High Resolution Investigations with the Scanning and Transmission Electron Microscope of Haemadsorption Binding Sites of Mumps Virus-infected HeLa Cells

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SUMMARY

Specific changes at the surface of HeLa cells infected with mumps virus were investigated in parallel with the scanning and transmission electron microscope.

The distribution of haemadsorption binding sites and virus-induced antigens at cell surfaces was simultaneously studied by labelling virus-specific antigens with peroxidase-conjugated antibodies after haemadsorption.

New information was obtained upon the three-dimensional aspect of the red blood cells, the topographical distribution of their binding sites on the infected cells, and the specific structures at the cell surface which are involved in the process of haemadsorption.

INTRODUCTION

Infection of cell cultures with myxo- and paramyxoviruses induces specific morphological and antigenic alterations at the surface of host cells (Shelokov, Vogel & Chi, 1958; Duc-Nguyen & Rosenblum, 1967; Duc-Nguyen, 1968; Mannweiler & Rutter, 1972). Morphological changes of the plasma membrane can be detected only with the transmission electron microscope, whereas the new virus-specific components present at the cell surface are easily recognized in the light microscope by means of immunofluorescence and adsorption of red blood cells (RBC). Studies done with the transmission electron microscope were mainly aimed at investigating the ultrastructural aspects of haemadsorption, but the techniques applied were not entirely suitable for drawing definite conclusions concerning the distribution of RBC on the whole surface of an infected cell as well as the relationship between antigenically modified areas of the host cell plasma membrane and the binding sites for the adsorbed erythrocytes. Furthermore, these technical inadequacies did not permit a clear distinction to be made between true adsorption and non-specific contact of the RBC with the plasma membrane.

The present paper shows the results obtained in parallel morphological investigations with the scanning electron microscope and the transmission electron microscope of mumps virus-infected HeLa cells after haemadsorption experiments. Flat embedding for studies with the transmission electron microscope prevents haphazard contact between adsorbed erythrocytes and non-specific parts of the plasma membrane of the infected cells in the preparation procedures, especially while culture cells are being harvested or centrifuged. Many of the above difficulties were overcome by simultaneous use of haemadsorption and labelling of the virus-induced antigens at the cell surface with peroxidase-conjugated
antibodies. Three-dimensional illustrations of the infected cells should provide new information regarding the topographical distribution of the adsorbed RBC on the different cells in the culture and the involvement of specific structures of the cell surface in the process of haemadsorption.

METHODS

Cell cultures. HeLa cells were used in all experiments. The cell culture was obtained from Flow Laboratories (England) and was further passaged in our laboratory. Cells were cultivated in medium 199 (M 199) (GIBCO) with 5% (v/v) foetal calf serum at 37 °C in 200 ml flasks and passaged with the aid of a trypsin solution (0.25 g % (w/v) in balanced salt solution, BSS). For the morphological investigations, 2 ml suspensions of 4 × 10⁶ cells were seeded on to coverglasses in 8 mm × 50 mm glass tubes and infected with mumps virus 24 h later.

Virus. The Enders strain of mumps virus (American Type Culture Collection) was cultivated in chicken embryos and adapted to HeLa cells by 10 blind passages (Mannweiler & Rutter, 1972). Titration of the adapted virus was performed in HeLa cells after incubation for 10 days at 37 °C. The end-point titre was estimated by haemadsorption according to the method of Kärber (1931) and expressed as TCID₅₀/ml.

Infection of cell cultures. The medium of the confluent cell cultures was decanted and 0.5 ml of virus suspension was added to the cell layers. Input multiplicity was approx. 1 TCID₅₀/cell. After incubation for 1 h at room temperature, the unadsorbed virus was removed by washing three times with BSS. The cultures were further incubated at 37 °C in fresh medium.

Haemadsorption procedures. The technique described by Henle (1969) was employed. At different intervals after inoculation the cultures were washed with BSS and each coverglass culture was overlaid with 1 ml of a 0.5% (v/v) chicken erythrocyte suspension in M 199. Following incubation for 30 min at room temperature, the unadsorbed erythrocytes were removed by repeated washings with BSS.

Anti-mumps sera. Allantoic fluid of infected chicken embryos was concentrated by centrifuging at 25000 g for 40 min. The virus suspension was mixed with complete Freund’s adjuvant (Difco) at a ratio of 1:1(v/v). Rabbits received intramuscular injections of the emulsion into different sites. After 5 weeks the animals received 3 weekly intravenous booster injections and were bled 7 days later. The serum obtained was inactivated at 65 °C for 20 min, titrated by haemadsorption inhibition, and stored in samples of 0.1 ml at −20 °C.

Anti-rabbit γ-globulin antibodies were induced in guinea-pigs. Rabbit γ-globulin obtained on DEAE cellulose was mixed 1:1(v/v) with complete Freund’s adjuvant and inoculated intramuscularly into the backs of well-developed animals, each animal receiving approx. 10 mg. After 5 weeks the animals were given another 2 intraperitoneal injections of 3 mg rabbit γ-globulin each and were bled 7 days after the final inoculation. The sera were inactivated and pure anti-rabbit γ-globulin antibodies obtained by absorption and elution of insoluble rabbit γ-globulin according to the method of Avrameas & Ternynck (1969). The antibodies were conjugated with horseradish peroxidase (Sigma) (Avrameas, 1969) by means of glutaraldehyde and stored in small samples at −70 °C.

Labelling of mumps antigens. All infected HeLa cell cultures with or without haemadsorption were washed with BSS at room temperature and fixed for 5 min with a cold solution of 4 g % (w/v) formaldehyde and 0.25% (v/v) glutaraldehyde in 0.1 M-phosphate buffer, pH 7.2, the formaldehyde being freshly prepared from paraformaldehyde (Graham & Karnovsky, 1966). After fixation the cells were washed with BSS. The coverglasses were
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removed from the tubes and each was overlaid with 0.1 ml rabbit anti-mumps serum diluted 1:10. After incubation for 5 min at room temperature the cells were washed 5 times with BSS, overlaid with 50 μg of guinea-pig anti-rabbit γ-globulin antibodies in 0.1 ml BSS and kept at room temperature for another 5 min. The unadsorbed antibodies were eliminated by a thorough washing and the cells were fixed with ice-cold 1:7 % (v/v) glutaraldehyde solution in sodium cacodylate buffer. After 20 min the fixative was removed by repeated washings. Deposits of peroxidase-conjugated antibodies were made visible by cytochemical methods (Mannweiler & Rutter, 1973b).

Normal HeLa cell cultures treated as described above or mumps virus-infected cultures stained with rabbit anti-influenza serum instead of rabbit anti-mumps serum served as controls.

For electron microscopy the cell cultures were washed twice with pre-warmed buffer and fixed with 1:7 % (v/v) glutaraldehyde in sodium cacodylate buffer, pH 7.2. For studies with the scanning electron microscope the cultures were fixed for 1 h to 20 days at 4 °C, washed again, and fixed for 4 h in 2 g % (w/v) CrOsO₄ solution (Dalton). They were dehydrated in alcohol, air-dried or dried by the critical point method (Frigen 11 and Frigen 13; Cohen, Marlow & Garner, 1968, or Frigen 11, amylacetate and CO₂; Anderson, 1951) and vacuum-evaporated by carbon and gold or palladium, respectively. Micrographs were taken by Stereoscan S₄-10 or Autoscan (Siemens) at 20 to 35 kV with 100 to 200 μm aperture and 40 to 45° tilt angle.

For studies with the transmission electron microscope the cultures were fixed in the same glutaraldehyde solutions for 20 min, washed and fixed for 40 min in 2 g % (w/v) CrOsO₄ (Dalton). After dehydration in alcohol the preparations were embedded in ERL-4206 (Spurr, 1969). To prevent displacement of cell processes and erythrocytes while scraping the cells from the coverslip and during centrifuging, the cells were flat embedded. The thin sections were stained with lead citrate and uranyl-acetate solutions and examined in a Siemens Elmiskop I. Uninfected HeLa cell cultures treated in the same way served as control for the different preparation procedures and for exclusion of contamination with mycoplasma.

RESULTS

Due to the low input multiplicity only 20 to 30 % of the cells of a culture were infected simultaneously. The adsorbing cells in the cultures are focused in small groups. A single cell is able to adsorb one or several RBC (Fig. 1 and 4a). As seen in the scanning electron microscope, the erythrocytes appear homogeneously spherical or ovoid with no severe deformation. The erythrocytes are bound to the cell either by short cell processes or by several small protuberances and cytoplasmic folds at circumscribed areas at the surface of the HeLa cell (Fig. 1 and 2). However, they may also be attached by larger thin cell processes extending from the cell border (Fig. 3; see also Mannweiler & Rutter, 1973a,b). Apparently, the duration of infection has no bearing on the shape and number of the binding sites.

Similar results were obtained by investigations with the transmission electron microscope. Using flat embedding, the adsorbed RBC are not displaced in the course of the embedding procedures and their shape and binding sites at the cells are readily discerned (Fig. 4 and 5). Haemadsorption occurs only where the plasma membranes are morphologically altered in their ultrastructural composition. Part of the unit membrane complex becomes darker and more osmophilic (Fig. 4b). At the cytoplasmic part, specific tubular or rod-like structures are deposited which when sectioned transversely appear as small circular structures (Fig. 5).

Occasionally the RBC are bound to cell protuberances by thin pseudopodium-like processes extending from the area of contact with the erythrocytes. This type of deformation
Fig. 1. (a) HeLa cell of a culture 72 h after infection with mumps virus and haemadsorption with unfixed erythrocytes; erythrocytes are attached to the cell body by small cytoplasmic processes →; critical point dried with Frigen. (b) Higher magnification of →.
Fig. 2. HeLa cell of a culture 14 h (a) and 48 h (b) after infection with mumps virus. The culture was pre-stabilized for 5 min by 1% formaldehyde solution prior to haemadsorption with unfixed erythrocytes and air-dried.

and extrusion of the erythrocytes is visible only in the transmission electron microscope on thin sectioning (Fig. 5).

Preparations incubated with peroxidase-labelled immune sera following haemadsorption reveal different morphological aspects at the adsorption sites. Either the contact between the plasma membranes of the extruding cell process and the adsorbed erythrocyte is so close as to leave no space for adhesion of labelled antibody, or a margin remains for adhesion. In the latter case, a broad zone of strongly osmophilic material is seen between the two
parallel plasma membranes which continues where the margin between the two adjacent cells (RBC and HeLa cell) develops into larger spaces and contact ceases altogether (compare right and left part of Fig. 5).

In haemadsorption experiments not all antigenically altered areas are labelled by adsorption of erythrocytes. Antigens stained by peroxidase-labelled immune sera are distributed over the cell surface in patches. Morphological alterations of the unit membrane complex are always detectable at these sites. In the adjacent cytoplasm specific tubular nucleocapsid-like structures can be found. Sometimes the plasma membrane forms a strikingly straight and rigid line in comparison with the slight undulation of the unstained parts of the plasma membrane (Fig. 6a). In general, these labelled parts of the plasma membrane are evaginating in different shapes. Most conspicuous in the cytoplasm beneath these areas are strands of very fine fibrillar structures which are mainly oriented parallel to the cell surface (Fig. 6b).

No positive haemadsorption is observed in uninfected control cells after washing 3 times with buffer. No peroxidase-positive deposits are found at the plasma membranes of these cells or on mumps virus-infected cells incubated with rabbit anti-influenza serum.

**DISCUSSION**

Due to the relatively low input multiplicity used in these investigations it was possible to examine infected and uninfected control cells in the same culture under exactly identical preparation conditions. As observed in the scanning electron microscope, the shape and structure of erythrocytes remained essentially unchanged after positive haemadsorption. Even pre-incubation of erythrocytes with formaldehyde prior to haemadsorption had no appreciable effect on the shape and structure of the adsorbed RBC or on the shape and number of the attachment sites of RBC (K. Mannweiler & G. Rutter, unpublished obser-
Fig. 4. (a) HeLa cell of a culture 60 h after infection with mumps virus; haemadsorption followed by incubation with peroxidase-labelled specific immune serum. (b) Part of a cell of the same preparation as in (a) without incubation with labelled immune serum. E, part of an erythrocyte.

vation). The pseudopodium-like extrusions at the bottom of the deposited erythrocytes (Fig. 5) occasionally observed in ultrathin sections in the transmission electron microscope were not detectable.

Examination of ultrathin sections in the transmission electron microscope of flat embedded tissue cultures shows that the erythrocytes were attached only at sharply circumscribed areas of larger extrusions of the cell surface and that the ultrastructure of these parts
Fig. 5. Same preparation as in Fig. 4(a) showing positive (left portion) and negative (right portion of cell protuberance) labelling of specific antigen after haemadsorption at the morphologically specifically altered plasma membrane.

of the plasma membranes of the infected cell was always specifically modified. These findings demonstrate a circumscribed patch-like distribution of haemagglutinin and probably of other mumps virus antigens in the plasma membrane of HeLa cells late after infection with a relatively low input multiplicity. Our experiments failed to reveal attachment of RBC to morphologically unaltered plasma membranes of infected HeLa cells over longer segments, as described for cell cultures infected with myxo- and paramyxoviruses (Hotchin et al. 1958; Duc-Nguyen, 1968). However, due to careful flat embedding after haemadsorption the association of RBC with the membrane of infected cells was not mechanically influenced during the embedding procedures. Thus, the induction of false contact between RBC and infected culture cells was prevented. Virus-like particles so far have not been found to cause the attachment of RBC to the culture cell surface (cyto-/virus-haemadsorption, Hotchin et al. 1958). These findings were unexpected because comparative investigations revealed virus titres of these cell cultures to have approx. $10^6$ TCD$_{50}$/ml; probably the absence of virus particles is the result of washing before the haemadsorption procedure.

Incubation with peroxidase-labelled specific immune sera after haemadsorption clearly revealed that not all specifically stained plasma membrane areas on the cells could be demonstrated by attachment of erythrocytes. In our opinion, this could be explained by the
size of RBC in comparison with the altered plasma membrane areas and/or by labelling of virus antigens other than haemagglutinin with antibodies against the whole virus. These labelling experiments also demonstrated some different kind of contact between adsorbed erythrocytes and ultrastructurally modified areas of the plasma membrane of the extruding surface of infected cells (Fig. 5). It remains to be seen whether biologically different types of attachment are related to the two morphological aspects or whether these are simulated by sectioning sites. In the first case, the areas where the peroxidase material has been deposited would demonstrate a more loose binding of RBC to the virus antigen at the cell surface similar to the first step in the haemagglutination process of myxo- and paramyxoviruses.

Fig. 6. HeLa cell of a culture 60 h after infection with mumps virus after incubation with peroxidase-labelled immune sera. (a) Labelled plasma membrane in a strikingly straight and rigid line, as compared with non-labelled parts of plasma membrane. (b) Large extrusion of labelled parts of plasma membrane. Circular and rod-like nucleocapsid-like structures in the adjacent cytoplasm; circumscribed bundle of microfibrils at the base of this area (→).
In our opinion, it is very reasonable to assume that firm attachment of the adsorbed RBC takes place only at those areas which are inaccessible to antibody molecules between the two attached plasma membranes. The labelling procedures were done after fixation of the cell with aldehyde. We therefore estimate that the topographical distribution of virus antigens at the cell surface was not affected by incubation with specific antivirus antibodies (Rutter & Mannweiler, 1973). By the morphological changes in the plasma membrane structure, these areas are readily identified in the transmission electron microscope. This is in contrast to our findings with HeLa cells after non-permissive infection with A9PR8 influenza virus (Mannweiler & Rutter, 1974), where 20 h after infection almost the entire plasma membrane is labelled by peroxidase-conjugated antivirus antibody, although in most cases the plasma membranes revealed no ultrastructural alterations other than in those areas where virus-specific budding processes occur.

In our HeLa cell mumps virus system, peroxidase-positive material so far has been demonstrated only at the morphologically altered areas of the plasma membrane. Some parts of the membrane are characterized by proceeding in a conspicuously straight line (Fig. 6a). Apparently there is not only an antigenic alteration of this sector of the plasma membrane, but also a physiological rigidity of the specifically altered membrane portion. This rigidity may be also responsible for the strikingly small areas of attachment of RBC to the infected cell surface.

Further investigations will have to elucidate whether the extensive extrusion processes of virus-induced antigens at the cell surfaces are due to a rigidity of the ‘differentiated plasma membrane’ in these sections and whether they are similar in nature to the capping phenomenon in lymphocytes after contact with anti-γ-globulin antibodies (de Petris & Raff, 1972; Raff & de Petris, 1973). Particularly interesting is the striking accumulation of fine fibrillar structural elements in the cytoplasm beneath these areas. Morphologically they are very similar to microfibrils discussed as functionally important structures for the cell and plasma membrane mobility (Wessels et al. 1971). Their location at the cell surface may also have functional implication with respect to the movement of virus antigen at the host cell surface (Rutter & Mannweiler, 1973).

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