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# Fusion of Erythrocytes by Sendai Virus Studied by Immuno-Freeze-Etching

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Extensive fusion of human erythrocytes agglutinated by Sendai virus was observed after 30 s of incubation at 37 C. Electron microscopy of thin sections failed to reveal the presence of virions, viral fragments, or discrete viral antigens reactive with ferritin-labeled antibody at the sites of fusion. Immuno-freeze-etching of membrane surfaces demonstrated the dispersal of viral envelope antigens from what appeared to be original sites of viral attachment. Virus-induced clustering of membrane glycoproteins was interpreted as resulting from interaction of viral antigens with membrane receptor proteins and forming the structural basis for fusion of membranes with one another.

Lysis of human erythrocytes by Sendai virus was the subject of recent investigations based on immunochemical and immunoferritin techniques (8), as well as on light and electron microscope observations of thin-sectioned and negatively stained materials (2, 4). We have also demonstrated the sequential stages of viral fusion of human erythrocytes by electron microscope examination of thin-sectioned material and replicas of freeze-etched preparations (3). The use of ferritin-labeled antibody in conjunction with this latter technique (immuno-freeze-etching) appears to be useful in studying the distribution of erythrocyte membrane structural proteins (antigens), some of which have already been otherwise identified by different workers (7a, 14, 17). By the technique of freeze-etching, a characteristic rearrangement of erythrocyte membrane proteins has been shown to occur during the process of viral fusion (3). These observations have now been extended with the use of ferritin-labeled viral antibodies to delineate the distribution and incorporation of viral antigens in the plasma membrane at various stages of the fusion process. The use of mature human erythrocytes excludes cell-dependent factors which must be taken into account in interpreting analogous observations with nucleated cells (15). Our results on erythrocyte fusion reported here support the concept of the membrane as a fluid-lipid system (16) in which glycoproteins can move freely. They also suggest a new interpretation of the general mechanism by which cellular membranes are fused by Sendai virus.

## MATERIALS AND METHODS

**Virus.** Sendai virus (obtained from the World Influenza Center, Mill Hill, London) was grown in the chorioallantoic sac of 10-day-old chicken embryos inoculated with 0.2 ml of diluted ($10^{-3}$) infective chorioallantoic fluid (CAF) from previous passages. After 3 days, CAF, with a hemagglutinin (HA) titer of 2,560, was harvested and clarified by low-speed centrifugation. The virus was then pelleted for 60 min at 45,000 × *g* on a cushion of 60% sucrose and suspended in small volumes of phosphate-buffered saline (PBS), pH 7.2. This concentrated virus was dialyzed against a mixed salt buffer (MSB: 145 mM $Na^+$, 4 mM $K^+$, 2.5 mM $Ca^{2+}$, 1 mM $Mg^{2+}$, 126 mM $Cl^-$, 27 mM $HCO_3^-$, 0.5 mM $SO_4^{2-}$, 1 mM $HPO_4^{2-}$, 900 mg of D-glucose per liter (pH 7.4). For the experiments, the virus was diluted with MSB to a concentration of 8,000 hemagglutinating units (HAU) per ml.

**Erythrocytes.** Human erythrocytes from samples of blood freshly drawn into ACD solution (group O, Rh positive) were washed four times in MSB. The washed erythrocytes were used on the same day for absorption of antisera or for the fusion experiments.

**Antisera.** Rabbits were immunized by three weekly intramuscular injections of 2 ml of virus (100,000 HAU) in complete Freund adjuvant. An intracutaneous booster of the same mixture was given 2 weeks after the last injection. Animals were bled 2 weeks after this booster injection. Each serum was inactivated (56 C, 30 min) and exhaustively absorbed with washed human group O erythrocytes. The globulin fractions were conjugated with ferritin according to methods previously described (5).

**Fusion experiments.** The fusion experiments were carried out essentially as reported previously (3). A 0.2-ml amount of washed erythrocytes were suspended at 4 C in 10 ml of MSB containing 8,000 HAU of Sendai virus per ml. After 30 min, the agglutinated cells were centrifuged and washed twice in MSB at 4 C. After the last centrifugation, the supernatant fluid was removed and 1 ml of MSB was added to the sediment, which was left undisturbed. Samples were taken after 0, 30, 120, 240, and 600 s of incubation at 37 C and chilled quickly in an ice bath just prior to direct fixation for electron microscopy or immunoreaction with ferritin-labeled antibody prior to chemical fixation.

**Immunoreaction.** A 0.5-ml amount of ferritin-labeled antibody (protein concentration 2 mg/ml) was added to the samples of chilled virus-erythrocyte mixtures. The agglutinated cells were resuspended by gentle pipetting and left for 30 min with occasional shaking at 20 C. After this period, the cells were washed free of unreacted antibody by three washings in cold MSB and subsequently prepared for electron microscopy. As controls, cells were incubated with solutions containing free ferritin (protein concentration above 10 mg/ml) or guinea pig anti-rabbit IgG conjugated with ferritin in place of the specific Sendai virus antiserum.

**Electron microscopy.** After the specific immunoreaction, or after incubation with free ferritin or unrelated ferritin-labeled antibodies used as controls, the washed cells were fixed. Cells used for thin sectioning were fixed in a mixture of 3% glutaraldehyde and 3% acrolein in cacodylate buffer (pH 7.2), washed in two changes of 0.1 M cacodylate buffer containing 0.18 M sucrose, and postfixed in 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) at 4 C. The fixed material was left overnight in a 4% solution of uranyl acetate before dehydration and embedding in plastic. Similar fixation of cells used for freeze-etching experiments was stopped after 20 min by two washings in PBS at 4 C followed by three more washing procedures in distilled water. After final centrifugation, the cells were frozen and replicated after 3 min of deep-etching in a Balzers freeze-etching apparatus according to standard methods (11). The freeze-etching of cells suspended and frozen in distilled water after reaction with labeled antibodies and chemical fixation is referred to hereinafter as immuno-freeze-etching. Other samples were frozen without prior chemical fixation in a medium consisting of PBS (pH 7.4) containing 20% glycerol as described in a previous report (3).

## RESULTS

All experiments were initiated by adsorption of virus to erythrocytes at 4 C for 30 min, centrifugation of agglutinated cells, and replacement of unadsorbed virus with MSB. From this point on, different experimental conditions governed the progress of fusion as observed by light and electron microscopy. Temperature was a critical factor. No fusion occurred at or below 20 C. In the experiments involving subsequent incubation of virus-agglutinated cells for various times at 37 C, the reaction could be stopped by quick rechilling. Thereafter, incubation for 30 min at 20 C did not alter the extent of interactions between virus and cells beyond the point reached during the experimental period at 37 C. For this reason, reactions with ferritin antibody could be carried out at 20 C on stabilized virus-cell mixtures. The agglutination and fusion of erythrocytes by Sendai virus as visualized by phase contrast microscopy after 30 min at 37 C has already been described (3). However, by electron microscopy, the first morphological evidence of cell-cell fusion was encountered as early as 30 s after the change of temperature to 37 C. Membranes of agglutinated erythrocytes lying parallel to one another and separated by a gap normally 10-nm wide were bridged by initial connections between the two cytoplasms (Fig. 1). A striking characteristic of these loci of primary fusion was the absence of viral particles or of viral antigen recognizable by reaction with specific ferritin-labeled viral antibody. On free, unapposed segments of membrane observed after 30 min at 37 C (not shown), many virions were still detectable. These absorbed particles were discretely tagged with ferritin antibody, and in a few instances viral envelope had already started to fuse with the cellular membrane. This is suggested in the upper left corner of Fig. 1, where a few ferritin antibody molecules are seen to be reactive directly with the plasma membrane. The incidence of specific reactivity of antiviral antibody with plasma membrane increased markedly on further incubation at 37 C, during which at least 50% of the viral particles became merged with the surface of the cell. The primary sites at which this fusion between viral envelope and cell membrane had occurred were recognized in thin sections by the presence of nucleocapsid remnants just beneath segments of the cell surface which had become widely reactive with ferritin antiviral antibody. Once the viral envelope had become fully integrated into the plasma membrane, reactivity with viral antibody was no longer restricted to these sites of initial viral incorporation, but became widely distributed over the membrane independently of residual nucleocapsid (Fig. 2). This observation was confirmed by the replication of cellular surfaces with the immuno-freeze-etching method. Cells which were reacted in the standard way with virus at 4 C and subsequently with ferritin-labeled antibody at 20 C revealed virions at-

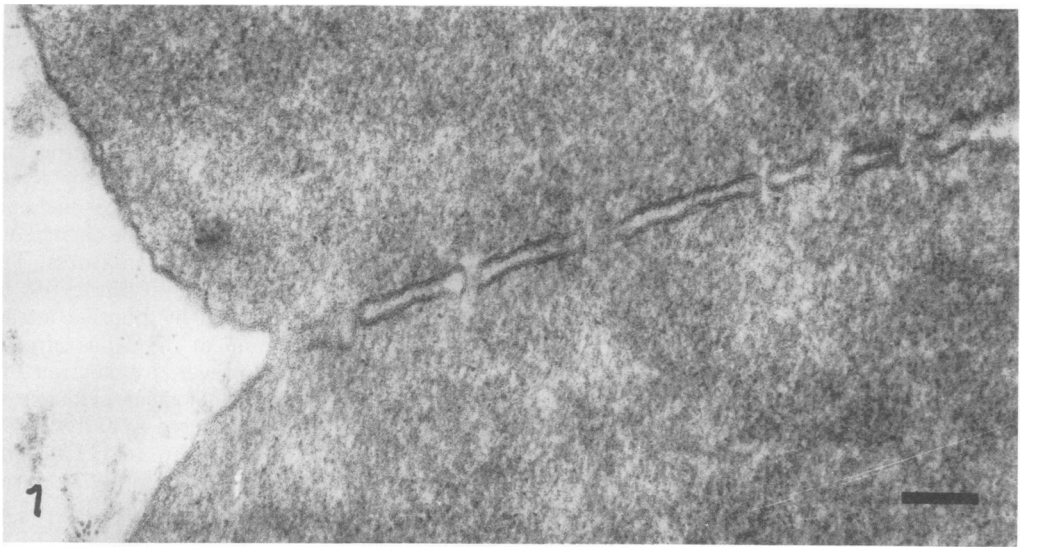



FIG. 1. *Human erythrocytes, after reaction with Sendai virus at 4 C, incubation at 37 C for 30 s and reaction with ferritin-labeled viral antibody. Note the absence of any viral structures or ferritin-tagged viral antigens at the sites of fusion and the simultaneous connection of membranes with one another at several points. ×80,000. Bar, 100 nm.*

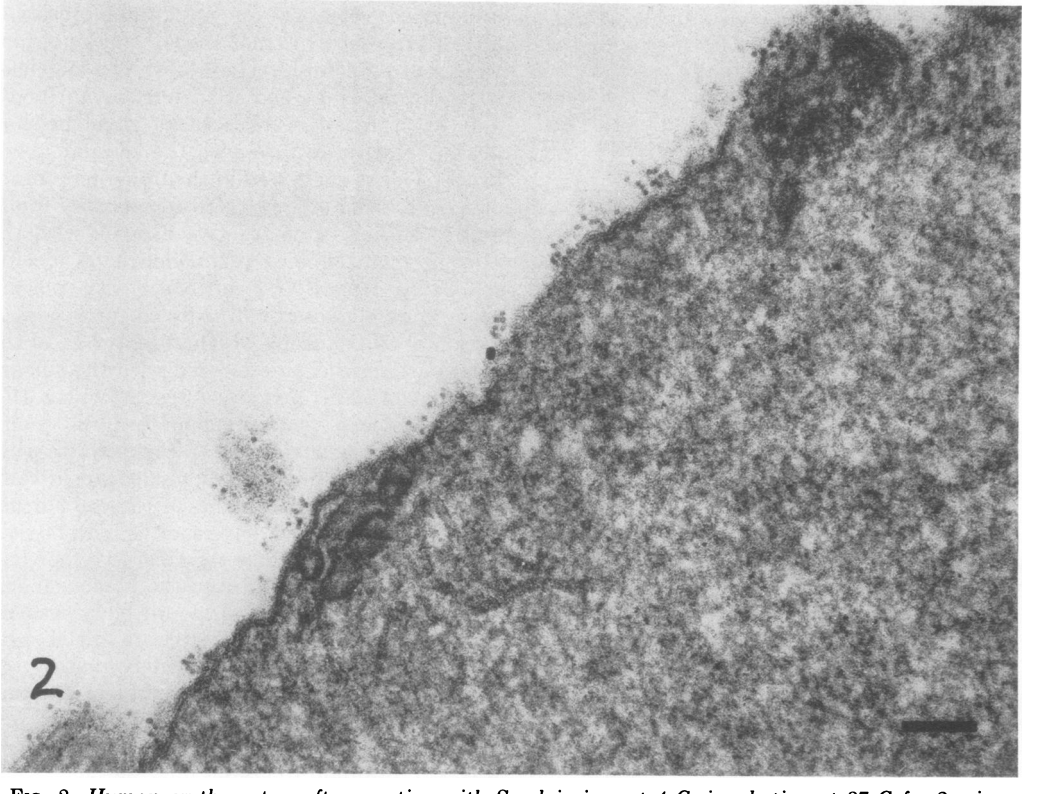



FIG. 2. *Human erythrocytes, after reaction with Sendai virus at 4 C, incubation at 37 C for 2 min, and reaction with ferritin-labeled viral antibody. The points of initial fusion of viral envelope with the plasma membrane are indicated by the presence of viral ribonucleoprotein strands near the cell surface which is reactive with ferritin antibody. The random distribution of ferritin indicates reaction of antibodies with sites other than loci of initial fusion between virus and cell. ×100,000. Bar, 100 nm.*

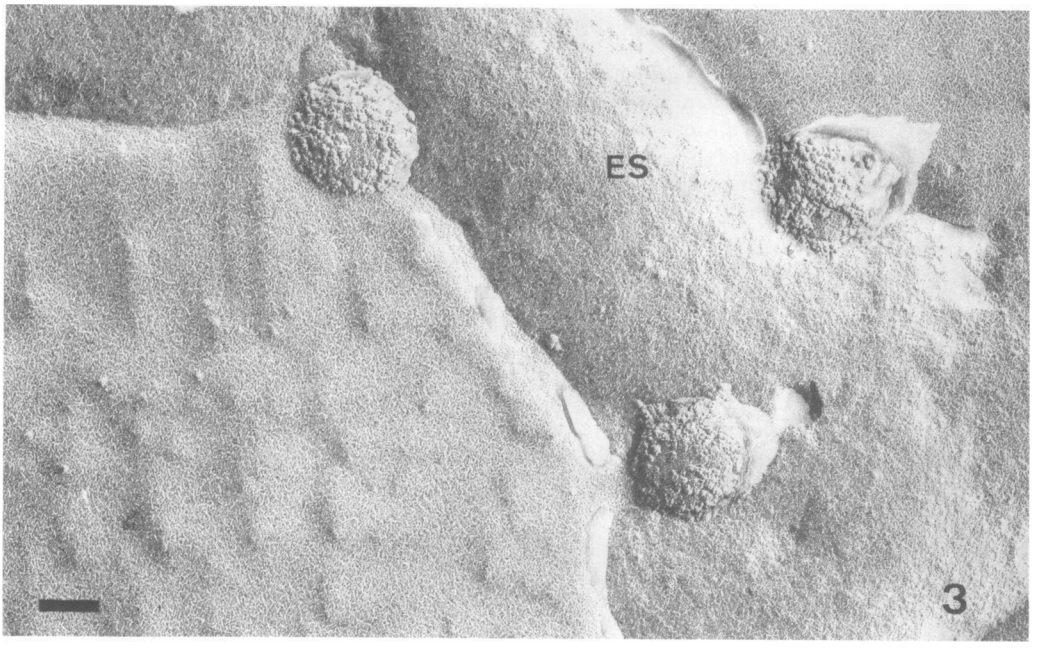
tached to the cell surfaces, but no fusion. The virions were covered with ferritin antibody appearing as particles with a diameter of 15 nm (Fig. 3). Elsewhere the surface of the cells remained smooth, indicating that it had not reacted with viral antibody. Cells similarly prepared, but incubated with either guinea pig antisera of unrelated specificity or with free ferritin, showed the same smooth surface; and the surface of virus particles had a more finely granular character, readily distinguished from the larger ferritin deposits. The merging of viral particles with the cellular surface was readily documented by the immuno-freeze-etching method and was observed after only 120 s of incubation at 37 C (Fig. 4). Ferritin antibodies tagging viral antigens were found in clusters of varying size and in random distribution over the plasma membrane. In contrast to the discretely and heavily tagged virions seen in Fig. 3, clusters of 2 to 50 molecules of ferritin antibody are seen in Fig. 4, 5, and 6 on the external surface (ES) of the membrane and reflect the disintegration of the viral envelope after its incorporation into the plasma membrane. On the outer fracture face (OFF), typical intramembranal particles 5 to 10 nm in diameter are seen. These particles have been rearranged into a pattern which is different from their normal random distribution and which results in the appearance of intervening smooth patches, referred to previously as "plaque-like" areas (3). This aggregation of intramembranal particles increased with prolonged incubation at 37 C. Specimens frozen in PBS containing 20% glycerol, instead of revealing surfaces exposed by sublimation (ES), revealed almost exclusively inner or outer fracture faces (IFF, OFF) of cells agglutinated and partially fused by virus. Figures 7 and 8 show portions of two apposed cell membranes, the upper of which, exposing the inner fracture face, has been broken away in some areas to reveal the underlying outer fracture face of the other cell. The clustering of intramembranal glycoprotein particles was a common finding after 10 min of incubation of cells and virus at 37 C (Fig. 7, 8) and became apparent on both aspects of the membranes, the grouped patterns on the IFF resembling those on the OFF. It was occasionally possible to detect areas in which the rim formed by the IFF leaflet became merged with the OFF leaflet (Fig. 7, arrows).

The sequence of events observed in our experiments based on electron microscope examination of thin sections, freeze-etching, and immuno-freeze-etching can be summarized as follows. (i) Cells are agglutinated at 4 C by the virions. (ii) Incubation at 37 C rapidly results in extensive fusion of the agglutinated cells by interconnection of membranes (Fig. 1). (iii) Thin sections of these primary sites of intercellular bridging do not reveal the presence of any virions, viral fragments, or discrete viral antigens reactive with specific ferritin-labeled viral antibody (Fig. 2). (iv) Thin sections (Fig. 2) and immuno-freeze-etching (Fig. 5, 6) revealed the merging of the viral envelope with the plasma membrane, followed by rapid dissemination of viral antigens from the loci of primary viral attachment to secondary sites, identified by their reaction with ferritin-labeled viral antibody. The sites of initial viral attachment remain reactive with labeled antibody. (v) Incubation at 37 C caused progressive agglutination of intramembranal particles as seen in replicas of freeze-etched preparations of agglutinated-fused erythrocytes. This process was recognizable after 2 min at 37 C (Fig. 4, OFF area) and became a dominant feature within the first 10 min (Fig. 7, 8).

Figures 9 and 10 represent schematically two- and three-dimensional concepts, respectively, of the interaction of Sendai virus with erythrocyte membranes and the reaction of ferritin antibody with viral components. The sketches, based on interpretation of freeze-etched preparations, illustrate virus adsorption (Fig. 9A, B, 10A), the incorporation into the membrane of viral envelope fragments and their gradual disintegration (Fig. 9C, 10B), the centrifugal migration of viral antigen through the membrane, and the resulting clustering of the intramembranal particles as seen on the fracture faces of the cellular membrane (Fig. 10B).

## DISCUSSION

Interpretation of the foregoing results takes advantage of the concept of the membrane as a "fluid mosaic" model (16) in which the glycoprotein is represented schematically as globular particles randomly interspersed within a bilayer of lipids and lipoproteins. These intramembranal globular particles, which have been shown to be mobile within the membrane (13), represent the more hydrophobic portions of glycoprotein complexes, the distal hydrophilic ends of which bear the sialic acid-containing receptors for myxoviral hemagglutination (10, 17). Extracted in soluble form, this glycoprotein is a potent inhibitor of viral hemagglutination and a substrate for viral neuraminidase (8). The primary interaction required for hemolysis-fusion would therefore appear to be between the viral antigens (hemagglutinin-neuraminidase com-



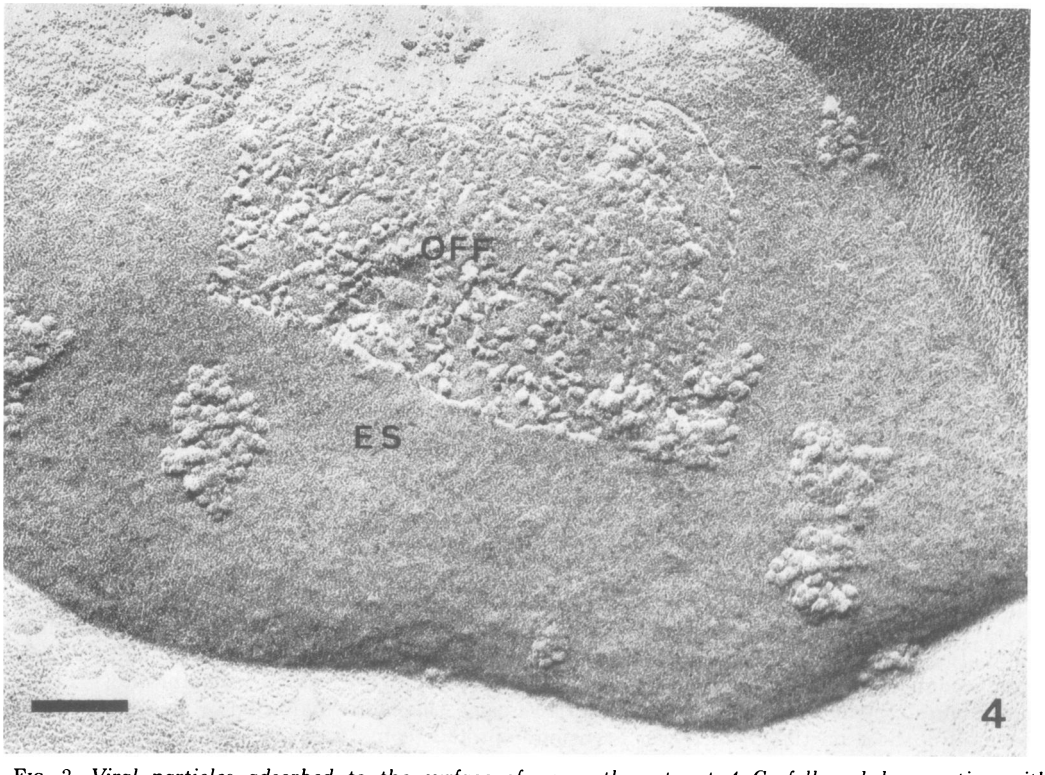
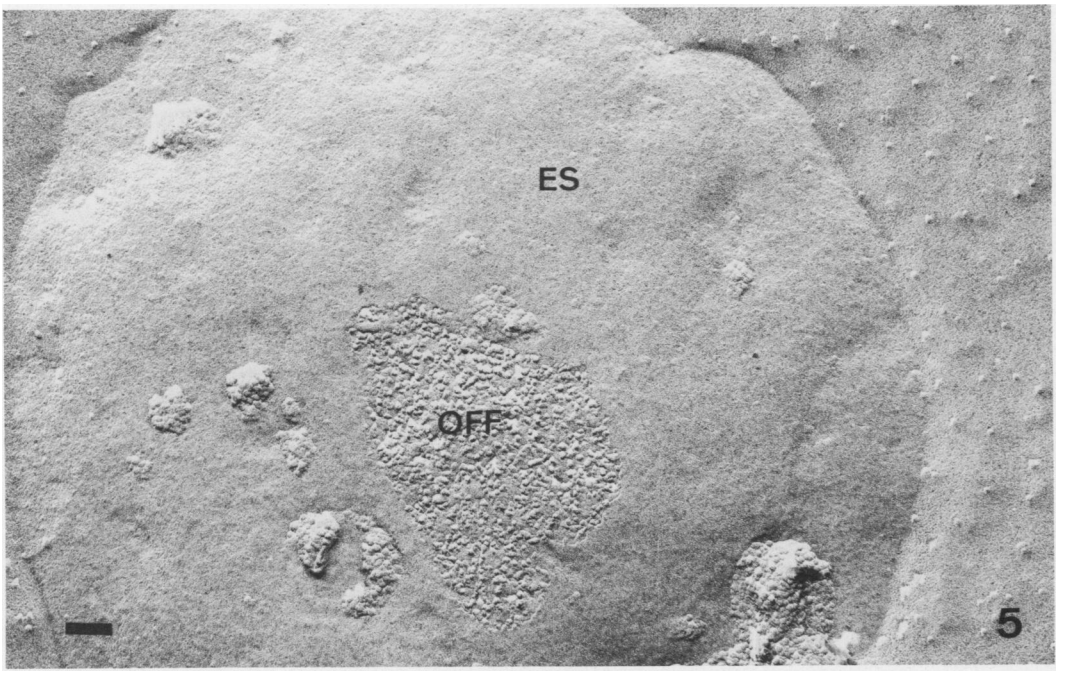




FIG. 3. *Viral particles adsorbed to the surface of an erythrocyte at 4 C, followed by reaction with ferritin-labeled antibody. No fusion between virus and cell membrane is observed in these samples prepared at temperatures below 37 C. The exposed surfaces of the virions are covered with particles of ferritin-antibody, whereas the external surface (ES) of the cell, revealed by sublimation of the medium, is smooth and is unreactive with antibody. ×80,000. Bar, 100 nm.*

FIG. 4. *Same as Fig. 3, after 2 min of incubation at 37 C. Virus has merged with the cellular surface and no longer appears as a round particle adsorbed to the surface as in Fig. 3. The presence of viral antigens in the membrane is indicated by dispersed clusters of ferritin antibody. Abbreviations: ES, external surface; OFF, outer fracture face of membrane. ×125,000. Bar, 100 nm.*



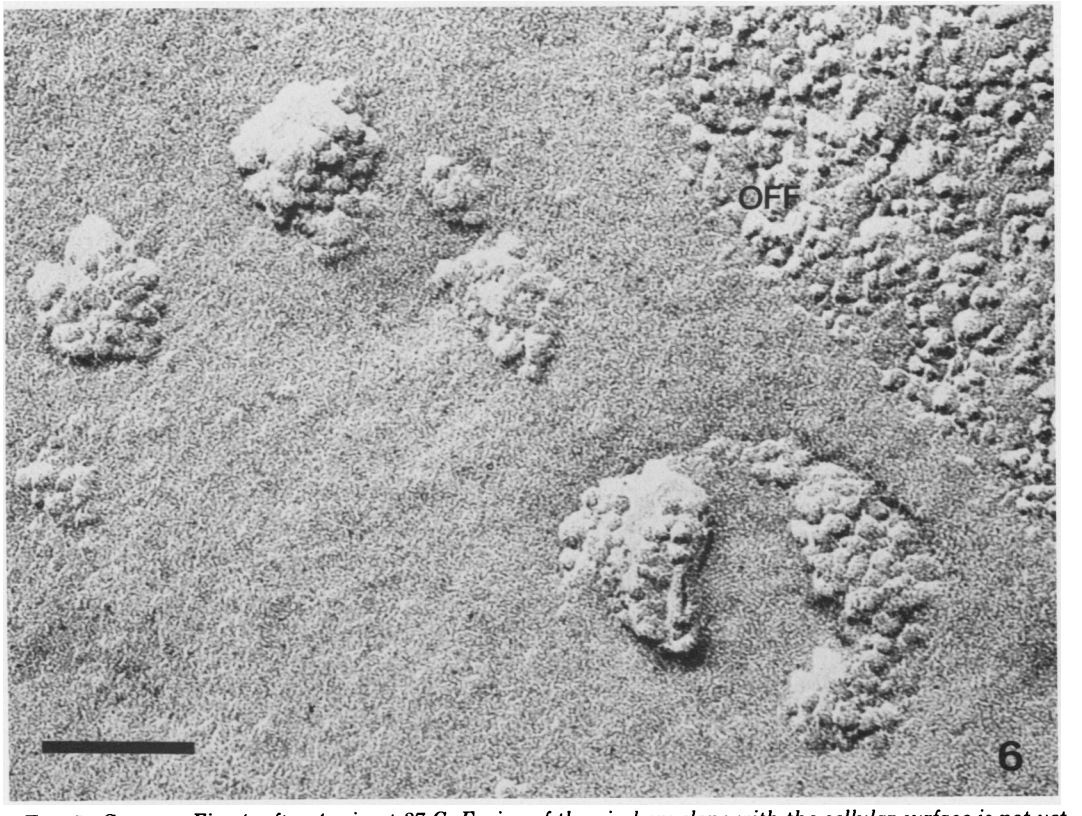
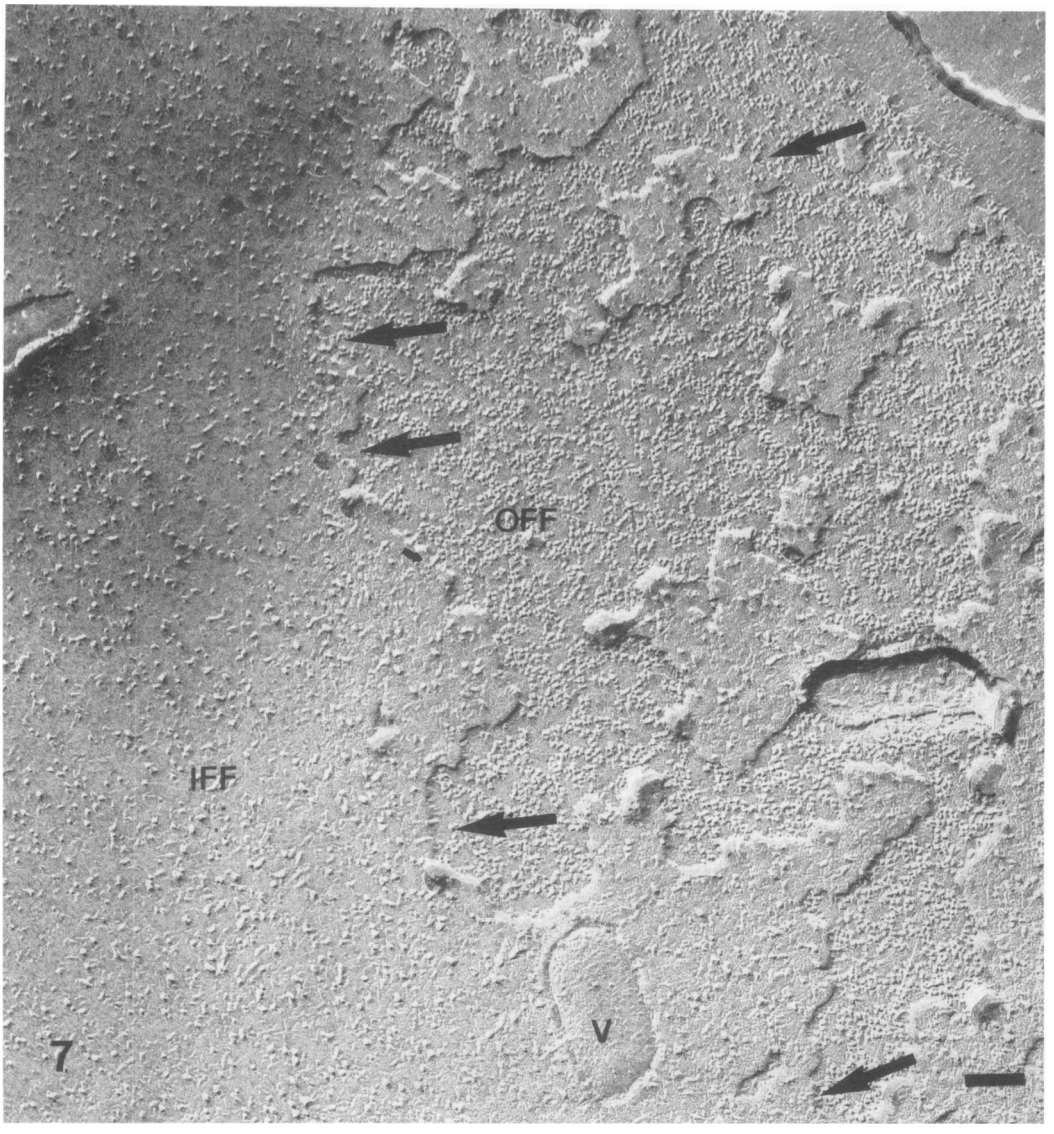




FIG. 5. *Same as Fig. 4, after 4 min at 37 C. Fusion of the viral envelope with the cellular surface is not yet complete.* ×*60,000. Bar, 100 nm.*

FIG. 6. *Detail of Fig. 5.* ×*200,000. Bar, 100 nm.*



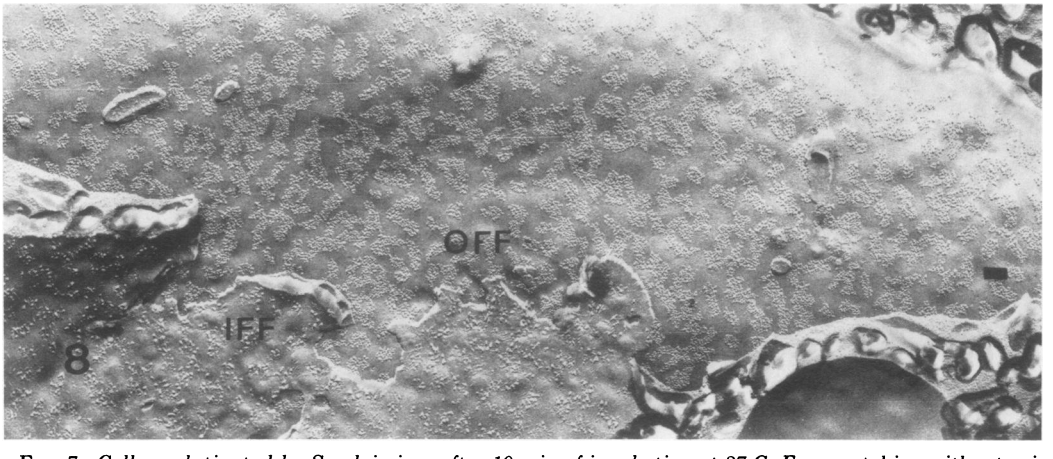




FIG. 7. *Cells agglutinated by Sendai virus after 10 min of incubation at 37 C. Freeze-etching without prior immunoreaction and chemical fixation (specimen frozen in medium containing 20% glycerol). The overlying cell shows the inner fracture face (IFF), the underlying membrane shows the outer fracture face (OFF), and both display aggregation of the membrane glycoprotein particles. Arrows indicate zones in which the fusion between the IFF and OFF leaflets appears to have occurred. ×100,000. Bar, 100 nm.*

FIG. 8. *Same as Fig. 7, 10 min of incubation at 37 C. Note clustering of membrane particles. ×30,000. Bar, 100 nm.*

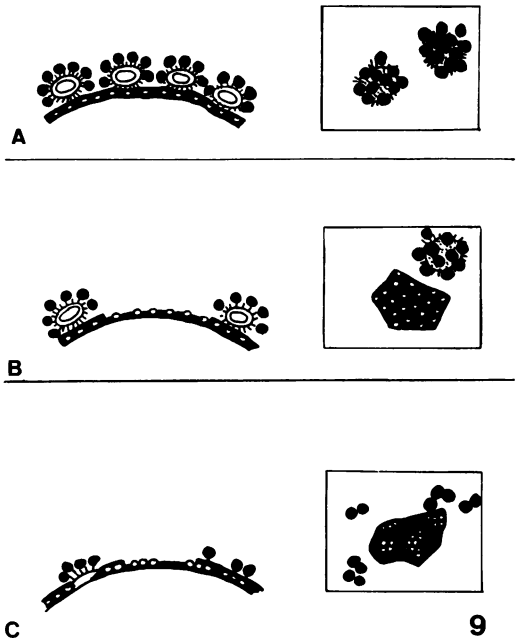



FIG. 9. *Schematic representation of the immuno-freeze-etching of Sendai virus interacting with red blood cell membranes. A, Virus particles adsorbed at 4 C (left, cross-section; right, views from above). Surfaces of virions are covered with ferritin-labeled antibody. Membrane glycoproteins appear as white particles. B, Same as A, after fracture of the membrane by freeze-etching. The intramembranous particles are exposed by fracturing of the surface. Virus-attached particles of ferritin antibody are present only at "etched" external surface exposed by sublimation. C, Same as B, after incubation at 37 C. The viral envelope is still tagged after its incorporation into the membrane. The superior view shows ferritin antibody attached to dispersed viral antigens.*

plex) on the one hand and, on the other, this receptor glycoprotein. The interaction occurs first between intact virions and the cell surface (2) and then somehow within the membrane between the disrupted viral envelope components and the intramembranal glycoprotein particles. The latter reaction is reflected in the aggregation of glycoprotein particles observed within the membranes of fused cells, although a direct agglutinating action of viral subunits on the intramembranal glycoprotein cannot be proven from the data at hand. Correlated with membrane particle aggregation is the dispersal of viral antigen from the sites of primary viral attachment to other areas of the membrane. This dispersal was signaled by the reactivity of ferritin-antiviral antibody not only at the sites of primary viral attachment but also at widely scattered foci on the cell surface, as demonstrated by immuno-freeze-etching (Fig. 4, 5, 6).

Fusion-hemolysis, then, is the secondary result of these interactions. The degree to which cell-cell fusion limits or balances hemolysis depends on the extent and intimacy of initial contact between virus-bearing cells. The extent of either reaction is influenced in turn by the relative concentration of virus and cells in the mixture. The displacement of the glycoprotein particles by clustering represents a basic structural change necessary for the occurrence of fusion or lysis, favored by the formation of "plaque-like" areas with lipid micelles. The latter are considered to be an ideal type of conformation for the establishment of fusion between membranes (9). On the other hand, the clustering of proteins in apposed membranes may also favor fusion by allowing interdigitation of the proteins, as suggested by others (18). The areas in which proteins are clustered represent weakened segments of the membrane through which hemoglobin and ions escape.

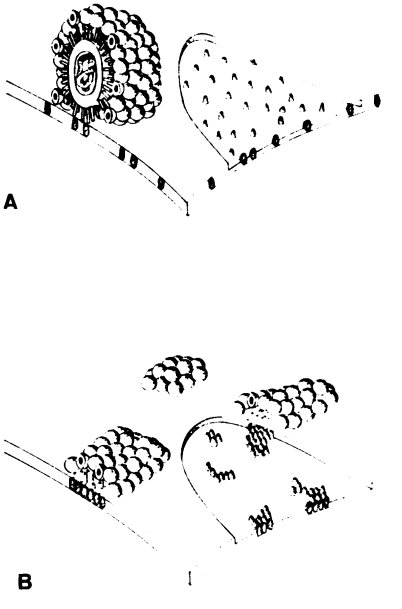



FIG. 10. *A, Schematic representation of Sendai virus after adsorption to an erythrocyte membrane and reaction with virus-specific ferritin-labeled antibody, as seen by immuno-freeze-etching. Membrane-intercalated particles represent the glycoproteins of the membrane. B, Same as A, after incubation at 37 C. The viral envelope has fused with the plasma membrane. The disintegration of virion and the migration of viral antigens within the plane of the membrane result in the agglutination of the cellular proteins which represent receptors for the viral structures. Particle clustering is seen in the fractured part of the membrane.*

The foregoing interpretation, supported by the consistent absence of morphologically recognizable viral remnants at the sites of fusion, represents a substantial departure from previously held concepts of the fusion mechanism (6, 7, 12). Okada (12) postulated the direct mediation by virions, or viral envelope fragments above a critical size, first in cell-cell bridging and then in reconnection of the fused viral and cell membrane leaflets. In actual fact, such relationships have never been demonstrated with an electron microscope either in our investigation or in those of others.

The temperature dependence of these intramembranal reactions also supports our interpretations. Temperatures below 15 C stopped the reaction and prevented cell fusion. It is known that, at or below 15 C, viscosity of membrane phospholipids inhibits their free diffusion and intermixing with proteins (16). This would explain why the observed aggregation of particles, and hence fusion, were prevented at temperatures below 15 C.

Moreover, fusion can be prevented by reaction of the cellular surface (before or after viral adsorption) with concanavalin A, which inhibits the mobility of the membrane glycoprotein particles (T. Bächi, unpublished data). Recent reports have described similar aggregation of particles induced by chemical agents. The membrane particles could be reversibly clustered by acid pH (13), by partial tryptic digestion of the membrane (17), or by treatment with hypotonic media (14). It is conceivable that any of these chemical treatments concomitantly cause structural modifications which could induce cell fusion in the absence of virus (1). However, the irreversibility of the aggregation induced by Sendai virus under strictly physiological conditions is to be contrasted with the more drastic conditions needed to induce similar changes by these nonviral reagents.

Endogenous cell fusion occurring during maturation of paramyxoviruses and pseudomyxoviruses, as well as antibody-mediated fusion of cells infected with parainfluenza virus type 2, can readily be explained by the same hypothesis. This latter virus is not ordinarily highly syncytiogenic. However, in the presence of either anticellular or antiviral antibody, cells in the early stages of infection become fused with one another (19). In both endogenous and antibody-mediated fusion of infected cells, rearrangement of intramembranal particles may logically be assumed to occur. This favors the fusion of cells held in apposition to one another either by nascent virions in the membrane or, as in the latter instance, by antibody.

## ACKNOWLEDGMENTS

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