THE ROLE OF CELL SWELLING AND HAEMOLYSIS IN SENDAI VIRUS-INDUCED CELL FUSION AND IN THE DIFFUSION OF INCORPORATED VIRAL ANTIGENS

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SUMMARY

The role of the haemolytic activity of Sendai virus in cell-cell fusion has been examined in monolayers of human erythrocytes and erythrocyte ghosts fused with either haemolytic or non-haemolytic virus. Morphological observations indicate that cell swelling and haemolysis is a distinct event in cell-cell fusion irrespective of whether it is virally induced or, in the case of non-haemolytic virus, experimentally induced. Osmotic swelling appears to be the driving force by which cells which have established sites of membrane fusion expand such sites to form poly-erythrocytes. Immunofluorescent labelling of viral antigens incorporated into the erythrocyte membrane as a result of viral envelope-cell fusion indicates that diffusion of antigens in the plane of the membrane is restricted in intact erythrocytes and resealed erythrocyte ghosts but not in haemolysed erythrocytes or unsealed ghosts. A perturbation of the erythrocyte membrane resulting from osmotic lysis appears to form a prerequisite for the lateral diffusion of viral elements.

INTRODUCTION

The attachment of Sendai virus to human erythrocytes is usually followed by fusion between the viral envelope and the erythrocyte membrane (Morgan & Howe, 1968). According to the type of virus, this mode of interaction has a different effect on erythrocytes: whereas virus grown in embryonated eggs and harvested 72 h after infection induces haemolysis and fusion of the cells (Bachi & Howe, 1972) virus harvested 24 h after infection does not induce these dramatic alterations (Homma, Shimizu, Shimizu & Ishida, 1976). Freezing and thawing of this latter type of Sendai virus results, however, in the production of particles which behave like haemolytic virus (Homma et al. 1976). Thus, physical rather than chemical differences, may be responsible for the failure of this virus to induce haemolysis. The haemolytic property of 72-h virus appears to be due to the fact that fusion of viral envelopes with the erythrocyte membrane induces a permeability change in the membrane (Pasternak & Micklem, 1973), the net effect of which is entry of water, cell swelling and haemolysis (Hosaka & Shimizu, 1977; Knutton et al. 1976; Poste & Pasternak, 1978); 24-h virus does not induce such a membrane permeability change and so does not induce cell swelling and is nonhaemolytic (Knutton & Pasternak, 1979). Previous morphological
and biochemical studies have led to the conclusion that an increase in passive permeability is an important event in Sendai virus-induced cell fusion and that cell swelling is the mechanism whereby cells that have established sites of membrane fusion expand such sites to form fused cells (Knutton, 1978; Knutton & Pasternak, 1979).

It is well known that following the fusion of haemolytic virus with a cell plasma membrane there is lateral diffusion of viral antigens away from the site of primary attachment (Bachi, Aguet & Howe, 1973; Bachi, Deas & Howe, 1977; Maeda et al. 1977). Freeze-fracture studies of erythrocytes treated with non-haemolytic virus, however, have suggested that such a rapid diffusion of incorporated viral antigens does not occur in the absence of cell swelling and haemolysis (Knutton, 1979). In order to investigate further the involvement of cell swelling and haemolysis in both cell-cell fusion and the diffusion of viral antigens, we have examined erythrocytes and erythrocyte ghosts fused with both haemolytic and non-haemolytic preparations of Sendai virus.

MATERIALS AND METHODS

Erythrocyte monolayers

Monolayers of human erythrocytes (Type O) were prepared as previously described (Bachi, Eichenberger & Hauri, 1978) by allowing a suspension of washed erythrocytes in phosphate-buffered saline containing Ca\(^{++}\) and Mg\(^{++}\) (PBS) (Dulbecco & Vogt, 1954) to settle on to poly-L-lysine-coated plastic Petri dishes or glass coverslips. Unattached cells were removed by washing 3 times with PBS. Monolayers of unsealed 'leaky' erythrocyte ghosts were prepared from monolayers of intact erythrocytes by replacing the PBS with hypotonic (10 mosmol phosphate, pH 7.4) buffer and washing 3 times with the same buffer. Resealed ghost monolayers were prepared from unsealed ghost monolayers by replacing the hypotonic buffer with PBS containing 2 mM ATP and incubating for at least 1 h at 37 °C (Steck & Kant, 1974). Cell monolayers were stored at 4 °C until used. In some experiments erythrocytes and resealed ghosts were exposed to hypotonic buffer subsequent to treatment with Sendai virus. Attached monolayers of cells were used in these studies for convenience of manipulation and photomicroscopy. Corresponding studies carried out in suspension gave identical results indicating that poly-L-lysine attachment did not affect any of the observations.

Sendai virus

Sendai virus was grown in the chorioallantoic sac of 10- or 11-day-old embryonated chick eggs and harvested from the allantoic fluid either after 24 or 72 h. After a clarification at low speed, the virus was pelleted (20 000 g, 20 min) and resuspended in PBS at a concentration of 10-20 000 haemagglutinating units per ml (HAU/ml) and stored at 4 °C until used. Haemolysis of different batches of 24-h virus was assayed (see below). All preparations of 24-h virus exhibited some haemolysis and this was found to vary from batch to batch. For the experiments described in this paper we arbitrarily chose an haemolysis figure of 5 % or less as representing a good batch of non-haemolytic virus. In a few experiments more haemolytic batches of 24-h virus (5–15 % haemolysis) were used. 72-h virus was routinely very haemolytic (> 80 % haemolysis). Some batches of 24-h virus were subjected to 10 cycles of freezing and thawing by immersing test tubes containing 1-ml aliquots of the virus suspension alternately into a mixture of dry ice/ethanol and then into a waterbath at 37 °C (Homma et al. 1976). Frozen and thawed 24-h virus became as haemolytic (> 80 % haemolysis) as 72-h virus.

Haemolysis titrations

The percentage haemolysis induced by different virus preparations was determined as previously described using monolayers of erythrocytes (Bachi et al. 1978).
Haemagglutination

Haemagglutination assays were performed with freshly washed chicken erythrocytes according to standard techniques.

Phase-contrast microscopy

Fusion of cells was monitored by phase-contrast microscopy. 1-ml aliquots of Sendai virus (~300 HAU/ml) in either PBS or 10 mosmol phosphate buffer were added to erythrocyte or erythrocyte ghost monolayers on Petri dishes and incubated for 30 min at 4 °C. After rinsing the cells with buffer at 4 °C a second incubation for 1 h at 37 °C was carried out. In some experiments the isotonic buffer was replaced with hypotonic buffer following the 37 °C incubation. Monolayers were examined with a Zeiss Invertoscope D. Time-lapse sequences of micrographs were recorded by placing monolayers incubated with virus at 4 °C onto a heated microscope stage at 37 °C and recording micrographs at 30-s intervals.

Antisera

FITC-anti Sendai virus antibody was prepared as previously described (Bachi et al. 1978). Antisera to egg-grown Sendai virus was raised in rabbits. The Ig fraction was obtained from heat-inactivated serum by ammonium sulphate precipitation and conjugated with FITC according to standard methods. Alternatively, the IgG fractions of the serum were digested with mercaptopeptidase (Sigma, St Louis, U.S.A.) (Stanworth & Turner, 1973). After the reaction the Fc fragments and undigested IgG molecules were eliminated by passing the mixture over a protein A-Sepharose 4 B column (Pharmacia, Uppsala, Sweden). The Fab fragments were then purified on Sephadex G-50 and DEAE-cellulose and dialysed against PBS. Antibodies with a molar protein:fluorescein ratio of from 1:4 to 1:7 were used for immune reactions.

Immune reactions

Immune reactions were carried out as previously described (Bachi et al. 1978). Non-specific background staining to the charged substratum could be significantly reduced or eliminated by incubating cell monolayers with buffer containing heparin (50μg/ml) prior to immune labelling. Erythrocyte or erythrocyte ghost monolayers on glass coverslips which had been incubated with Sendai virus either at 4 or 37 °C were placed in a humidity chamber at 4 °C and stained with FITC-anti Sendai virus antibody or FITC-anti Sendai virus antibody Fab fragments (approximately 0.1 ml) diluted in the appropriate isotonic or hypotonic buffer. Each immune reaction was stopped by three 5-min washings with buffer at 4 °C. After the last wash the coverslips were mounted on microscope slides and immediately examined with a Zeiss 18 or Leitz Ortholux microscope with incident light fluorescence illumination. Photographs were taken with a Neofluor 50 or 63 objective on Ilford HP5 film.

Electron microscopy

Scanning and freeze-fracture electron micrographs of virally treated erythrocytes and erythrocyte ghosts were prepared as previously described (Knutton, 1977; Knutton, Jackson & Ford, 1977).

RESULTS

Human erythrocytes adsorbed onto poly-L-lysine coated Petri dishes or coverslips form essentially confluent monolayers (Bachi et al. 1978) and adsorption of Sendai virus at 4 °C does not result in any change in the appearance of cells by phase-contrast microscopy (Fig. 1 A). Scanning electron micrographs, however, do reveal the virus particles bound to the erythrocyte surface (Fig. 4 A). The fate of erythrocytes following
Fig. 1. Phase-contrast micrographs showing monolayers of human erythrocytes treated with non-haemolytic Sendai virus at 4°C for 30 min (A) and 37°C for 1 h (C, E) and subsequently exposed to hypotonic medium (a, d, f); E and F show the same cells before and after hypotonic lysis. Fused cells are not seen at 4°C (A, B); at 37°C extensive cell fusion is only seen following hypotonic lysis. × 700.
a subsequent incubation at 37 °C depends on the preparation of virus used. 24-h virus does not induce haemolysis or any change in the appearance of most cells (Fig. 1 c) although a few cells can sometimes be detected as being fused (Fig. 1 c, arrow). If such cell monolayers are now exposed to hypotonic medium, polyerythrocyte formation ensues and there is total haemolysis (Fig. 1 D). Fig. 1 E, F show the same cells treated with 24-h virus before (Fig. 1 E) and after hypotonic lysis (Fig. 1 F). In this experiment some cell swelling and haemolysis had occurred during treatment with the virus producing recognizable fused cells which can be used as a reference for locating the same cells before and after hypotonic lysis (Fig. 1 E, arrowhead). Many cells which prior to lysis cannot be detected as being fused (Fig. 1 E, arrows) give rise, following hypotonic lysis, to typical fused cells (Fig. 1 F, arrows). Osmotic haemolysis alone, however, is not sufficient to induce polycythrocyte formation and exposure of erythrocyte monolayers alone or monolayers with adsorbed virus at 4 °C to hypotonic medium simply produces single haemolysed erythrocytes (Fig. 1 B). Incubation of erythrocytes with haemolytic 72-h virus or frozen and thawed 24-h virus at 37 °C results in the direct formation of haemolysed polycythrocytes similar to those illustrated in Fig. 1 D, F.

The temporal sequence of events taking place during erythrocyte fusion induced by haemolytic Sendai virus is illustrated in the time-lapse sequence of micrographs shown in Fig. 2. Fusion of 4 erythrocytes is illustrated and is initially detected by light microscopy at small (< 1 μm diameter) regions of close cell contact (Fig. 2 B, D, F, arrows). Subsequent events involve a progressive expansion of the initial cell–cell bridges as cells swell (Fig. 2 C–E) until cell rupture and haemolysis occurs (Fig. 2 G, H). In suspension, osmotic swelling would result in the formation of a single spherical
Fig. 3. Phase-contrast micrographs showing monolayers of unsealed (A) and resealed erythrocyte ghosts (B–D) incubated at 37 °C for 1 h with haemolytic (A, B) and non-haemolytic Sendai virus (C, D). Fused cells are only observed with resealed ghosts (B, D) and with non-haemolytic virus only when cells are subsequently exposed to hypotonic medium (D). × 700.

Fused cell but, in this case, attachment of the cells to the coverslip prevents this occurring.

The involvement of cell swelling in virus-induced cell fusion was investigated further by studying the interaction of Sendai virus with erythrocyte ghosts (Fig. 3). Neither 72- nor 24-h virus induced the formation of polycytoe ghosts when incubated at 37 °C with ‘leaky’ unsealed ghosts (Fig. 3 A), even though freeze-fracture observations show that fusion of viral envelopes with erythrocyte ghost membranes
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Fig. 4. Scanning (A, B) and freeze-fracture electron micrographs (C) showing monolayers of erythrocytes (A, B) and a preparation of erythrocyte ghosts treated with Sendai virus. Virus particles bind randomly to the erythrocyte surface at 4 °C (A) and after 1 h at 37 °C a few virus particles still remain adsorbed (B). Fusion between viral envelopes (sv), identified by their characteristic morphology (Knutton, 1977), and unsealed erythrocyte ghosts (—) can be seen by freeze fracture (C). A, B, × 6500; C, × 70,000.

does occur under these conditions (Fig. 4C). The same incubation of resealed ghosts with 72-h virus resulted in the direct formation of polyerythrocyte ghosts (Fig. 3B) whereas with 24-h virus (Fig. 3C) polyerythrocyte ghost formation only occurred following a subsequent treatment with hypotonic medium (Fig. 3D). As regards cell–cell fusion, therefore, resealed ghosts behave like intact erythrocytes.

A few virus particles which have neither fused and become incorporated into the
Figs. 5-8. Direct immune fluorescence staining of Sendai virus antigens with anti-viral antibody (Figs. 5, 6, 8) or anti-viral antibody Fab fragments (Fig. 7). × 700.

Fig. 5. Erythrocyte monolayers incubated with haemolytic Sendai virus. Virus particles adsorbed to erythrocytes at 4 °C are stained with a granular fluorescence (A) and hypotonic lysis does not alter this staining pattern (B). After fusion at 37 °C for 10 min (C) a more diffuse fluorescence is seen in the fused (arrows) and haemolysed cells (arrowheads) whereas after 1-2 h all cells display uniform fluorescence (D).

erythrocyte membrane nor eluted from the cell surface can still be seen bound to the cell surface following a 37°C incubation of erythrocytes with either 24- or 72-h Sendai virus (Fig. 4B).

Direct labelling of viral antigens with FITC-anti Sendai virus antibody or FITC-anti Sendai virus antibody Fab fragments was used to assess the distribution of viral
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Fig. 6. Erythrocyte monolayers incubated with non-haemolytic Sendai virus. Erythrocytes incubated for 1 h at 37 °C retain a granular fluorescence (A) whereas a diffuse (c) or uniform fluorescence (D) is observed if cells are subsequently lysed in hypotonic buffer for 1–2 h at 4 °C (c) or 37 °C (D). A mixture of both granular (asterisk) and diffuse staining cells (arrowheads) is seen with more haemolytic (10–15 % haemolysis) batches of 24-h virus (B).

antigens. Depending on the virus preparation used and the incubation conditions the distribution of fluorescence tended to be either granular or diffuse and this was unchanged if Fab fragments were used rather than whole antibody molecules. Diffuse staining could be further subdivided into either diffuse or uniform; the change from diffuse to uniform being both time and temperature dependent. The observed fluorescence distribution seen with resealed ghosts (Fig. 8A, C, D) was the same as that observed for intact erythrocytes (Figs. 5A, 6A, D) and will not be described separately.
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Fig. 7. Monolayers of erythrocytes incubated with 24-h Sendai virus (10–15% haemolytic). A mixture of granular (asterisk), diffuse (arrowheads) and uniformly fluorescent cells (arrows) is seen after 1 h at 37 °C (A, C) whereas a subsequent hypotonic lysis for 1–2 h at 37 °C results in all cells having a uniform fluorescence (D). The equivalent phase-contrast micrograph of C is shown in D.

Adsorption of Sendai virus to erythrocytes at 4 °C (Fig. 5A) results in a very granular distribution of fluorescence and this staining pattern is unchanged if cells are subsequently exposed to hypotonic medium (Fig. 5B). Erythrocytes incubated with 24-h virus at 37 °C also exhibit a granular fluorescence (Fig. 6A) although it is a finer granularity than that seen at 4 °C (cf. Figs. 5A, 6A).

Under conditions where swelling and lysis occur, however, a diffuse fluorescence is observed. When erythrocytes are incubated at 37 °C with haemolytic 72-h virus one
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Fig. 8. Monolayers of resealed (A, C, D) and unsealed (B) erythrocyte ghosts incubated with non-haemolytic Sendai virus. Granular fluorescence is seen at 4 °C (A). With resealed ghosts granular fluorescence is retained following a 1-h incubation at 37 °C (C) whereas uniform fluorescence is seen following a subsequent hypotonic lysis for 1–2 h at 37 °C (D). Unsealed ghosts incubated for 1 h at 37 °C exhibit diffuse or uniform fluorescence (B).

initially observes a markedly reduced granular fluorescence and a more diffuse fluorescence especially in the fused (Fig. 5c, arrows) and haemolysed cells (Fig. 5c, arrowheads) while continued incubation for 1–2 h results in all cells having a uniform fluorescence (Fig. 5d). Some residual granularity, however, is always apparent in most cells (Figs. 5d, 6d). A similar change from a granular to a diffuse fluorescence is seen when erythrocytes (Figs. 6c, d, 7b) or resealed ghosts (Fig. 8d) which have been
incubated at 37 °C with 24-h virus are subsequently exposed to hypotonic medium. The temperature dependence of the change from diffuse to uniform fluorescence is illustrated in Fig. 6c, d. Lysis at 4 °C results in a change from a granular to a diffuse fluorescence (Fig. 6c) but uniform fluorescence is only seen when lysis is carried out and cells incubated at 37 °C (Fig. 6d).

The difference in fluorescence distribution between intact and swollen or haemolysed cells is also clearly illustrated in experiments using Sendai virus which is neither totally haemolytic nor non-haemolytic. Figs. 6b, 7a, c, d are from experiments using batches of 24-h virus which induced 5–15% haemolysis. Following a 1–2-h incubation at 37 °C many erythrocytes are unaltered whereas others are fused and/or swollen (Fig. 7d, arrowheads) or fused and/or haemolysed (Fig. 7d, arrows). Comparison of the phase-contrast image with the corresponding fluorescence image (cf. Fig. 7c and d) shows that the unaltered cells retain a granular fluorescence (Figs. 6b, 7a, c, asterisks), swollen yet unhaemolysed cells a more diffuse fluorescence (Figs. 6b, 7a, c, arrowheads) and haemolysed cells a uniform fluorescence (Fig. 7a, c, arrows).

‘Leaky’ ghosts exhibit a diffuse or uniform fluorescence when incubated at 37 °C with Sendai virus irrespective of which virus preparation is used (Fig. 8b).

DISCUSSION

The events occurring during the interaction of Sendai virus with cell surfaces and particularly erythrocyte membranes are determined by the properties of the viral envelope. Sendai virus, according to its growth conditions, can occur in 3 different states: (1) that of an infectious haemolytic particle (Fukai & Suzuki, 1954); (2) that of an infectious non-haemolytic particle (Homma et al. 1976); and (3) that of a non-infectious non-haemolytic particle (Homma, 1971). The failure of the latter class of particles to develop the biological activities under discussion lies solely in the inactive state of their protein F0, the cleavage of which is required to enable viruses to fuse with a cell membrane (Homma & Ohuchi, 1973; Scheid & Choppin, 1974). Viral envelope–cell fusion, a property of infectious virus particles, however, does not in itself suffice to induce cell–cell fusion. The observations presented in this paper indicate that the ability of Sendai virus to fuse erythrocytes depends on 2 distinct events: (1) fusion of the membranes of 2 adjacent cells, a virally mediated event which is known to require virus–cell fusion; and (2) induction of permeability-induced cell swelling. Both haemolytic and non-haemolytic virus preparations can induce membrane fusion (Knutton, 1978; Homma et al. 1976) but the lytic property of the virus appears to be essential to bring about cell fusion.

Electron microscopy of erythrocytes treated with non-haemolytic Sendai virus has shown that the membranes of some cells have fused and that cells are connected by small cytoplasmic connexions (Knutton, 1979b) but because such sites of fusion are below the resolution of the light microscope such cells cannot be detected as being fused (Fig. 1f, arrows). That typical fused cells can be produced from cells treated with non-haemolytic virus by exposure to hypotonic medium (Fig. 1f, arrows) is
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direct evidence that fusion precedes swelling and that cell swelling is the driving force which expands cells which have established sites of fusion to form polycythocytes. The studies with erythrocyte ghosts also confirm the role of cell swelling in cell–cell fusion. Resealed ghosts are osmotically sensitive and so, like intact erythrocytes, swell to produce polycythocyte ghosts; ‘leaky’ unsealed ghosts, on the other hand, are not osmotically sensitive and so polycythocyte ghosts are not produced by any virus treatment. It has been known for some time that Sendai virus will fuse erythrocytes but not erythrocyte ghosts (Peretz, Toister, Lasler & Loyter, 1974). Only if the ghosts are first resealed and can be osmotically swollen will fused cells be produced on subsequent treatment with virus.

Previous morphological observations have indicated that the interaction of haemolytic Sendai virus with the erythrocyte membrane is as follows: at 4 °C virus particles are adsorbed randomly to the erythrocyte surface (Fig. 4A) (Büchi et al. 1977; Knutton et al. 1977). Warming to 37 °C induces fusion of many virus particles (Apostolov & Almeida, 1972; Morgan & Howe, 1968), other particles do not fuse and elute from the surface (Volsky & Loyter, 1979) while a small percentage neither fuse nor elute and remain adsorbed (Fig. 4B) (Knutton et al. 1977). Prolonged incubation at 37 °C results in disintegration of the fused viral envelopes and lateral diffusion of viral antigens away from sites of fusion (Büchi et al. 1973, 1977). The distribution of fluorescence seen in erythrocytes treated with haemolytic Sendai virus is readily interpreted in terms of this sequence of events. A granular fluorescence reflects the staining pattern of intact virus particles which have not disintegrated. Diffusion of viral antigens away from sites of fusion is reflected in a more diffuse and ultimately uniform fluorescence.

Our studies have shown that diffusion in the plane of the membrane of non-haemolytic Sendai virus antigens is restricted unless experimental osmotic lysis is induced. Incubation of virus and cells at 37 °C does not alter the granularity of the fluorescence characteristic of adsorbed virus, indicating that there has been no significant diffusion of viral antigens away from sites of fusion. Subsequent hypotonic lysis results in a diffuse or uniform fluorescence in a manner identical to that observed in cells treated with haemolytic virus. Preparations of non-haemolytic virus which do induce some swelling and haemolysis present, accordingly, a mixture of fluorescence patterns (Figs. 6B, 7A, C) and comparison with the equivalent phase-contrast micrographs clearly demonstrates that diffusion of antigens to reach a uniform distribution occurs faster in haemolysed cells than in swollen but unhaemolysed cells. Thus, the restriction of mobility appears to be a function of cell swelling irrespective of whether it is of viral or experimental origin. The observation of Maeda et al. (1977) on the different fate of viral antigens incorporated into Ehrlich ascites cells treated with haemolytic virus in the presence and absence of saccharides can also be interpreted in a similar manner since high concentrations of saccharides prevent virally-induced cell swelling (Knutton, 1979a; Knutton & Pasternak, 1979).

Our observations imply that osmotic swelling and lysis perturbs the structural organization of either the erythrocyte membrane and/or the incorporated viral envelope so as to allow diffusion of viral antigens. Recent evidence does suggest that
24-h Sendai virus has a more rigid structure than 72-h virus (Kim, Hama, Miyake & Okada, 1979). However, the observation that diffusion of viral antigens does not appear to be restricted in sealed ghosts suggests that erythrocyte membrane perturbations, possibly involving spectrin/actin interactions (Nicolson, 1976), are involved and have already occurred during the hypotonic lysis used to prepare the ghosts.

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REFERENCES


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