Journal of Supramolecular Structure 9:441–452 (1978)
Normal and Abnormal Red Cell Membranes 293–304

Redistribution of Intramembrane Particles of Human Erythrocytes Induced by HVJ (Sendai Virus): A Prerequisite for the Virus-Induced Cell Fusion

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Aggregation of intramembrane particles of human erythrocytes was found to be induced by HVJ (Sendai virus) under conditions which lead to cell fusion. Degree of polyerythrocyte formation was compared under a variety of conditions with extent of cluster formation observed with the same preparations. Both structural changes of the membranes, ie, fusion and clustering of the particles, behaved very similarly under widely different virus-to-cell ratios and over the time course of cell fusion. Furthermore, by inclusion of high concentrations of antispectrin antibodies within the ghosts, inhibition of clustering of intramembrane particles and hindrance of virus-induced cell fusion were found to occur simultaneously. Antibodies by themselves did not induce aggregation of particles under isotonic conditions, whereas particle clustering could be induced under hypotonic conditions at antibody concentrations causing partial cross-linking of spectrin molecules.

In conclusion, clustering of intramembrane particles seems to be required for virus-induced fusion of human erythrocytes.

Key words: cell fusion, human erythrocytes, Sendai virus, clustering of intramembrane particles, antispectrin antibody

Intramembrane particles (IMP) observed by freeze-fracture electron microscopy in human erythrocyte membranes have been identified as cell surface glycoproteins [1, 2]. In reconstitution experiments [3] similar particles have been observed with liposomes containing purified band 3* dimers, and a possible association of glycophorin or other surface proteins with IMP has also been suggested [1, 2, 4]. By freeze-fracture studies of Sendai virus-induced fusion of human erythrocytes, Bächi and co-workers [5, 6] discovered that aggregated distribution of IMP was observed with extensively fused cells in contrast to

*The major polypeptides of the erythrocyte membrane are designated by Steck's nomenclature [39].

Abbreviations: HVJ, hemagglutinating virus of Japan (Sendai virus); HAU, hemagglutinating unit; TBS, tricine-buffered saline (140 mM NaCl, 5.4 mM KCl, 40 mM tricine-NaOH, pH 7.6); BSA, bovine serum albumin; IMP, intramembrane particles; PF and EF, protoplasmic fracture face and extracellular fracture face, respectively. Nomenclature of Branton et al. [25]

Received May 4, 1978; accepted August 7, 1978.

0091-7419/78/0903-0441$02.30

their random distribution in untreated erythrocytes. Because this clustering of IMP was induced by action of the virus, and this change seems to expose protein-free areas on the cell surface which may be sites of fusion of lipid bilayers, this process was thought to be a prerequisite of virus-induced cell fusion [6, 7].

On the other hand, the virus has been known to induce several changes on human erythrocytes; since only some of them may be actually related to cell fusion, critical studies will be required to draw final conclusions about involvement of these phenomena in virus-induced fusion of erythrocyte membranes. Hemagglutination [8, 9] and probably fusion of the viral envelope to the cell membrane (envelope fusion) [10–12] seem to be required for formation of polyerythrocytes, whereas hemolysis, which is induced by fusion of some but not all types of viral envelope to erythrocytes [13, 14], and virus-induced fluidity changes of lipid bilayers [15, 16] may not be required for cell fusion, although these are also induced by the virus.

Therefore, we started a closer examination of the relationship between cluster formation of IMP and fusion of adjacent cell membranes to find out whether the former is required for the latter or not. To this end, the following experiments were undertaken: 1) comparison of the virus-to-cell ratio required for cell fusion and that needed for aggregation of IMP; 2) comparison of the time course of the fusion of erythrocyte ghosts and that of clustering of IMP; 3) inhibition of clustering of IMP by antispectrin antibodies and its relationship to the inhibition of the virus-induced fusion of erythrocyte ghosts. The results of these experiments will be reported together with the effect of salt concentrations on mobility of the IMP-spectrin complex.

MATERIALS AND METHODS

Cells and Virus

Human erythrocytes were obtained from a blood bank (Midori Jugi) and used within four weeks after they had been drawn. Hemagglutinating virus of Japan (HVJ, Sendai virus), kindly propagated in Dr. Y. Okada's laboratory, was grown in the allantoic sac of chick embryos and purified as elsewhere described [17, 18]. Hemagglutination titer was determined by Salk's pattern method [19].

Preparation of Ghosts and Fusion Experiments

Ghosts were prepared according to Dodge et al [20] with 5 mM sodium phosphate (pH 8.0) as hemolyzing buffer. Packed ghosts were washed once with the lysing buffer, incubated with four volumes of tricine-buffered saline (TBS; 140 mM NaCl, 5.4 mM KCl, 40 mM tricine-NaOH, pH 7.6) containing 5% bovine serum albumin (BSA) and other proteins when indicated at 0° for 10 min, and then resealed by incubation at 37° for 60 min as described previously [21]. Albumin-loaded ghosts and intact erythrocytes were washed twice with TBS and suspended in TBS. Fusion experiments were carried out as follows: Cells or ghosts were mixed, to give a final concentration of 2%, with indicated amounts of the virus at 0° for 15 min to allow agglutination; then the aggregates were incubated at 37° for 30 min unless stated otherwise. Degree of fusion was observed by a Nikon-phase contrast microscope.

Electron Microscopy

The samples were fixed by the addition of an equal volume of 4% glutaraldehyde prepared with the same medium and warmed to reaction temperature. This process was

performed for 40 min at 37° or for 2 h at 4°. After three washes with the incubation medium, the fixed samples were suspended in 20% (v/v) glycerol in physiologic saline at 4° over night. Then freeze-fracture was performed under standard conditions with a Hitachi freeze-fracture apparatus. Electron micrographs were taken with a Hitachi HU-12 electron microscope.

Preparation and Purification of Antispectrin Antibodies

Spectrin was extracted from ghosts by dialysis against 1 mM ethylenediaminetetracetic acid (EDTA), pH 10, at 4° for 18–20 h and further purified by gel filtration on Sepharose 4B. Antibodies against spectrin were produced in rabbits. The antisera were first fractionated by ammonium sulfate precipitation and then purified by a spectrin-Sepharose column (2–3 mg spectrin per milliliter of packed gel) prepared by the method of Cuatrecasas [22]. Antispectrin antibody thus purified was 63% precipitable with purified spectrin and formed a single precipitin band on immunodiffusion against solubilized erythrocyte membrane and purified spectrin. Antispectrin F_{ab} fragments were made and purified by the procedure of Porter [23]. Antigen-binding activity of the F_{ab} fragments was checked by ability to prevent precipitation of spectrin-[^{125}I] antibody complex near the equivalence point. Control immunoglobulin (IgG fraction) was prepared form non-immunized sera by ammonium sulfate precipitation and DEAE-cellulose column chromatography.

Reagents

Bovine serum albumin (fraction V) and N-[tris(hydroxymethyl)methyl] glycine (tricine) were purchased from Sigma Chemical Co. Sodium iodide I^{125} was obtained from Dai-ichi Chemical Co. Iodinated bovine serum albumin was prepared by a chloramine-T method [24].

RESULTS

Aggregation of IMP and Polyerythrocyte Formation Induced by Different Amounts of HVJ

As shown in Fig. 1(A–D), fusion frequency of human erythrocytes observed after incubation for 30 min is dependent on the amounts (in hemagglutinating units – HAU) of the virus added A, 50 HAU; B, 100 HAU; C, 400 HAU; D, 800 HAU); further incubation up to 2 h has not appreciably changed the fusion profile. Freeze-fracture observation of the protoplasmic fracture face (PF) [25] of the membrane revealed that the degree of aggregation of the particles roughly paralleled that of polyerythrocyte formation, as shown in Fig. 1 (E, 50 HAU; F, 400 HAU; G, 800 HAU). Incubation at low temperature neither induced cell fusion nor stimulated redistribution of IMP (Fig. 1 H), although a ragged region was sometimes observed on fracture faces of the virus-agglutinated cells. At very low virus doses, such as 50 HAU/ml, when fused samples still contained many unfused single cells, some difference in degree of clustering was evident from fracture face to fracture face. An area showing average clustering is shown in Fig. 1 E as typical of the effect of virus treatment at 50 HAU/ml.

Time Course of Erythrocyte Ghost Fusion and Cluster Formation of IMP

As reported previously [21], erythrocyte ghosts loaded with bovine serum albumin could be fused by virus treatment, although larger amounts of virus were needed to attain the same level of fusion as for intact erythrocytes. The rate of the fusion process was similar to that of erythrocyte fusion. As shown in Fig. 2 (A–D), degree of fusion reached

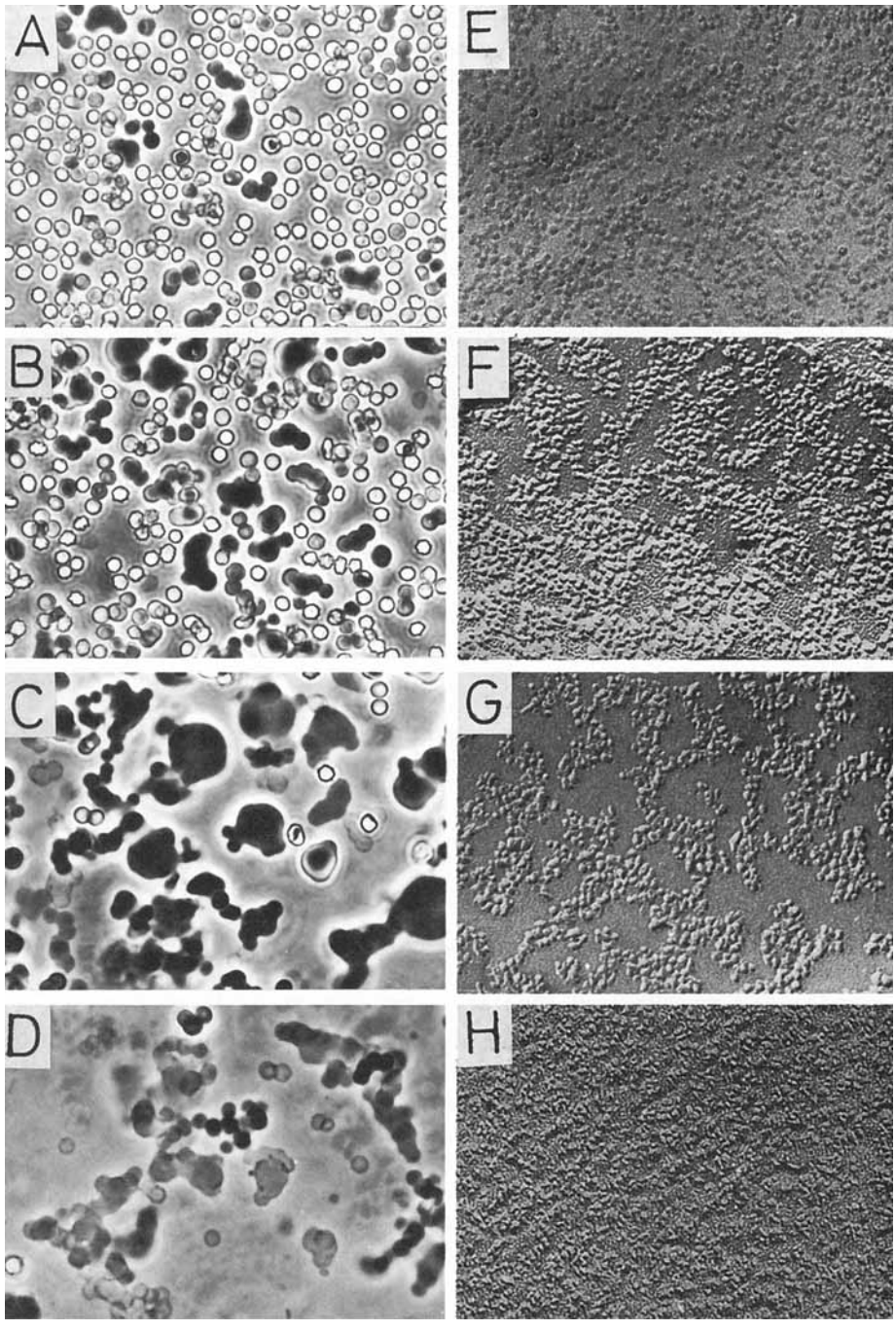

Fig. 1. Comparison of degree of cell fusion with that of cluster formation of intramembrane particles of different virus-to-cell ratios. Intact cells were agglutinated by different concentrations of HVJ for 15 min in an ice bath and subsequently incubated at 37° for 30 min except for H. A, E) 50 HAU/ml; B) 100 HAU/ml; C, F) 400 HAU/ml; D, G, H) 800 HAU/ml; H) no incubation at 37°. A–D) Phase microscopy, × 350; E–H) PF of freeze-fractured sample, × 100,000.

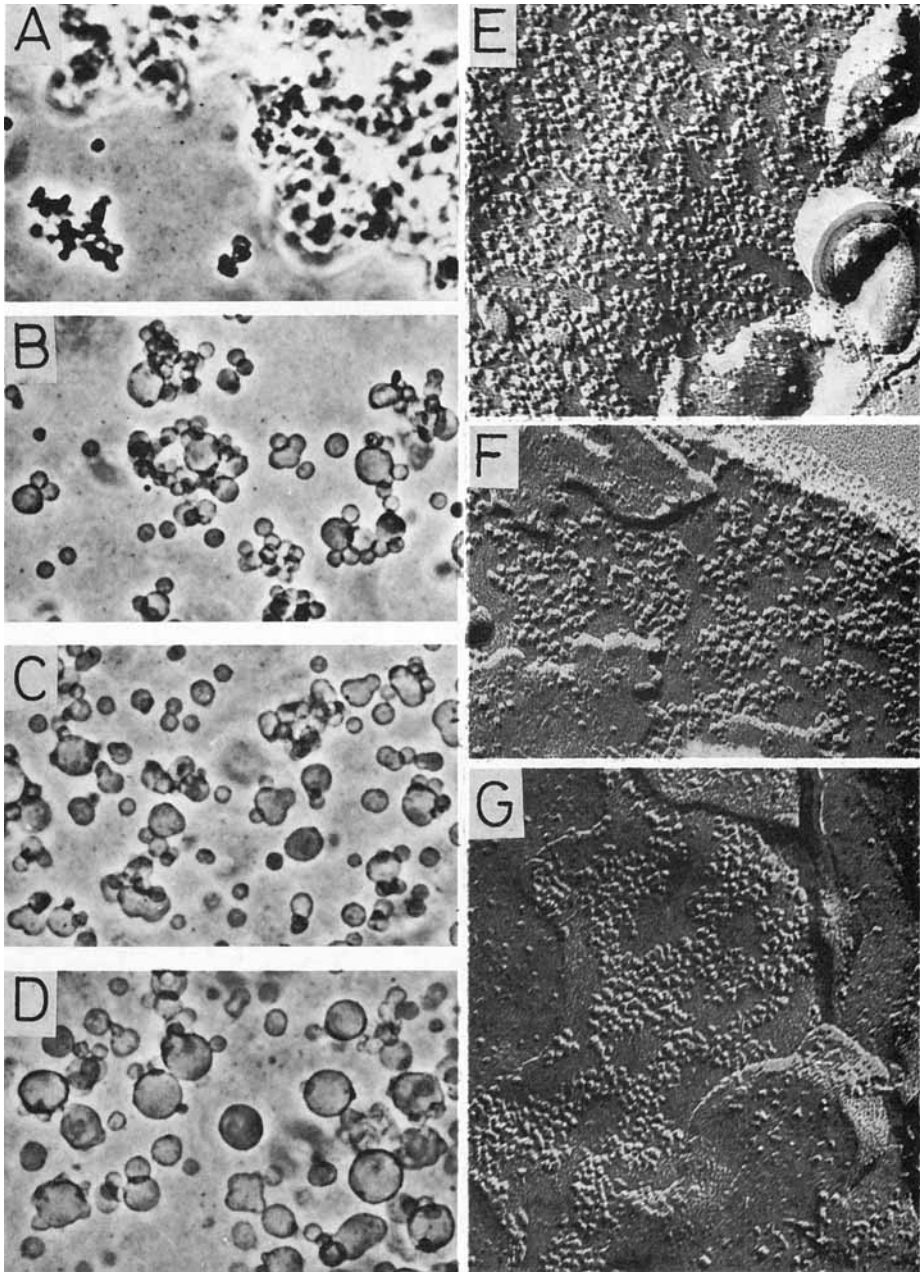

Fig. 2. Time course of the fusion reaction of BSA-loaded ghosts. The ghosts were agglutinated by HVJ (6,000 HAU/ml) for 15 min in an ice bath, then incubated at 37° for A) 0 min; E) 3 min; B) 5 min; C) 10 min; D, F, G) 30 min. A–D) Phase microscopy, × 350; (E–G) PF of freeze-fractured sample, × 100,000; E, F) samples were fixed with 2% glutaraldehyde at 37° for 40 min; G) reaction was stopped by cooling in an ice bath for 30 min, and then fixing with 2% glutaraldehyde was performed for 2 h at 4°.

maximum after 30–60 min incubation at 37°. When the virus-cell aggregate was incubated at 37° for 3 min (Fig. 2E), some redistribution of IMP on the PF could already be detected. Longer incubation (30 min) at 37° resulted in more extensive clustering of IMP (Fig. 2F). Cooling of the fused ghosts to 4° before fixing seems to enhance clustering (Fig. 2G), as recently reported in the case of Ca^{2+}-induced membrane changes of chicken erythrocytes [26], but similar cold treatment of unfused cells showed no appreciable clustering (Fig. 1H).

Inhibition of Virus-Induced Fusion and Clustering of IMP by Antispectrin Antibody Included Within Ghosts

As reported preliminarily [21], antispectrin antibody sequestered in ghosts at a concentration of 1 mg/ml (Fig. 3B) and 4 mg/ml (Fig. 3C) greatly inhibits virus-induced fusion. Figure 3A represents a typical fusion profile of ghosts loaded with bovine serum albumin. Inclusion of IgG fraction prepared from control rabbit sera has no inhibitory effect, as is shown in Fig. 3D. The requirement of relatively high concentrations of antibody (1–4 mg/ml) for inhibition suggests that extensive cross-linking of spectrin by the anti-

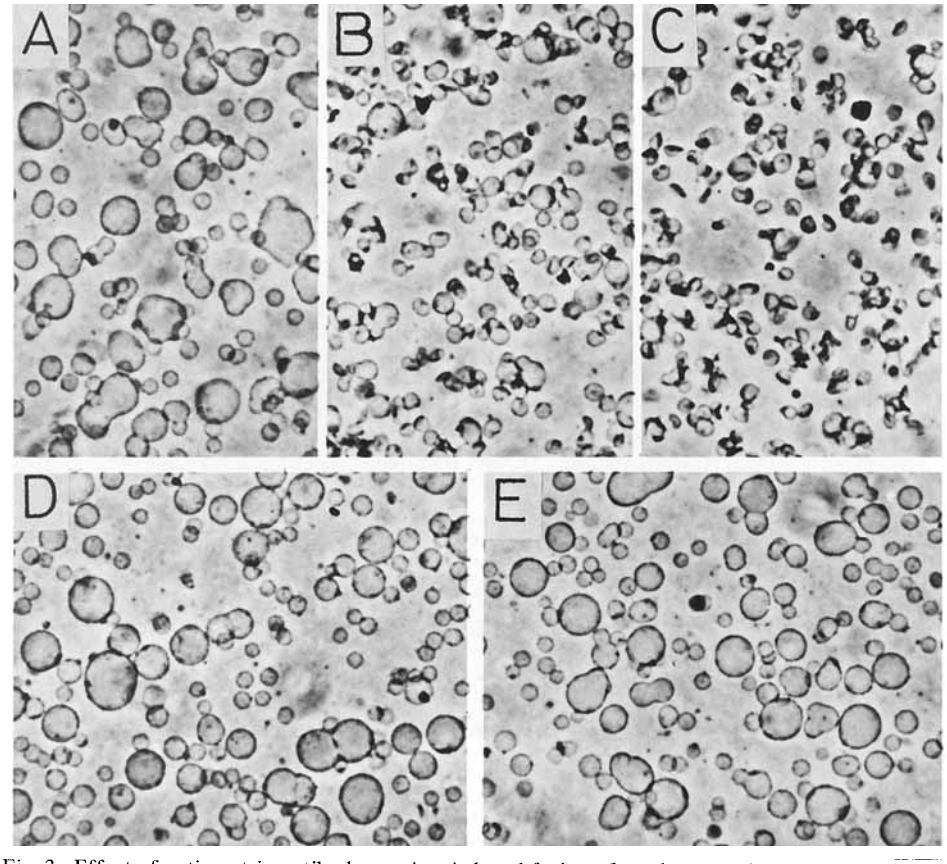

Fig. 3. Effect of antispectrin antibody on virus-induced fusion of erythrocyte ghosts. Ghosts were loaded with 5% BSA containing the following proteins at indicated concentrations by the method described in Materials and Methods. A) No further addition; B) antispectrin antibody, 1 mg/ml; C) antispectrin antibody, 4 mg/ml; D) control immunoglobulin, 4 mg/ml; E) Fab fragments prepared from the antibody, 4 mg/ml. These ghosts were agglutinated by HVJ (10,000 HAU/ml) for 15 min in an ice bath and then incubated at 37° for 30 min. Phase microscopy, × 350.

body might be necessary for inhibition. This assumption was further substantiated by the fact that monovalent F_{ab} fragments loaded at 4 mg/ml did not affect the fusion reaction (Fig. 3E).

Distribution of IMP was studied in inhibited and control samples. As shown in Fig. 4A, fused ghosts containing IgG fractions from control sera exhibited nonrandom distribution of IMP, whereas no such clustering was observed in ghosts loaded with antispectrin antibody at 5 mg/ml (Fig. 4B). Therefore, cross-linking of spectrin with high concentrations of the antibody under the conditions employed seems to restrict the movement of IMP, and this immobilization in turn may lead to the inhibition of virus-induced cell fusion.

Changes Induced by the Virus on External Fracture Face

The extracellular fracture face (EF) of human erythrocyte membranes has fewer IMP than the PF; therefore, it is rather difficult to detect such redistribution as might be induced by the virus. But upon closer examination, pits or depressions corresponding to the places where particles might have been pulled out would be detectable on the EF. As shown in Fig. 5A, the distribution of such pits on EF of fused preparations was nonrandom. In contrast, nonfused preparations had randomly distributed pits and IMP on their EF (Fig. 5B).

Effect of Antispectrin Antibody by Itself on Distribution of IMP:

Several years ago Nicolson and Painter [27] reported that antispectrin by itself induced clustering of cell surface anionic sites (sialic acid residues) in hypotonic conditions. Since we postulated that inhibition rather than induction of cluster formation is responsible for inhibition of virus-induced fusion, the effects of the antibody by itself on distribution of IMP were studied under several conditions. Treatment of ghosts by antibody under the hypotonic shock conditions employed by Nicolson and Painter (Fig. 6, A and B), and simple addition of the antibody to Dodge-type leaky ghosts [20] in the hemolyzing buffer (Fig. 6, C and D). both resulted in cluster formation of IMP at the antibody concentrations optimal for clustering (0.2–1.0 mg/ml). Thus, previous experiments [27] were substantiated by the freeze-fracture method.

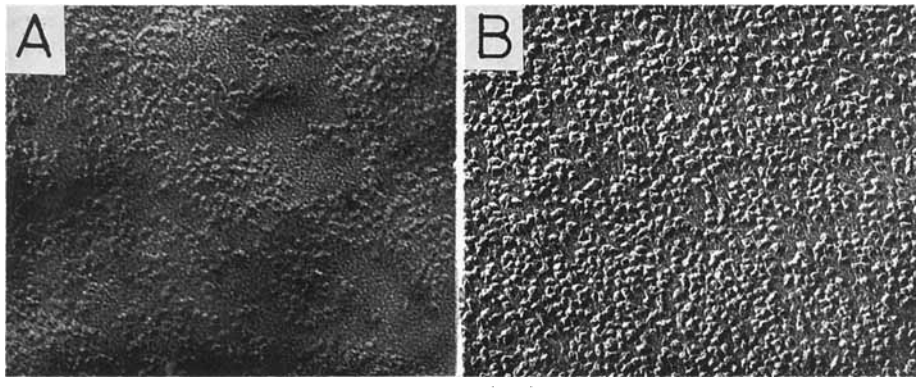

Fig. 4. Effect of antispectrin antibody on the distribution of intramembrane particles of HVJ-treated erythrocyte ghosts. The ghosts, loaded with BSA containing 5 mg/ml of antispectrin antibody (B), were agglutinated with HVJ (8,000 HAU/ml) and then incubated at 37° for 40 min with 2% glutaraldehyde, samples were freeze-fractured as described in Materials and Methods. PF at a magnification of 100,000.

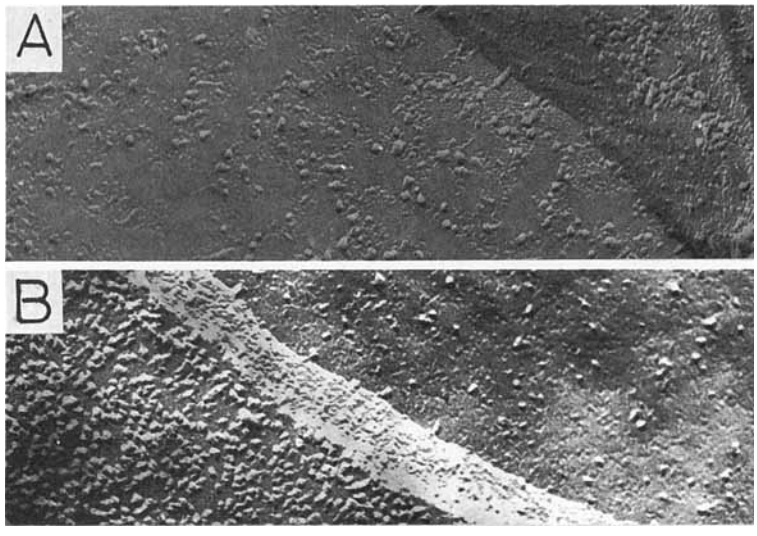

Fig. 5. EF of fused and unfused erythrocyte membranes. A) Fusion conditions were as described in legend of Fig. 1G. A piece of PF can be seen at upper right corner. B) Fusion was inhibited by antispectrin antibody as described in Fig. 4B. Random distribution of IMP on PF can be seen at lower left side, and EF is shown at upper right side.

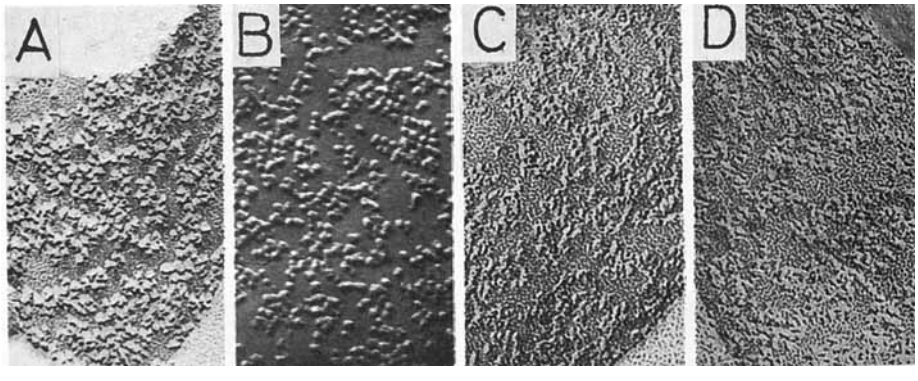

Fig. 6. Redistribution of intramembrane particles by antispectrin antibody in hypotonic conditions. A, B) Ghosts were prepared and processed as described by Nicolson and Painter [27] with slight modification. To pelleted ghosts which were incubated in an isotonic medium (300 PB: 0.05 M sodium phosphate–0.6% sodium chloride buffer, pH 7.5, 300 mOsm) over night at 4°, 10 volumes of 15 PB (300 PB diluted 1:20, pH 7.5) containing antispectrin antibody was added and incubated at 37° for 30 min. After cooling to 4°, the ghosts were washed twice in 15 PB and fixed in 2% glutaraldehyde for 40 min at 37°. Concentrations of the antibody used were 0.2 mg/ml (A) and 1.0 mg/ml (B). C, D) To ghosts prepared by the method of Dodge et al [20], antispectrin antibody in 5 mM sodium phosphate (pH 7.5) was added to give a final concentration of 1.0 mg/ml. After indubation at 37° for 30 min, the samples were fixed by the addition of equal volumes of 4% glutaraldehyde for 40 min at 37°. Freeze-fracturing was performed as described in Materials and Methods. PF was shown at × 100,000.

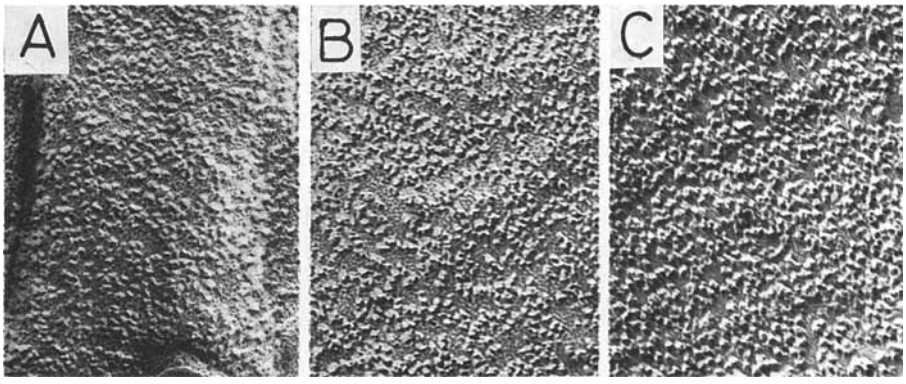

Fig. 7. Distribution of intramembrane particles of ghosts loaded with antispectrin antibody. The ghosts were loaded with 5% BSA containing the indicated concentrations of antispectrin antibody as described in Materials and Methods. Incubation conditions and freeze-fracturing were as described for fusion experiments except that no virus was added. The antibody concentrations employed were (A) 0.2 mg/ml, (B) 1 mg/ml, (C) 5 mg/ml. PF was shown at × 100,000. A ragged appearance was sometimes observed on fracture faces.

However, similar treatment of ghosts with antibody in isotonic salts containing BSA used for the fusion experiments had no effect on redistribution of IMP (Fig. 7, A–C). Therefore, stability of spectrin meshwork on the internal surface of erythrocyte membranes [3, 27–31] may be influenced by the medium composition, and in isotonic conditions partial cross-linking of spectrin by the antibody may not affect distribution of spectrin.

DISCUSSION

Addition of HVJ to erythrocyte suspensions induced several changes in erythrocytes. Amont them, cell agglutination, hemolysis, and redistribution of IMP have been related so far to fusion reaction. Agglutination of cells seems to be a prerequisite for cell fusion, since specific inhibition of hemagglutinating activity of the virus resulted in loss of cell fusion and hemolysis [8, 9]. Furthermore, release of sialic acid by neuraminidase treatment from the cell surface also inhibited both the hemagglutination and fusion reactions [32].

The relationship between hemolysis and cell fusion is more complex. There are several lines of evidence which indicate that fusion of the viral envelope to the cell membrane (envelope fusion) is directly or indirectly responsible for hemolysis [13, 14, 21]. Envelope fusion seems to be required for cell fusion as well as hemolysis, since virus preparations incapable of fusing with the cell membrane either by specific removal of F-glycoprotein through trypsin treatment [33] or by propagation of the virus in cells lacking processing activity (limited proteolysis of F-glycoprotein to an active form) [10] also lose the capacity of cell fusion, hemolysis, and infectious activities.

But envelope fusion does not always induce hemolysis, especially in the case of virus preparations harvested at an early stage of propagation in fertilized eggs. Freeze-thaw or hypotonic treatment of the virus preparations extensively enhanced hemolysis without

affecting fusion activity. Furthermore, several other processes, such as sonication [34], heating at 45° [35], and incubation with very low concentrations of glutaraldehyde [36] were found to inhibit cell fusion without affecting hemolysis.

Similar analysis of the relationship between cluster formation of IMP and cell fusion may be required to decide whether IMP aggregation is a prerequisite to cell fusion. Sendai virus-induced redistribution of IMP was first discovered by Bächi and co-workers [5, 6]; however, use of too high concentrations of virus (10–20 times our virus-to-cell ratios as shown in Fig. 1D and G) has been criticized [37]. But, as detected in this study, the appreciable redistribution of IMP with virus levels as low as 50 HAU/ml and the roughly parallel increase of clustering and cell fusion with increase of virus-to-cell ratio seem to substantiate Bächi and Howe's findings.

On the basis of extensive morphologic studies on fusion of the viral envelope to human erythrocytes, Knutton [37] claimed that he did not always observe redistribution of IMP in his preparations, although he noticed clustering of IMP after longer incubation At present we do not know the exact reason for this apparent discrepancy, but one possibility is that his virus strain has rather low frequency and slow rate of cell-to-cell fusion under the conditions employed, although hemolytic activity may be high with his virus preparations. If so, it is possible that freeze-fracture of a mixture of polyerythrocytes and unfused cells would reveal a spectrum of PF of different degrees of IMP aggregation, as described above. Selection of fracture face at an early stage of virus-cell interaction ie, invagination of the viral envelope (a step before occurrence of the virus-cell fusion) might be possible only under these conditions.

In our hands, the initial, rapid phase of hemolysis, which may represent fusion of the viral envelope to the cell membrane, is almost complete within 3 min after onset of incubation at 37° [18]. Invagination of the viral envelope could not be observed in so short a time as the 3 min incubation set by this rapid reaction. Furthermore, some redistribution of IMP seems to occur within this time, as shown in Fig. 2E. Therefore, the rate of the reaction of the virus with the cell membrane seems to be much faster with our virus preparations and under the conditions of our study.

As also shown in a preliminary communication [21], agglutination of cells by the virus and lysis of resealed ghosts (equivalent to fusion of the viral envelope to the cell membrane) were not affected by the inhibition of cluster formation of IMP by anti-spectrin antibodies; thus redistribution of IMP seems to participate at the latter stage of cell fusion, ie, fusion of adjacent cell membranes. In support for this assumption, measurement of envelope fusion by a spin-labeling technique [11, 12] also confirmed the absence of inhibition of this reaction by antispectrin antibody sequestered within ghosts (K. Sekiguchi and K. Kuroda, unpublished observations). As discussed previously [21], induction of redistribution of IMP has also been reported in many cases of membrane fusion phenomena.

As discussed above, clustering of IMP seems to be a necessary step for virus-induced cell fusion; however, it has also been reported that similar aggregation of IMP can be induced by anti-spectrin antibody at low concentrations which might cross-link spectrin only partially when added to erythrocyte ghosts under hypotonic conditions [27]. Because the viral induction of IMP clustering was performed in isotonic salts containing bovine serum albumin, control experiments without addition of the virus were done in the isotonic fusion medium. No such redistribution of IMP was observed by the antibodies by themselves at any concentrations tested. Thus, lateral mobility of spectrin-IMP complex seems to be influenced by the medium employed. Enhanced mobility of spectrin in hypotonic

conditions is consistent with higher extractability of spectrin at hypotonic conditions [38, 39]. Clustering of both surface anionic sites (sialic acid residues) [27], and IMP (this report) by antispectrin antibodies further substantiated the well-documented assumption that IMP correspond to a complex of band 3 dimer and glycophorin [1–4].

The questions that need to be addressed next would seem to be the following: 1) What component of the virus is responsible for the redistribution of IMP; 2) what component of erythrocyte membranes is first affected by virus treatment and then brings about the clustered configuration of IMP in the membrane; 3) what and how many components of erythrocytes participate in this reaction; 4) is aggregation of IMP for induction of cell-to-cell fusion or is some change in lipid bilayers also required for fusion of adjacent cell membranes?

ACKNOWLEDGMENTS

We are indebted to Professor Ryo Sato of our institute and to Professor Yoshio Okada of the Research Institute for Microbial Diseases for valuable discussions. Electron microscopy was done in Professor Y. Tashiro's laboratory at Kansai Medical University. We are grateful to him and to Dr. Shiro Matsuura of Kansai Medical University for kindly allowing us to use their equipment and for their help in electron microscopy.

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