STUDIES OF MEMBRANE FUSION

I. PARAMYXOVIRUS-INDUCED CELL FUSION, A SCANNING ELECTRON-MICROSCOPE STUDY

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SUMMARY
Fusion of erythrocytes and HeLa cells with Sendai and Newcastle disease viruses has been studied by scanning electron microscopy. Most virus particles are spherical but vary in diameter from ~200 to ~600 nm. At 4 °C virus particles bind randomly to the cell surface and at high cell densities cross-linking of adjacent cells by virus particles results in cell agglutination. Cell-cell fusion takes place when the agglutinated cell suspension is warmed to 37 °C. Fusion is initiated at sites of cell-cell contact and is accompanied in all cases by cell swelling. In the case of suspension HeLa cells, virally mediated cell swelling involves an ‘unfolding’ of cell surface microvilli and results in the formation of smooth-surfaced single or fused cells. With erythrocytes, swelling results in haemolysis. There is a dramatic reduction in the numbers of virus particles bound to cells following fusion.

INTRODUCTION
Ever since Okada (1958) reported that ultraviolet-inactivated Sendai virus could fuse cells when used at high multiplicities this technique has been widely used for the production of somatic cell hybrids (Harris, 1970; Ephrussi, 1972). The fusion of cells with enveloped viruses provides a convenient system with which to investigate the process of cell and membrane fusion and numerous morphological studies have been directed towards this end. However, these morphological studies have not resolved conflicting hypotheses whether fusion occurs directly between cells or via an intermediate step of fusion with the viral envelope (Apostolov & Almeida, 1972; Bächli & Howe, 1972; Bächli, Auget & Howe, 1973).

As part of a detailed morphological investigation into the mechanism of virus-induced cell fusion (Knutton, 1976, 1977) we have examined the changes in cell surface morphology which occur during the interaction with virus and during the fusion process and in this paper we present scanning electron-microscope observations on the fusion of erythrocytes and HeLa cells by Sendai and Newcastle disease viruses.
METHODS

Cell fusion

Human erythrocytes (Type O) from samples of blood freshly drawn into heparin were washed 3 times in Hanks' balanced salt solution (Hanks' BSS) (Hanks & Wallace, 1949). A 2% suspension of washed erythrocytes was used for fusion experiments. HeLa cells were grown on plastic Petri dishes in minimal essential medium (Eagle, 1959) containing 10% newborn-calf serum. Exponentially growing cultures were removed from culture dishes with trypsin, washed and resuspended at a concentration of $6 \times 10^6$ cells/ml in Hanks' BSS. Newcastle Disease virus was a donation by Dr D. J. Alexander, Central Veterinary Laboratories, Weybridge, Surrey; Sendai virus (from the Sir William Dunn School of Pathology, University of Oxford) was grown in 11-day-old chick embryos, harvested after 72 h and clarified by centrifugation. Virus was stored under liquid nitrogen at a concentration of 20000 haemagglutinating units/ml (HAU/ml) until used.

For fusion experiments, the virus was ultraviolet-inactivated and diluted with Hanks' BSS to a concentration of 1000 HAU/ml for the fusion of erythrocytes and 2000 HAU/ml for the fusion of HeLa cells; 1 ml of virus was added to 1 ml of the washed cell suspension at 4 °C. After 30 min the agglutinated cells were transferred to a water bath at 37 °C and samples taken after various time intervals up to 60 min. Fusion was stopped by either the addition of an equal volume of 6% glutaraldehyde in Hanks' BSS or by chilling to 4 °C.

Scanning electron microscopy

For scanning electron microscopy cells were allowed to settle on gelatin-coated glass coverslips (Vial & Porter, 1975) and immediately fixed with 3% glutaraldehyde in Hanks' BSS for 30 min. In some cases cells were postfixed for 1 h with 1% buffered osmium tetroxide prior to being dehydrated through a graded series of acetone solutions. The coverslips were then transferred to liquid carbon dioxide and critical point dried. Finally the coverslips were mounted on aluminium stubs, coated with a thin layer of gold, and the specimens examined in a Cambridge Type II stereoscan.

Observations

Sendai (SV) and Newcastle disease virus (NDV) are essentially spherical particles but vary in size from $\sim 200$ to $\sim 600$ nm in diameter (Fig. 1). When the virus is allowed to interact with erythrocytes previously attached to a substratum, virus particles are seen randomly distributed over the entire cell surface (Figs. 2, 3). In some instances, particularly with NDV, virus particles form deep invaginations in the cell surface and sometimes deform the erythrocyte membrane and cross-link different parts of the same cell (Fig. 3, arrow). Sendai virus particles bind to the surface HeLa cells in a similar manner. In suspension, HeLa cells are spherical and highly villated (Fig. 6) and virus particles bind to both microvilli and non-microvillous membrane (Fig. 7) and, in some instances, crosslink microvilli to the same cell (Fig. 8, inset).

The interaction of cells at high density with virus at 4 °C results in cell agglutination. Agglutination of cells can be seen to result from the crosslinking of adjacent cells by virus particles (Figs. 4, 5, arrows). This is more difficult to see with NDV because these virus particles tend to become embedded in the erythrocyte membrane (Fig. 5).

Erythrocytes essentially retain their biconcave disk morphology although many cells do become distorted during agglutination (Figs. 4, 5). HeLa cells remain spherical
(Fig. 8) but with these cells agglutination appears to involve the interdigitation and crosslinking of microvilli on adjacent cells by virus particles (Fig. 9). This is more clearly seen in stereo images (Fig. 10).

Cell-cell fusion is initiated by warming an agglutinated cell suspension to 37 °C. With erythrocytes the first noticeable change after only a few seconds at 37 °C is the swelling of many cells to a spherical shape and the first cell-cell fusion is apparent after about 1 min (Fig. 11). Continued incubation leads to extensive cell fusion as well as total haemolysis of both fused and unfused cells. Cells agglutinated with SV tend to remain agglutinated on incubation at 37 °C. Fusion under these conditions tends to result in the formation of large polyerythrocytes comprising many individual erythrocytes after incubation for times longer than about 5 min (Fig. 12). When NDV was used to fuse cells a marked disaggregation of the agglutinated erythrocytes took place on warming to 37 °C. As a result, NDV fused erythrocytes comprising more than two or three individual erythrocytes are very rarely seen even after long periods (up to 60 min) of incubation at 37 °C (Fig. 13). Numerous SV particles but only a few NDV particles remain bound to both fused and unfused erythrocytes following a 60-min incubation at 37 °C (Figs. 12, 14). In either case there are significantly fewer particles bound to fused erythrocytes when compared to erythrocytes agglutinated at 4 °C.

Incubation of a SV-agglutinated HeLa cell suspension at 37 °C also leads to cell fusion although the time course for HeLa cell fusion differs from that of erythrocytes. Extensive HeLa cell fusion is only seen after about a 30-min incubation at 37 °C (Fig. 15) although a few fused cells can be seen as early as 5 min. Many of the morpho-
Figs. 2, 3. Immobilized erythrocytes which have been allowed to interact with Sendai and Newcastle disease virus, respectively. Virus particles bind randomly to the cell surface. Newcastle disease virus forms deep invaginations in the erythrocyte membrane and sometimes cross-links different parts of the same cell (Fig. 3, arrow). Fig. 2, ×13000; Fig. 3, ×14000.

Figs. 4, 5. Erythrocytes agglutinated at 4 °C with Sendai and Newcastle disease virus, respectively. Agglutination results from the cross-linking of adjacent cells by virus particles (arrows). Fig. 4, ×7000; Fig. 5, ×6500.
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Fig. 6. The surface of HeLa cells is covered with many \( \sim 0.1 \) \( \mu m \) diameter and up to \( \sim 2 \) \( \mu m \) long microvilli. \( \times 7250 \).

Fig. 7. An immobilized HeLa cell which has been allowed to interact at 4 °C with Sendai virus. The spherical virus particles (arrows) bind randomly to both microvilli and non-microvillous membrane. \( \times 7500 \).

Figs. 8, 9. HeLa cells agglutinated at 4 °C with Sendai virus. HeLa cells remain spherical and agglutination appears to result from the interdigitation and cross-linking of microvilli on adjacent cells. Small spherical 'blebs' can be seen on many cells following interaction with virus at 4 °C. Fig. 8, \( \times 1750 \); inset, \( \times 7250 \); Fig. 9, \( \times 7000 \).
logical changes described for erythrocytes occur with HeLa cells. On incubation at 37 °C there is some disaggregation of the agglutinated cells with the result that when cell fusion does occur polykaryons comprising more than three or four individual HeLa cells are uncommon. Cell swelling is also a characteristic feature of HeLa cells, whether fused or unfused. With highly villated cells, swelling involves an 'unfolding' of cell surface microvilli and the formation of large, smooth-surfaced single or fused cells (Figs. 15, 18). During cell swelling, microvilli, which originally have a uniform diameter of ~0.1 μm expand at their bases (Fig. 16, arrowheads). Continued expansion results in the complete loss of cell surface microvilli (Fig. 17). By scanning electron microscopy many swollen cells appear to have damaged membranes (Fig. 18) although most are still viable and will attach and spread within 3–4 h if returned to monolayer culture conditions. There are some virus particles still bound to the cells after a 60-min incubation at 37 °C although, as with erythrocytes, the number is far fewer than are bound to cells at 4 °C (Fig. 18).

DISCUSSION

The random distribution of virus particles bound to both erythrocyte and HeLa cell surface implies that receptors for these viruses are present and randomly distributed on the surface of both cell types. Little is known about the receptor for paramyxoviruses in eukaryotic cells but in the erythrocyte membrane paramyxovirus receptors reside, at least in part, with the major sialoglycoprotein, glycophorin which is known from freeze-fracture studies to be randomly distributed within the mem-
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At 37 °C the first fused cells (arrow) are seen after about 1 min (Fig. 11). Other unfused erythrocytes are swollen and spherical. After 30 min (Fig. 12) many large pol erythrocytes are seen. Numerous virus particles remain bound to both fused and unfused cells. Both x 4750.

Figs. 13, 14. Erythrocytes fused with Newcastle disease virus at 37 °C for 30 min. Poly erythrocytes comprising more than two or three individual erythrocytes are rarely seen. After 30 min, haemolysis of most cells has occurred. Few virus particles (arrow) remain bound to fused cells. Fig. 13, x 1600; Fig. 14, x 8700.

brane (Tillack, Scott & Marchesi, 1972). NDV appears to deform the erythrocyte membrane much more than does SV. This probably reflects differences between virus haemagglutinins since the erythrocyte receptors are almost certainly the same for both types of virus. Many haemagglutinin ‘spikes’ are present in the envelope of paramyxoviruses which explains why these viruses are capable of causing cell aggluti-
Fig. 15-18. HeLa cells fused with Sendai virus.

Fig. 15. Low-power micrograph showing HeLa cells fused with Sendai virus at 37 °C for 30 min. Fused cells (arrows) comprising more than three or four individual HeLa cells are only occasionally seen. Both fused and unfused cells are swollen and have lost their microvilli. x 400.

Fig. 16. Early stage during cell-cell fusion. Fusion of the 2 cells has occurred at the site of cell-cell contact (arrows). Even at this stage most microvilli have been lost and those that remain are swollen and extended at their bases (arrowheads). x 3200.

Fig. 17. Late stage during the fusion of 3 HeLa cells. All cell surface microvilli have been lost as a result of cell swelling. x 3500.

Fig. 18. Many highly swollen fused cells appear to have damaged and torn membranes. A few virus particles (arrows) are still bound to cells following a 60-min incubation at 37 °C. x 3500.
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Agglutination results from the cross-bridging of adjacent cells by virus particles. With highly villated cells agglutination appears to involve the interdigitation and cross-linking of microvilli on adjacent cells, observations which confirm previous ultrastructural studies (Schneebberger & Harris, 1966; Harris, 1970).

Several virally mediated changes take place when an agglutinated cell suspension is warmed to 37 °C. These changes involve disaggregation of the agglutinated cells, cell swelling, cell-cell fusion and, in the case of erythrocytes, cell haemolysis. The observations that cell disaggregation occurs and that few virus particles are seen bound to cells following incubation at 37 °C is consistent with the knowledge that paramyxoviruses possess, in addition to the haemagglutinin, a neuraminidase which is capable of destroying the sialic acid-containing receptors (Choppin & Compans, 1975). Although many virus particles elute from cells on warming to 37 °C, other virus particles are known to fuse and become incorporated into the plasma membrane of both erythrocytes (Bachi et al. 1973; Shimizu, Shimizu, Ishida & Homma, 1976; Knutton, 1976) and HeLa cells (Knutton, manuscript in preparation). During such virus-cell fusion events there is an increase in passive membrane permeability (Pasternak & Micklem, 1973, 1974) and it is this which results in the osmotic cell swelling reported here. Both erythrocytes and HeLa cells increase in volume and assume an essentially smooth spherical shape. During the swelling of HeLa cells microvilli are lost. Calculations show that the cell surface area present in the form of microvilli allows highly villated cells like HeLa to swell and approximately double in volume without lysis (Knutton et al. 1976). The absence of extra cell surface area in the form of microvilli may explain why there is haemolysis of erythrocytes but not of HeLa cells on incubation with virus. Many of the highly swollen HeLa cells appear to have torn or damaged membranes when observed by scanning electron microscopy but this does not appear to be the result of cell lysis. Complementary thin section and freeze-fracture studies (Knutton, manuscript in preparation) show that such cells do, in fact, have intact plasma membranes. Membrane damage to these highly swollen cells, therefore, appears to be an artifact of the preparative technique and most likely occurs during the critical point drying stage.

A few virus particles remain bound to cells even after incubations at 37 °C for up to 60 min. Such virus particles may be defective in some way in that they neither fuse with nor elute from the cell surface.

Data presented in the accompanying paper (Knutton, 1977) suggests that cell-cell fusion may be initiated by the simultaneous fusion of one or more virus particles with 2 adjacent cells. Although the resolution of the scanning electron microscope is too low to reveal such virus-cell fusion events it is clear from the observation of early and intermediate stages of fusion that cell-cell fusion is initiated at sites of cell contact between agglutinated cells. Cell swelling appears to be the important event which then leads to the ‘unfolding’ of the 2 cell membranes and the formation of a single fused cell. Using SV large polyerythrocytes comprising many individual erythrocytes are formed whereas with NDV, fused erythrocytes comprising more than two or three individual erythrocytes are rarely seen. These observations suggest that with NDV
rapid elution of virus and cell disaggregation takes place before extensive cell-cell fusion can occur. The results also suggest a similar rapid elution of SV from HeLa cells. Although the actual mechanism of virus-induced cell fusion is probably the same, the time-course for fusion to occur and the extent of fusion clearly depends on such factors as the cell type, the type and strain of virus and the virus:cell ratio used.

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REFERENCES


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