Further Investigations on the Mode of Entry of Vaccinia Virus into Cells

By ANNABELLA CHANG AND D. H. METZ

National Institute for Medical Research,
Mill Hill, London NW7 1AA, England

(Accepted 31 March 1976)

SUMMARY

Earlier results indicating that vaccinia virus entered L cells by a process of direct fusion between the virus envelope and the plasma membrane of the cell have been confirmed and extended using immuno-ferritin conjugates to locate virus antigens on the host cell surface. After fusion, components of the virus envelope become rapidly dispersed in the plasma membrane. Fusion has also been observed as the predominant mode of entry of vaccinia virus into HeLa cells.

INTRODUCTION

It was recently reported (Armstrong, Metz & Young, 1973) that vaccinia virus appeared to enter cultured cells by a process involving direct fusion of the virus envelope with the plasma membrane of the cell within minutes after adsorption. These findings were in contradiction to the previously accepted view that phagocytosis or 'viropexis' is an obligatory event in vertebrate poxvirus infections (Dales, 1965), although virus-cell fusion has been demonstrated for a pox-related insect virus (Granados, 1973).

Since the observations on fusion have so far been based only on examination of thin sections of suspension cultures of infected mouse L cells, this paper reports an extension of these studies using immuno-ferritin labelling to locate virus antigens in both thin sections of cells and on replicas of the cell surface. Observations have also been extended to monolayers as well as suspension cultures, and to HeLa as well as to L cells.

METHODS

Cells and viruses. The source and growth of L cells and the propagation and purification of vaccinia virus, strain WR, have been described elsewhere (Metz, Esteban & Danielescu, 1975). HeLa cells, obtained from Dr J. Williams, Institute of Virology, University of Glasgow, were propagated in monolayer cultures and put into suspension culture on the day before infection. All cells were free from mycoplasma contamination as judged by the fluorescence stain of Russell, Newman & Williamson (1975).

Infection of cells. Infection of L cells in suspension culture has been described previously (Armstrong et al. 1973; Metz et al. 1975). Briefly, L cells concentrated to 10⁷/ml in Pucks saline A plus 20 mM-Mg²⁺ plus 1% foetal calf serum were infected with vaccinia virus at an added multiplicity of 500 particles/cell for 15 min at 37 °C and stirred. Immediately following the adsorption period, designated 'zero time', the cells were diluted five- or tenfold in warm Eagle's medium containing 5% calf serum. HeLa cells in suspension culture were
infected likewise. For infection of monolayers, 50 mm Petri dishes containing 13 mm diam.
glass coverslips were seeded with L cells in Eagle’s medium containing 10 % calf serum.
When the monolayers were just confluent the medium was aspirated, the dishes rinsed once
with Pucks saline containing 20 mM-Mg²⁺ and 1 % foetal calf serum, the 1.5 ml of this
medium containing sufficient purified vaccinia virus to give an added m.o.i. of about 1000
particles/cell, was applied. Dishes were incubated in an atmosphere of 5 % CO₂, with
occasional rocking, at 37 °C.

Preparation of ferritin-conjugated anti-vaccinia antibody. Anti-vaccinia (strain IDH) anti-
serum was kindly provided by Dr E. Boulter, Microbiological Research Establishment,
Porton. It had been prepared in a sheep by inoculating intracellular vaccinia virus which
had been grown on rabbit skin, then inactivated by irradiation and purified on a sucrose
gradient. The neutralizing titre of the serum was 1:20000. The globulin fraction was pre-
cipitated by 40 % saturated ammonium sulphate then applied to a DEAE-cellulose column
(Whatman DE52) and the IgG fraction eluted with 17 mM-sodium phosphate buffer, pH 7.6
(Williams & Chase, 1968). Horse spleen ferritin (Calbiochem) was purified by repeated
recrystallisation in cadmium sulphate solution until all amorphous material had been
removed (Howe, Morgan & Hsu, 1969). The ferritin and sheep IgG were conjugated using
glutaraldehyde (DePetris & Raff, 1972). A control conjugate was similarly prepared using
IgG from normal sheep serum. The conjugates were used at a concentration of 6 mg
ferritin/ml.

Labelling of cells. Samples of cells infected in suspension were cooled rapidly to 4 °C by
the addition of ice-cold Earle’s saline. All subsequent manipulations were carried out at 4 °C.
Cells were centrifuged (200 g for 3 min), washed twice in phosphate-buffered saline (PBS)
and once in PBS plus 2 % (w/v) bovine serum albumin (BSA). The pellet, containing approx.
1 to 2 × 10⁷ cells, was resuspended in 50 µl of ferritin-conjugated antibody and incubated
for 60 min at 4 °C. Unreacted conjugate was then removed by washing the cells three times
in PBS plus 2 % BSA, and once in PBS alone.

When cells were infected as coverslip cultures, unadsorbed virus was removed by rinsing
with PBS and then PBS plus 2 % BSA. The ferritin-conjugated antibody was then applied
to the coverslips for 60 min at 4 °C, followed by final washing as above, and immediately
fixed for electron microscopy.

Electron microscopy. For the preparation of thin sections, cell pellets were fixed in a
mixture of glutaraldehyde and osmium tetroxide as described by Hirsch & Fedorko (1968),
dehydrated with graded ethanol and embedded in Spurr’s resin. Sections were stained with
uranyl acetate and lead citrate (Reynolds, 1963), prior to examination in a Philips EM 300
microscope. Coverslip cultures were fixed in situ, then scraped from the glass and pelleted
by centrifugation and thereafter treated as described above.

For the preparation of replicas, labelled cells on coverslips were fixed in 1 % glutar-
alddehyde in PBS at 4 °C for 60 min, rinsed with PBS and then water. In some experiments,
fixation preceded immuno-ferritin labelling. Following dehydration in graded ethanol and
absolute acetone, the coverslip, with cells attached, was mounted on a specimen stage of a
Balzers High Vacuum Freeze-Etch Unit (BAF 301) and a platinum–carbon replica of the
cell surface was prepared as described by Nermut (1973). The replica was floated off and
cleaned in concentrated sodium hypochlorite, rinsed in distilled water and mounted on a
400 mesh copper grid.
Vaccinia virus with its characteristic morphology is readily recognized in thin sections (Armstrong et al. 1973), and anti-vaccinia antibody conjugated to ferritin was seen to have attached to the envelope of all virus particles on the exterior of the cell (Fig. 1), but not to the host cell membrane at sites remote from virus entry nor was label detected on the surface of uninfected control cells that had been exposed to the conjugate (not shown).

Since the ferritin was conjugated to antibodies prepared against the whole vaccinia virus and not to the purified virus envelope, the conjugate may be capable of labelling internal virus antigens as well as the virus envelope, but under the experimental conditions described here, internal virus antigens are not accessible to the conjugate, so only the virus envelope is specifically labelled.

The control conjugate of ferritin coupled to normal sheep IgG did not label either the virus or cell surface.

Fusion of the virus envelope with the plasma membrane of L cells

Previously conventional thin section data suggested that fusion between the virus envelope and the plasma membrane of L cells occurred within 15 min of mixing virus and cells, in suspension culture, at 37 °C (Armstrong et al. 1973). With the use of the ferritin-labelling technique, observations using thin sections confirm and reinforce these findings. Fig. 1 shows in the lower part two ferritin-labelled viruses adsorbed on to the surface of the cell, while the virus at the top shows a very early stage in the fusion between the virus envelope and the plasma membrane of the host cell. Fig. 2 and 3 show a later stage after fusion, with the lateral bodies of the virus beginning to disperse (Fig. 2). This is followed by a transfer of the virus core into the host cell cytoplasm (Fig. 5) with the virus envelope losing its original shape and flattening into the plane of the cell membrane. It would appear from the common observation of images such as that of Fig. 5, and also from surface replicas (Fig. 9 and 10) that, following fusion, the virus envelope becomes associated with the host plasma membrane; there is no evidence to suggest that the virus envelope enters the cell with the virus core.

Fusion with HeLa cells

Examination of the mode of entry of vaccinia virus into HeLa cells cultured in suspension has yielded results essentially identical to those obtained with L cells. Fusion is readily detected at zero time p.i. (Fig. 4). Very rarely, we have also detected intact virus particles in vacuoles within the cytoplasm at this time. Presumably these entered by a process of phagocytosis.

The fate of the fused virus envelope

Immuno-ferritin labelling of infected cells examined in thin sections permitted some investigation of the fate of the virus envelope antigens following their insertion into the cell membrane. While it was easy to locate the inserted envelope immediately after fusion, when it was still intact and the prominent virus core was still adjacent to the cell margin as in Fig. 5, at later times, when the core had migrated further into the cytoplasm, recognition of the fused virus envelope at the cell surface became difficult. In retrospect we concluded that this was due partly to the problem of detecting the dispersed envelope at the cell margin in the absence of an adjacent virus core structure as a readily identifiable landmark, and partly due to the observation of cell membrane in one dimension only so that only a small
Fig. 1–4. Thin sections of vaccinia infected cells reacted with ferritin-antibody conjugate.

Fig. 1. Ferritin-labelled virus particles adsorbed on the surface of L cells. The virus at the top shows an early stage of fusion with the cell membrane.

Fig. 2 and 3. Ferritin-labelled virus at a later stage of fusion with L cells. Lateral bodies (LB) are beginning to disperse in Fig. 2.

Fig. 4. Labelled vaccinia particle fused with HeLa cells.
Fig. 5. After fusion, the virus core (VC) migrates into the host cytoplasm and the virus envelope (VE) labelled with ferritin (F) is inserted into the cell membrane (CM).

As it proved impracticable to prepare satisfactory replicas from cells cultured in suspension, monolayers of infected L cells were used instead. Replicas of the surface of uninfected cells are relatively smooth (Fig. 6). Vaccinia virus particles are readily identified in replicas of infected cells which had been reacted with conjugate (Fig. 7); the characteristic brick shape with approximate dimensions 230 × 320 nm is within the range observed by others (200 to 250 × 300 to 350 nm; Westwood et al. 1964; Nermut, 1973). The ferritin label is clearly seen on the surface of the virus particles (Fig. 7 and 8).

With replicas, in contrast to sections cut in favourable planes, it is not possible to distinguish unequivocally between virus particles adsorbed on the cell surface, and those which have just fused. The value of replicas, on the other hand, is in providing images such as those in Fig. 8 and 9 which suggest a progression comparable to that seen in thin sections, in which the virus envelope first fuses and then flattens into the plane of the host cell plasma membrane (Fig. 5). The antigens of the virus envelope disaggregate and then disperse into small patches on the surface of the cell (Fig. 10).

The use of monolayer cultures for the preparation of replicas does not allow the degree of synchrony of infection that is possible with suspension cultures. Thus, from samples taken at both 15 min and 60 min after addition of virus it was possible to see in replicas all the various stages from adsorption to dispersal shown in Fig. 7 to 10. However, certain
Fig. 6. Uninfected cell.

Fig. 7. Brick-shaped, ferritin-labelled virus particles (V) on cell surface. Some smaller clusters of ferritin (↑) seen on the cell surface represent dispersed virus envelope.

Fig. 8. A labelled virus (arrowed) either adsorbed or in the very early stages of fusion.

Fig. 9. A patch of ferritin particles on cell surface indicative of a fused virus envelope, comparable to that of Fig. 5.

Fig. 10. Ferritin particle in smaller patches. After insertion into the host membrane, the virus envelope disaggregates and disperses in small patches.
Vaccinia entry

qualitative differences were discernible; very few small patches of fused virus envelope were detected after 15 min whereas after 60 min such small irregular shaped patches were quite common. Whether the cells were fixed or not before reaction with conjugate, the same results were obtained. Attempts to measure the dispersal of the ferritin label were unsatisfactory partly because the degree of labelling on the virus surface was found to be variable both in replicas of infected cell surfaces and also in control experiments using shadowed and unstained virus particles.

DISCUSSION

When L cells, cultured in suspension, are infected with vaccinia virus, virus RNA synthesis, polyribosome formation and protein synthesis occur with exceptional rapidity, all these being detectable within 30 min after the addition of virus (Metz & Esteban, 1972; Esteban & Metz, 1973; Metz et al. 1975). Thus, the biologically relevant processes of adsorption, penetration and uncoating of the virus particles must be completed well within this period. Results presented here and in a previous paper (Armstrong et al. 1973) leave little room for doubt that direct fusion of the virus envelope with the plasma membrane of the cell is responsible for penetration (first stage) uncoating. This is the case for both L cells and HeLa cells and for suspension and monolayer cultures alike. In this work phagocytosis of virus particles by L cells at zero time was never observed, and only very rarely was it seen in HeLa cells. Under our experimental conditions, at least, phagocytosis appears very unlikely to make an important contribution to initiation of vaccinia virus infection. In this respect vaccinia resembles certain other enveloped viruses where direct virus-cell membrane fusion has been observed. These include myxoviruses and paramyxoviruses (Hoyle, 1962; Meiselman, Kohn & Danon, 1967; Morgan & Howe, 1968; Morgan & Rose, 1968; Dourmashkin & Tyrrell, 1970; Bachi, Aguet & Howe, 1973; Kohn, 1975; Okada et al. 1975), rhabdoviruses (Heine & Schnaitman, 1969, 1971), and an insect poxvirus (Granados, 1973).

After fusion has occurred the virus envelope antigens disaggregate and become randomly dispersed as small patches of various size throughout the plasma membrane of the cell. Bachi et al. (1973) have reported very similar observations when Sendai virus fuses to erythrocytes.

Since the dispersion of virus antigens was observed irrespective of whether the infected cells were fixed or not before reaction with the conjugate, the possibility of patching due to the divalent antibody (DePetris & Raff, 1972) can be eliminated. Thus the clusters of ferritin observed give a true representation of the disaggregation of the virus envelope and dispersion in the host membrane.

In conclusion it is worth considering whether the virus envelope, dispersed in the cell membrane very soon after infection, could evoke a rapid host response in the infected animal. It has recently become apparent that at least part of the cell-mediated immune response to pox- and other virus infections is effected through specifically sensitized cytotoxic T lymphocytes (Blanden, 1974). It is particularly interesting that this response requires the matching of histocompatibility antigens of the target infected cell and the T cell (Blanden et al. 1975; Koszinowski & Ertl, 1975). In the poxvirus infected cell systems thus far examined the experimental arrangements are such that the cytotoxicity of the T cells is manifested against newly synthesised virus antigens at the surface of the infected cell. It will be interesting to see if a similar cytotoxic effect can be demonstrated against the virus antigens originating from the envelope of the infecting virion.
The authors wish to thank J. A. Armstrong and M. V. Nermut for helpful advice and discussion. The skilled technical assistance of Stephen Griffiths and Allan Douglas is gratefully acknowledged.

REFERENCES


(Received 5 February 1976)