Adsorption and Penetration of Enveloped and Naked Vaccinia Virus Particles

L. G. PAYNE* AND E. NORRBY

The Department of Virology, Karolinska Institutet, School of Medicine, Stockholm, Sweden

Received for publication 9 November 1977

The adsorption and penetration of intracellular naked vaccinia virus (INV) and extracellular enveloped vaccinia virus (EEV) were examined. The adsorption kinetics of INV and EEV were similar, but INV adsorption was found to be more sensitive to the adsorption environment than EEV. The PFU-to-particle ratio for the two virus particles indicated that EEV was approximately two times as infectious as INV. Kinetic studies at 37°C showed that EEV penetrated cells more rapidly than INV. Penetration of EEV was unaffected by incubation in phosphate-buffered saline, but was somewhat reduced by incubation at 22°C. In contrast, INV penetration was effectively eliminated by incubation in phosphate-buffered saline or by incubation at 22°C. In addition, INV but not EEV penetration was sensitive to treatment with sodium fluoride and cytochalasin B. These results are discussed with regard to the mechanism of INV and EEV penetration.

The early events in cell-animal virus interactions have been studied by biochemical methods, bioassays as well as electron microscopy. These methods have shown that adsorption of virus particles to cells is mediated by the interaction of virus attachment sites to cell receptors. Penetration of attached virus particles has been demonstrated to occur mainly by either phagocytosis of particles or fusion of envelopes with cell membranes (reviewed in 13; 23). The latter mechanism has been conclusively shown only for certain members of the paramyxovirus group (13).

The early interaction of vaccinia virus with cultured cells has been most extensively studied by Dales and associates (12, 14, 15). Penetration of vaccinia virus was shown to be mediated by phagocytosis of adsorbed virus particles. These studies were all performed using virus purified from mechanically disrupted cells. Although vaccinia is largely a cell-associated virus, a small proportion of mature virus particles may acquire an envelope either at intracellular membranes or at the cytoplasmic membrane (21). This envelope is different in structure and origin from the vaccinia unit membrane, which is synthesized de novo and independent of cellular membranes. The unit membrane is a basic structure of both intracellular and extracellular virions and is unique to the poxvirus group. The envelope is similar in structure and origin to the envelopes of other enveloped virus groups and is present only on extracellular vaccinia virus (1, 27). Recent work has also provided convincing evidence that intracellular and extracellular viruses differ markedly in their antigenic structure. Separate neutralizing antigens (1, 29; reviewed in 5) and the vaccinia hemagglutinin (27) were found associated with the envelope. On the basis of these structural and topological distinctions, we have initiated the use of the abbreviations INV and EEV to describe intracellular naked virus and extracellular enveloped virus, respectively (27).

These recent findings have provided an impetus to reexamine the adsorption and penetration of vaccinia virus with special emphasis on the EEV, which is of interest for two reasons. (i) The spread of poxvirus infections in vitro and in vivo is presumably by extracellular virus (4). (ii) The existence of enveloped and naked vaccinia viruses that are both infectious affords the unique opportunity to study the role of envelopes in these early events.

MATERIALS AND METHODS

Virus. The hemagglutinin-producing IHD-J strain of vaccinia virus was grown in stationary monolayer cultures of KB or HeLa cells. Cells for virus production were cultivated in Eagle minimal essential medium plus 5% calf serum and maintained in Eagle minimal essential medium plus 2% calf serum during virus replication. Cells were infected at a multiplicity of infection of 0.3 PFU/cell for 1 h at 37°C, and unadsorbed virus was removed by washing. Radioactively labeled virus was obtained by the inclusion of 3μCi of [3H]thymidine per ml during virus propagation. At 40 to 48 h postinfection, the cells and supernatant medium were separated by a 10-min centrifugation at 3,000 rpm. INV was released by Dounce homogenization of the infected cells after resuspension with distilled water for 10 min. Nuclei and large debris were sedimented by centrifugation at 3,000 rpm for 10 min. EEV was sedimented from the growth medium by
centrifugation at 10,000 rpm for 30 min in a Sorvall GSA angle rotor (Du Pont Instruments). The EEV-containing pellets were suspended in phosphate-buffered saline (PBS). Both INV and EEV were treated with a Rapidis (Ultrasonics Ltd.) Sonifier at 30 μm for 10 s before purification.

INV and EEV were purified either by equilibrium centrifugation in cesium chloride gradients or rate zonal sedimentation in sucrose gradients. Purification in cesium chloride was performed as previously described (27). Virus was centrifuged for 1 h in a Beckman SW40 rotor at 30,000 rpm on cesium chloride gradients formed by prelayering 1.30- to 3.0 ml, 1.25- to 4.0 ml, and 1.20- to 5.0 ml cesium chloride solutions. The INV and EEV bands were harvested and dialyzed overnight against PBS. INV and EEV were also purified by centrifugation on 12.5-ml linear 20 to 45% (wt/wt) sucrose gradients in an SW40 rotor at 15,000 rpm for 45 min. Virus bands were collected and frozen without dialysis.

Antisera. Antiserum to IHD-J vaccinia virus was produced by intramuscular immunization of rabbits with live cesium chloride-purified INV emulsified with Freund complete adjuvant. The addition of Freund adjuvant to live virus has been shown to greatly elevate antibody production (6). Each rabbit received 2.5 X 10^7 PFU. Antisera were collected 6 weeks after immunization.

Measurement of virus adsorption to cultured cells. Quantitation of virus adsorption was made by either plaque formation or attachment of [3H]thymidine-labeled virus to 2-day-old monolayer cultures of A-549 cells. This heteroploid human lung cancer cell line was provided by W. A. Nelson-Rees (University of California, Oakland). Cells in 33-mm plastic petri dishes were washed once with 2 ml of HEPES (N-2-hydroxyethyl piperezine-N'-2-ethanesulfonic acid)-buffered lactalbumin plus 1% calf serum, except where otherwise stated, before addition of 1.0 ml or 10 μl volumes of virus. The virus material used for adsorption was cesium chloride- or sucrose gradient-purified INV and EEV or unpurified EEV. Virus was diluted more than 1:1,000 before use. After a period of adsorption, the unattached virus was removed by a single 3-ml wash with PBS. In adsorption experiments using plaque formation as the mode of quantitation, 3 ml of a 0.3% agarose in HEPES-buffered lactalbumin plus 3% calf serum medium was added, and plaques were counted after 48 h at 37°C. In adsorption experiments measuring uptake of [3H]thymidine-labeled virus, the cells were washed a second time, and the pooled washes were filtered through 220-nm Millipore membrane filters to trap the unadsorbed virus particles for determination of radioactivity.

Measurement of virus penetration of cultured cells. The penetration kinetics of virus into A-549 cells were determined by measuring the rate at which cell-attached virus became resistant to inactivation by antiviral antibody. Virus was first adsorbed onto washed A-549 monolayers in 33-mm petri dishes for 30 min at 4°C to minimize virus penetration. Penetration was initiated by washing the cells with 4°C medium, followed by addition of medium prewarmed to the appropriate temperature. An alternative method was used for determination of effects of NaF and cytochalasin B on penetration. In these experiments, washed A-549 monolayers were pretreated for 30 min with the inhibitor, and virus was adsorbed for 5 min in the presence of the inhibitor. Unadsorbed virus was removed by an inhibitor-containing wash, and penetration proceeded in the presence of the inhibitor. The entire pretreatment, virus adsorption, and virus penetration procedure was performed at 37°C.

At appropriate times after commencement of penetration, 1 ml of a 1:10 dilution of antiserum directed against live virus or normal rabbit serum at 4°C was added, and the cells were incubated for 2 h at 4°C to stop further virus penetration and to effect a neutralization of unpenetrated virus. Prior to addition of agarose, the antiserum was removed by a single wash. Plaques representing penetrated virus were counted 48 h later.

Determination of the relative infectivities of INV and EEV. [3H]thymidine-labeled INV and EEV from the same Roux bottle (10° cells) of HeLa cells were analyzed in sucrose gradients for infectivity and [3H]thymidine content. Prior to centrifugation of INV released by Dounce homogenization, the virus-containing cytoplasmic fraction was adsorbed three times successively with packed rooster erythrocytes to remove any EEV that may occur in such preparations. EEV in the growth medium was pelleted as described above. Samples of 1 ml of both INV and EEV were sonically disrupted for 10 s at 30 μM before centrifugation at 15,000 rpm for 45 min on a 12.5-ml 20 to 45% sucrose gradient in SW40 tubes. A 1:100 dilution was made immediately after gradient fractionation and frozen for later quantitation of infectivity. The number of PFU was determined by adsorption of 10 μl for 30 min to monolayers of A-549 cells in 33-mm petri plates. The [3H]thymidine content was measured by the trapping of virus particles from 220 μl on 220-nm Millipore filters for liquid scintillation counting.

RESULTS

Adsorption of INV and EEV to monolayers of A-549 cells. An analysis of the attachment of INV and EEV to A-549 monolayers was performed by quantifying the number of infectious virus particles adsorbed that resulted in plaque formation. Table 1 shows the results of INV and EEV adsorption from 1.0-ml volumes of media of varying complexity to cells pretreated for 30 min with the corresponding media. Adsorption was most efficient in HEPES-buffered lactalbumin plus 1% calf serum for both INV and EEV. Both virus particles attached less efficiently as the complexity of the adsorption environment decreased, resulting in adsorption from a PBS medium of 43% EEV but only 6% INV.

A direct comparison of INV and EEV plaque counts in HEPES-buffered lactalbumin plus 1% calf serum approaches unity, whereas in PBS 7.5 times as much EEV adsorbed to the cell monolayer. Although both virus particles showed a dependence on Ca2+, Mg2+, and serum...
for adsorption, neither INV nor EEV could be removed from cells after adsorption in the presence of these three components by washing with serum-free medium or medium containing EDTA. A separate series of experiments clearly demonstrated that the decreased plaque formation shown in Table 1 was not due to elution of virus previously adsorbed in the various media.

To study the adsorption rate, INV and EEV in HEPES-buffered lactalbumin plus 1% calf serum were added to the monolayers in 10-μl volumes, and samples were taken at various times. Adsorption from a small volume delivered to the center of the petri dish avoids meniscus formation at the petri dish wall and the resultant reduction in adsorption efficiency. Figure 1 represents the adsorption kinetics of INV and EEV at 21°C. No essential difference was found for the adsorption rate of the two virus particles. Attachment was complete in 15 to 20 min in all such experiments. Similar results were obtained at 37°C. Under these conditions, the adsorption of CsCl-purified [3H]thymidine-labeled INV and EEV after a 30-min incubation was 90 and 94%, respectively.

Penetration kinetics of INV and EEV. The rate of INV and EEV penetration as judged by the acquisition of serum resistance was studied after the adsorption at 4°C of virus in HEPES-buffered lactalbumin plus 1% calf serum. Figure 2 illustrates the penetration of virus in the presence of HEPES-buffered lactalbumin plus 1% calf serum after a shift to 22 or 37°C. Under these conditions, the acquisition of serum resistance by EEV proceeded at a rate clearly distinct from that of INV. At 37°C EEV was always over 90% penetrated at 60 min. In the same 60-min time interval, INV was most often only 50 to 60% penetrated. Penetration of more than 90% of INV occurred between 3 and 4 h after initiation of penetration.

The penetration of INV and EEV at 22°C in HEPES-buffered lactalbumin plus 1% calf serum after adsorption at 4°C is also depicted in Fig. 2. The attainment of a serum-resistant state by EEV under these conditions, although slower than observed at 37°C, still gave 50 to 60% EEV penetration after 60 min at 22°C. In contrast, INV penetration after 60 min was greatly reduced at 22°C compared to 37°C, without any significant increase even at 120 min.

The results obtained at 37°C in Fig. 2 represent penetration of virus in an environment expected to permit the cell's full metabolic activities to proceed unimpaired. Figure 3 represents virus penetration under conditions anticipated to decrease the cell's metabolism. In such experiments, cells were washed twice and incubated for 30 min in PBS at 4°C before virus adsorption at 4°C in PBS. Virus penetration at 37°C was also in a PBS milieu. EEV penetration was
unaffected by this treatment, whereas INV acquired serum resistance at a significantly reduced rate compared to the HEPES-buffered lactalbumin plus 1% calf serum medium (see Fig. 2). INV was never seen to exceed 35% penetration at 60 min in such experiments.

Effect of specific inhibitors on INV and EEV penetration. We next examined the effect of inhibitors of cell function on the process of virus penetration. Table 2 shows INV and EEV penetration in the presence of 5 × 10⁻⁵ M sodium fluoride or 10 μg of cytochalasin B per ml. The inhibitors were present during a 30-min pretreatment and during virus adsorption for 5 min. Both inhibitors induced profound morphological changes which were reversible within 2 h. Virus adsorption was unaffected by these inhibitors. The quantity of EEV attaining serum resistance under these conditions was not significantly affected compared to controls. In contrast to EEV, INV penetration was consistently reduced by cytochalasin B and sodium fluoride. Corresponding results were obtained with cytochalasin D. Higher concentrations of cytochalasin B did not further reduce penetration, whereas higher concentrations of sodium fluoride were toxic to the cells.

Relative infectivities of INV and EEV. Quantitative estimation of the PFU-to-particle ratio for INV and EEV requires that each virus is homogeneous and that the virus suspension is composed of nonaggregated particles. We have employed incorporated [³H]thymidine as an indicator of the number of virus particles. Inherent in this approach is the assumption that equal amounts of thymidine label are present in INV and EEV. Virus released into the extracellular medium of stationary cultures was the source of EEV, since we have previously noted (27) that INV is not present under these conditions. We have found that intracellular virus released after Dounce homogenization is composed mainly of INV with varying quantities of EEV. To eliminate this contaminating EEV, advantage was
taken of the presence of hemagglutinin in EEV but not INV (27) by adsorbing extracted virus with rooster erythrocytes. Thus, the [3H]thymidine-labeled INV and EEV from a single Roux bottle of HeLa cells could be analyzed on sucrose sedimentation gradients after a brief sonic disruption.

Figure 4 depicts the infectivity and [3H]thymidine distribution obtained after centrifugation of INV and EEV at 15,000 rpm for 45 min on 20 to 45% continuous sucrose gradients. The absence of aggregates is indicated by the presence of a single peak in the center of the gradients and by the ratio of PFU to [3H]thymidine. The mean (± standard deviation) PFU-to-counts per minute ratios for the three peak fractions in Fig. 4A and B are 1,151 (±213) and 2,522 (±99), respectively. Furthermore, electron microscopic observation of over 300 virus particles in each peak fraction showed that 88% of the particles in Fig. 4A and 86% in B occurred as single particles. The morphology of the virus particles in the peak fractions of Fig. 4A and B were revealed by electron microscopy to be homogeneous populations of INV and EEV, respectively. Centrifugation of these virus particles on preformed CsCl gradients confirmed the electron microscopic observations, since particles from Fig. 4A banded at the characteristic density for INV (1.27 g/ml) while particles from Fig. 4B banded at the density of EEV (1.23 g/ml) (data not shown).

A comparison of the PFU-to-counts per minute ratios for INV (1,151) and EEV (2,522) can thus be used to calculate the relative infectivities of the two virus populations. In the experiment

![Graph](image)

**Fig. 3.** Penetration kinetics of INV (○) and EEV (●) at 37°C under conditions restricting cell function. The methods employed are the same as described for Fig. 2 except that cells were washed twice and incubated for 30 min in PBS plus 1% calf serum before virus adsorption at 4°C. Penetration proceeded at 37°C in a PBS medium.

<table>
<thead>
<tr>
<th>Virus particle</th>
<th>Treatment (conc)</th>
<th>No. of plaques developing after treatment with</th>
<th>% Non-neutralizable virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal rabbit serum</td>
<td>Rabbit antivaccinia serum</td>
</tr>
<tr>
<td>INV</td>
<td>Sodium fluoride (5 × 10^{-3} M)</td>
<td>70 ± 7</td>
<td>26 ± 3</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>75 ± 12</td>
<td>58 ± 7</td>
</tr>
<tr>
<td>EEV</td>
<td>Sodium fluoride (5 × 10^{-3} M)</td>
<td>67 ± 9</td>
<td>57 ± 10</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>69 ± 15</td>
<td>61 ± 14</td>
</tr>
<tr>
<td>INV</td>
<td>Cytochalasin B (10 μg/ml)</td>
<td>133 ± 9</td>
<td>65 ± 8</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>145 ± 19</td>
<td>116 ± 11</td>
</tr>
<tr>
<td>EEV</td>
<td>Cytochalasin B (10 μg/ml)</td>
<td>172 ± 14</td>
<td>149 ± 18</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>172 ± 11</td>
<td>154 ± 6</td>
</tr>
</tbody>
</table>

*a* Monolayer cultures in petri dishes were pretreated for 30 min, and viruses were adsorbed for 5 min, then removed by washing, followed by a 2-h penetration period. The entire procedure was performed at 37°C in HEPES-buffered lactalbumin plus 1% calf serum in the absence or presence of inhibitor.

*b* The numbers of plaques developing after a 2-h treatment at 4°C with normal rabbit serum or antivaccinia rabbit serum are recorded as the mean ± standard deviation of five petri dishes.
described here, EEV was 2.2 times as infectious as INV.

DISCUSSION

The adsorption of virus particles to cells is dependent on two factors. The first factor is the rate of collision between virus particles and cells, which, under the conditions of the experiments reported here, may be assumed to be equal for INV and EEV. The second factor is the environment in which adsorption takes place. A strong dependence of EEV on the adsorption environment has been previously noted (22). Our results are in agreement with these findings. The relatively lower dependence of EEV on the adsorption milieu is most probably an expression of a higher affinity for cell receptors. The existence of specific attachment site-receptor interactions for INV seems questionable to us. This doubt is based on the fact that in vitro (2), and presumably in vivo, it is EEV and not INV that is responsible for the spread of infection. As a consequence, EEV has been exposed to a selective pressure resulting in the development of attachment sites specific for cell surface receptors. The intracellular existence of INV excludes such a selection.

EEV was found to be twice as infectious as INV. We have shown that equal quantities of INV and EEV are adsorbed and penetrated. A difference in the infectivities of INV and EEV can be the result of a lower infectious-to-noninfectious particle ratio for INV than EEV or a difference in the efficiency of the uncoating process. The latter possibility requires further investigation.

Following virus attachment to susceptible cells, virus can penetrate by a number of different mechanisms (reviewed in 23). The mode of entry of vaccinia virus has been shown electron-microscopically to be by either phagocytosis (14) or fusion (3, 9, 19). These studies were made employing INV particles. Fusion of the INV unit membrane with the cytoplasmic membrane is detectable only during the first 15 min after adsorption (3) and therefore seems not to be the dominant mode of entry, since our data show that only 30 to 35% of INV have acquired serum resistance at this time. We have endeavored to study the ingress of both INV and EEV by following the acquisition of serum resistance. We found the penetration of the two virus particles to differ markedly. These dissimilarities may be summarized as follows. Under conditions permitting optimal cell function at 37°C, the kinetics of serum inaccessibility were significantly more rapid for EEV than INV. EEV penetration was unaffected by conditions expected to affect the cell’s metabolic function (penetration in PBS) or by inhibition of ATP generation by sodium fluoride. In sharp contrast to this independence of cell function, INV penetration showed a distinct dependence on cell activity. In addition, penetration of INV but not EEV was sensitive to cytochalasins which affect a variety of cell membrane activities. Furthermore, INV entry was significantly more temperature sensitive than EEV.

These findings with regard to INV penetration are in part supported by reports in the literature. Sodium fluoride treatment inhibits the acquisition of serum resistance (14). Cytochalasin D was reported (16) to reduce the penetration of vaccinia. The entry of INV after 1 h at 22°C was observed (2) to be only 5% of the penetration seen at 37°C.

The penetration of INV reported here and in the literature contrasts very sharply with that reported here for EEV and elsewhere for poliovirus. It has been shown that poliovirus penetration is unaffected by the presence of sodium fluoride (20). A similar lack of effect on poliovirus ingress was found (16) for cytochalasin B and D. Furthermore, incubation of poliovirus-cell complexes at 22°C did not inhibit the acquisition of serum resistance by adsorbed virus (24).

The effect of these treatments on cells has been documented. Sodium fluoride (10), cytochalasin B (30), and ambient temperature (28) have all been shown to decrease endocytosis by cells markedly. The use of the term endocytosis is meant to include ingestion of material by both pinocytosis and phagocytosis. Endocytosis can thus be defined as a cell-dependent function requiring energy and possibly intact microfilaments. The results reported here and elsewhere on the penetration of INV can therefore be interpreted as supporting a mechanism requiring the active cell function of endocytosis. This interpretation relegates the INV particle to a passive role during penetration.

Certain members of the paramyxovirus group have been shown to penetrate cells by fusion of the viral envelope with the cytoplasmic membrane (13). A general characteristic of fusion by

Fig. 4. Sedimentation of [3H]thymidine-labeled INV (A) and EEV (B) from the same Roux bottle in continuous 20 to 45% (wt/wt) sucrose for 45 min in an SW40 rotor at 15,000 rpm. INV was released from cells by Dounce homogenization and adsorbed with rooster erythrocytes, while EEV was concentrated from growth medium by centrifugation as described in the text. Infectivities of INV (C) and EEV (D) and radioactivity of INV (△) and EEV (○) were determined as described in the text.
paramyxoviruses and presumably other fusing enveloped virus groups is that this mode of entry is temperature dependent (7, 25, 26). Our data demonstrate that EEV is able to penetrate at this reduced temperature. It therefore seems less likely that EEV enters the cell by fusion. EEV could also penetrate by endocytosis. However, this also seems unlikely, since endocytosis is a cell function but EEV is still able to penetrate under conditions that restrict cell function (NaF, cytochalasin B, and reduced temperature) as discussed above. Thin-section electron microscopy should provide further information on these possibilities.

An alternative mechanism to fusion and endocytosis for EEV entry is a recent hypothetical model proposed for the penetration of rhinoviruses (8). This model centers on the specificity of the virus-cell membrane interaction. The initial interaction of virus and cell is mediated by the juxtaposition of a virus attachment site and a cell receptor. At this point adsorption may or may not be reversible. Irreversible adsorption is achieved by a progressive increase in the number of specific virus-cell interactions, which results in a steady increase in the amount of the virus particle surface that is in contact with the cell membrane. This encompasement of the adsorbed virus particle requires only the free movement of the cell receptors in the plane of the cell membrane, which is not susceptible to inhibitors of energy generation (18) or microfilament function. Furthermore, the effect of reduced temperature on penetration is as would be expected if the movement of receptors were due to simple diffusion in a lipid membrane whose viscosity increases at lowered temperature. Membrane protein movement at 22°C is 50% of that at 37°C and is completely eliminated at 15°C (18). Virus penetration would accordingly occur at a reduced rate; however, the reduction would be anticipated to be dependent on the receptor concentration at the cell surface and therefore to vary for different virus-cell systems. Encirclement of the virus particle by the cell membrane is complete when the virus particle attachment sites have progressively bound to the receptors in such a way as to result in the apposition of cell membrane at one point on the virus particle surface. Fusion of the apposing membranes culminates in the topological sequestration of the virus particle and renders it inaccessible to antisera.

Fundamental to our interpretation of the proposed model (8) is that EEV, poliovirus, and presumably other viruses are not passive passengers during penetration. Instead, we visualize the virus particle as actively contributing to its own penetration by interacting with specific cell membrane receptors and that it is this specific union which is responsible for virus attachment and acts as the driving force leading to virus penetration. Since pinocytosis, phagocytosis, and endocytosis are terms describing cell function which disregard the virus particles' activity during penetration, we prefer to use the term viropexis (17).

ACKNOWLEDGMENTS
The able technical assistance of Lillemor Borgen is gratefully acknowledged.

LITERATURE CITED
fussof the virus envelope with host cell membrane. Virol. 52:305-309.