membrane fusion in which cell membranes and cytoplasm of two adjacent cells become continuous only locally but most of juxtaposed cell membranes of two fusing cells still remain intact and, thus, occurrence of cell fusion could not be detected by phase-contrast microscopy.

This step can be easily detected from the spreading of the included fluorescent albumin. Since spreading of the fluorescent albumin over the aggregates could be observed even when the rounding process was not occurring either by inhibition with external albumin or inclusion of only trace amount of albumin (0.5%) within the ghosts, the swelling process does not seem to be a prerequisit for the local cell-cell fusion.

Both Stage IV and V represent the rounding process of polyghost-formation. Colloidal osmotic gradient across the cell membrane, indeed, may be the effective driving force for the swelling of the ghosts, but it should be noted that an osmotic gradient alone is not sufficient to allow the ghosts to swell, as is evident from the fact that albumin-loaded ghosts by themselves cannot swell without the aid of viral action. This may be because the osmotic gradient produced by included albumin is too weak to overcome

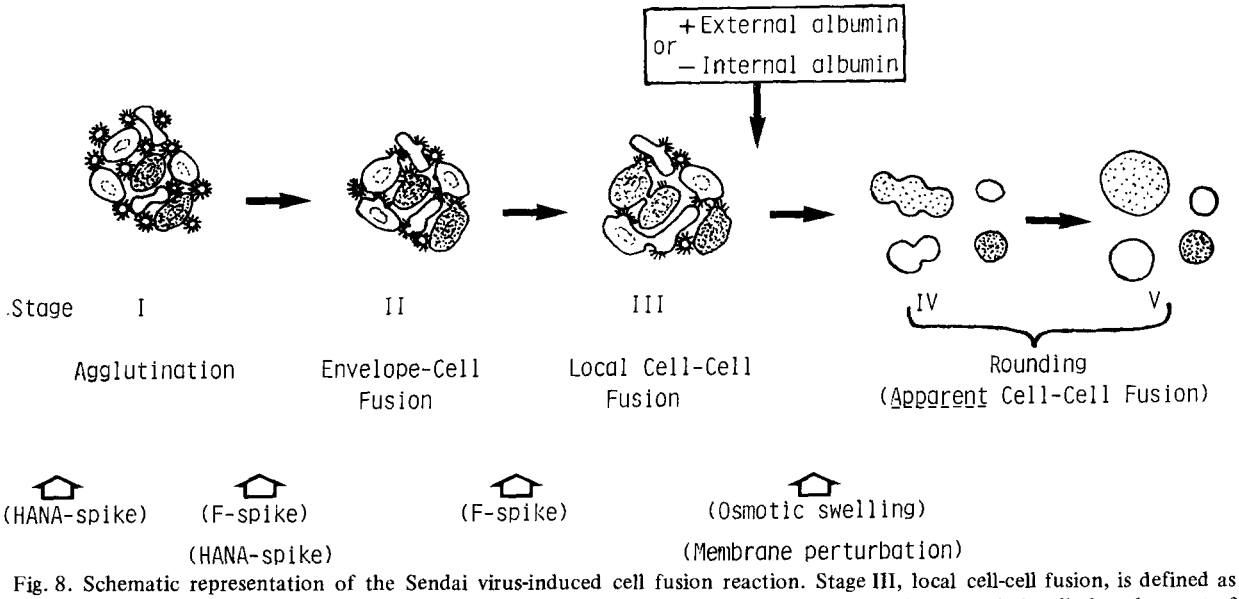

Fig. 8. Schematic representation of the Sendai virus-induced cell fusion reaction. Stage III, local cell-cell fusion, is defined as membrane fusion in which cell membranes and cytoplasm of two adjacent cells become continuous only locally but the most of jaxtraposed cell membrane of two fusing cells still remains intact and, thus, occurrence of cell fusion could not be detected by phase-contrast microscopy. Stage IV is a step in which polyerythrocyte formation can be detected by phase-contrast microscopy but rounding is stopped at an intermediate stage, i.e. polyerythrocytes of dumpbell shape. Large spherical polyerythrocytes (or polyghosts) are formed at Stage V, and this is the final step of the cell fusion reaction.

the constraint caused by spectrin meshwork to maintain the cell shape. The modification and possibly partical destruction of the spectrin meshwork seem to be required for the rounding of the locally-fused ghosts. The extensive redistribution of intramembrane particles accompanying the virus-induced cell fusion reaction [3,12,34], also suggested that such disintegration and loosening of the spectrin meshwork takes place during fusion reaction.

The overall cell fusion reaction is not observable whenever one of each sequential step is inhibited, therefore, it is important to identify which stage(s) is susceptible to a particular effector of the fusion reaction of the ghosts. Thus, externally added macromolecules do not affect local cell fusion (Stage III) but almost completely inhibit the rounding process (Stage IV). The apparent loss of fusion ability of the unloaded ghosts may also be due to blockade of the Step IV. Inclusion of albumin enables the ghosts to swell by producing colloidal osmotic gradient.

The inhibitory sites of the antispectrin antibodies are also of great importance, because the spectrin meshwork is considered to play an important role in the fusion of the erythrocytes. Previously, we have shown that antispectrin antibodies could inhibit both apparent cell fusion reaction and redistribution of the intramembrane particles [12]. The further detailed investigation indicates that antispectrin antibodies inhibit not only rounding but also initial local cell-cell fusion, although no inhibition of the envelope fusion could be observed (unpublished data). The separation between Stage II and Stage III is also observed in the fusion of other cell types. The addition of cytochalasins at an early stage of the virus-induced fusion of Ehrlich ascites tumor cells did not inhibit the envelope fusion but significantly inhibited cell-cell fusion, thus, resulting in dissociation of the aggregates into single cells (Refs. 35, 36, and our unpublished observation).

Beside these results, the importance of the rounding process in the practical measurement of cell fusion reaction by phase-contrast microscopy has to be stressed.

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