Significance of Extracellular Enveloped Virus in the \textit{in vitro} and \textit{in vivo} Dissemination of Vaccinia

By LENDON G. PAYNE

The Department of Virology, Karolinska Institute, School of Medicine, SBL S-105 21
Stockholm, Sweden

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SUMMARY

The significance of extracellular enveloped vaccinia (EEV) for the \textit{in vitro} and \textit{in vivo} dissemination of vaccinia virus was investigated. The quantity of \textit{in vitro} released extracellular virus correlated very closely with the ability of 13 vaccinia strains to cause long-range spread of infection (comet formation) in cell cultures infected at low m.o.i. but was not correlated with plaque size. The kinetics of virus spread after low m.o.i. was related to the amount of virus released from primary infected cells but not to their content of intracellular naked vaccinia (INV). Most extracellular vaccinia virus from IHD-J-infected RK-13 cells banded in CsCl density gradients as EEV (88\%) while very little banded as INV (2\%). Antisera to the envelope prevented comet formation while antisera to INV did not.

CsCl centrifugation of blood-borne extracellular virus from rabbits infected intravenously with vaccinia virus after cyclophosphamide treatment revealed that 64\% of the virus banded as EEV but only 11\% as INV. High \textit{in vitro} EEV-yielding vaccinia strains were able to spread from the respiratory tract to the brains of mice and cause death. Low \textit{in vitro} EEV-yielding vaccinia strains were generally not able to disseminate \textit{in vivo} or cause mouse mortality. The notable exception to this trend was strain WR, which, although releasing small amounts of virus \textit{in vitro}, could nevertheless very effectively disseminate \textit{in vivo}, causing a high rate of mouse mortality. Treatment with anti-envelope serum protected mice from a lethal vaccinia infection whereas antisera to inactivated INV did not. These results indicate that the \textit{in vitro} dissemination of vaccinia infection is mediated by EEV and implicate EEV as having a role in the \textit{in vivo} dissemination.

INTRODUCTION

Vaccinia virus exists in two infectious forms. Intracellular naked vaccinia (INV) is infectious and accumulates in large numbers inside the cell; for these reasons it is this cell-associated virion that has traditionally been studied (for review, see Moss, 1974). On the other hand, vaccinia virus naturally released into the extracellular milieu during the course of infection is surrounded by an envelope not present on INV (Appleyard \textit{et al.} 1971; Payne & Norrby, 1976). This virion referred to as extracellular enveloped vaccinia (EEV) is also infectious but usually only amounts to about 1\% of the total virus produced, although 25 to 35\% of the virus has recently been shown to be released from IHD-J vaccinia-infected RK-13 cells (Payne, 1979). The conversion of INV to EEV is mediated by an intracellular virion intermediate surrounded by two membranes derived from the Golgi apparatus, the outermost of which fuses with the plasma membrane releasing EEV composed of an...
internal virion wrapped in the inner Golgi membrane (Ichihashi et al. 1971; Morgan, 1976; Payne & Kristensson, 1979). Acquisition of the envelope results in the cleavage of some INV surface proteins that are absent from EEV (Payne, 1978, 1979). The envelope of EEV contains antigenic specificities distinct from INV (Appleyard et al. 1971; Turner & Squires, 1971; Prakash et al. 1977) and is responsible for a penetration of cells by EEV that is much more rapid than is seen with INV (Payne & Norrby, 1978). Nine glycoproteins and one non-glycosylated protein are present in the envelope (Payne, 1978, 1979). The vaccinia haemagglutinin is present in the envelope of EEV (Payne & Norrby, 1976) and has been identified as the 89K glycoprotein (Payne, 1979).

A role for extracellular virus in the spread of infection both in vitro and in vivo was proposed before the establishment of the existence of enveloped vaccinia virus (Boulter, 1969). At present, data supporting this proposition are based on antibody protection studies. Antisera containing antibody to both INV and EEV protect against virus spread in vitro and against various parameters of illness in vivo while antisera directed only against INV does not (Appleyard et al. 1971; Boulter et al. 1971; Turner & Squires, 1971; Boulter & Appleyard, 1973; Appleyard & Andrews, 1974).

Although antisera to live vaccinia prevent distant spread of virus in vitro, they do not prevent plaque formation (Appleyard et al. 1971). This implies that distant spread is mediated by extracellular virus while plaque formation in the presence of antibody is mediated by a cell-to-cell spread. In the present paper, the use of the terms ‘virus spread’ and ‘virus dissemination’ refer to the distant spread of virus. This paper provides further evidence for the importance of EEV for the in vitro and in vivo spread of infection.

**METHODS**

*Cells and virus.* RK-13 cells and HeLa cells were cultivated in Nunc plastic flasks (25 cm²) with MEM in Earle’s salts plus 5% foetal calf serum and penicillin-streptomycin. Cells were passaged 1 to 3 and infected on day 3 post-passage at 22 °C (for 1 h with either 5 p.f.u./cell or 150 p.f.u./flask). Unadsorbed virus was removed by two washes and 10 ml of medium was added to each flask. Maintenance medium for virus-infected cells was the same as for cell cultivation except that the foetal calf serum concentration was reduced to 1%. Time 0 was taken as the time of maintenance medium addition and incubation at 37 °C. At the end of infection, cells floating in the medium were sedimented at 3000 rev/min for 5 min, the sediment resuspended in 5 ml of distilled water and pooled with the remaining glass-attached cells. The supernatant medium was retained for plaque determination of extracellular virus infectivity. Intracellular virus was released by freeze-thawing and sonication before plaque titration.

Vaccinia strains Capetown, South Africa, Dairen, Lederle, Gallardo, Hall Institute White, Lafontaine, Tashkent and Venezuela were obtained from Dr K. Dumbell (Wright-Fleming Institute, St Mary’s Hospital, London, U.K.), strains IHD-J and IHD-W from Dr S. Dales (University of Western Ontario, London, Canada), strain WR from Dr E. Lycke (University of Göteborg, Göteborg, Sweden) and strain Lister from the State Bacteriological Laboratory, Stockholm, Sweden. All vaccinia strains were cultivated in HeLa cell Roux bottles (270 cm²) for production of stock virus and frozen in 10% sorbitol at −20 °C. Virus stock material was sonicated before use in experiments.

*Antisera.* INV and EEV from IHD-J-infected RK-13 cells were purified by two consecutive CsCl centrifugations (Payne, 1979). The envelope was purified as previously described (Payne, 1978). The protein content was determined by u.v. spectrophotometry (Joklik, 1962). Virus infectivity was eliminated by u.v.-light inactivation (Boulter et al.)
In vitro and in vivo significance of EEV 1971. One mg of inactivated INV, inactivated EEV, live INV and purified envelopes from 1 mg of EEV were homogenized in complete Freund's adjuvant and injected intramuscularly into the hind legs of adult rabbits. Live INV was administered to rabbits in complete Freund's adjuvant since it has been demonstrated to evoke antibody titres higher than can be obtained without the adjuvant (Boulter et al. 1971). Rabbits were given a booster dose intravenously 4 weeks later with the same quantity of material as was used for the primary immunization. Two weeks after the booster, the rabbits were exsanguinated.

Plaque and plaque reduction assays. Virus material was sonicated before plaque titration. All infectivity titrations were made with 3 or 4 Petri plates per dilution on RK-13 cells in 33 mm Petri dishes (Payne & Norrby, 1976). Plaque diameters were determined from enlarged photographs three times. INV for use in plaque reduction tests was purified by CsCl centrifugation to eliminate both EEV and soluble antigens that might compete for antibody binding. EEV was harvested 24 h p.i. but not purified. Both virus preparations were frozen in 10% dimethyl sulphoxide in medium until use. Virus neutralization enhancement antibody titres were determined by mixing twofold serum dilutions with an equal volume of INV or EEV. A 2 h incubation at 37 °C was followed by the addition of an equal volume of 1/20 diluted purified sheep anti-rabbit gamma globulin (Department of Immunology, State Bacteriological Laboratory) and incubation at 20 °C for 1 h. The virus–serum mixture was plaqued as described (Payne & Norrby, 1976). Between 50 and 100 plaques were obtained from controls with normal rabbit serum. Neutralization enhancement titres are expressed as the serum dilution giving a 50% plaque reduction.

Serum anti-comet activity. Two day-old RK-13 monolayers in 33 mm Petri plates were infected with 25 p.f.u. of IHD-J for 1 h at 37 °C and then overlaid with Hepes-lactalbumin plus 1% FCS. At 3 h p.i., the medium was replaced by new medium containing serial twofold dilutions of antisera. Petri plates were then incubated at 37 °C for 48 h. The anti-comet activity of a serum was taken as the highest dilution preventing distant spread (comet formation) of virus infection from primary plaques.

Animal experiments. Three-week-old mice weighing 14 to 15 g were infected intranasally, while ether anaesthetized, with 10^4 p.f.u. of virus in 10 μl. The mice were either observed during a 3 week period for mortality or sacrificed daily for virus titration of internal organs. The lungs, livers and brains were collected, macerated with a pestle and mortar and taken up in PBS as a 10% (w/w) suspension before plaque titration. In mouse protection experiments, 0.2 ml of rabbit antisera was passively transferred intraperitoneally at the time of intranasal infection. Rabbits were injected intraperitoneally with 150 mg/kg cyclophosphamide and infected intravenously 24 h later with 10^7 p.f.u. IHD-J vaccinia. The rabbits were exsanguinated 72 h p.i. The blood (150 to 200 ml) was mixed with sodium citrate (approx. 80 to 100 ml) to prevent coagulation. Cellular blood components were removed by sedimentation twice at 3000 rev/min for 10 min. Extracellular virus in the clear plasma was centrifuged at 15000 rev/min for 30 min in an SW27 rotor (Beckman). The sediment was easily resuspended in 1 ml PBS with light sonication before density gradient centrifugation.

Density gradient centrifugation. The extracellular virus from RK-13 cells infected with 5 p.f.u./cell and labelled with ^3H-thymidine (5 μCi/ml) was collected 72 h p.i. Cells floating in the medium were removed by centrifugation at 3000 rev/min for 5 min. The extracellular virus-laden supernatant was centrifuged in a GSA rotor (Sorvall) at 10000 rev/min for 30 min. The sediment was resuspended with sonication.

Extracellular virus from the blood of infected rabbits and from the medium of infected RK-13 cells was subjected to density gradient ultracentrifugation. CsCl gradients were formed by prelayering CsCl solutions of 1.30 (3 ml), 1.25 (4 ml) and 1.20 (5 ml) g/ml. Centrifugation was for 2 h at 30000 rev/min in an SW40 rotor (Payne & Norrby, 1976).
Comparison of virus spread (comet formation) in RK-13 cell cultures. Cell monolayers were infected with (a) Lederle and (b) IHD-J as described in Methods and photographed 48 h p.i.

The gradients were harvested dropwise and monitored for infectivity or radioactivity and density.

RESULTS

Comparison of 13 vaccinia strains for in vitro and in vivo virus spread

Thirteen vaccinia strains were investigated with regard to parameters of virus dissemination in cell cultures and mice. A very simple but elegant means of monitoring virus spread in vitro is by the formation of comets (Appleyard et al. 1971). In this test, cell monolayers infected at low multiplicities can be qualitatively monitored for distant spread of virus from the primary infected cells. Fig. 1 shows RK-13 cell monolayers infected with either vaccinia strain Lederle or IHD-J. Infection with IHD-J vaccinia (Fig. 1b) produced a primary plaque or comet head and a series of secondary plaques forming the comet tail. In contrast, infection with Lederle vaccinia (Fig. 1a) resulted in the formation of well-demarcated plaques with little sign of tail formation.

Table 1 shows the relationship of the amount of virus released from RK-13 cells and HeLa cells infected with 13 vaccinia strains to various parameters of in vitro and in vivo virus spread. The vaccinia strains are ordered according to the quantity of virus released from RK-13 cells. This cell system was previously shown to release as much or more virus than is obtained from HeLa cells (Payne, 1979) which was largely confirmed here. There was generally very little correlation in the amount of virus released from RK-13 cells to the quantity of intracellular virus produced. IHD-J, for example, synthesized only twice as much intracellular virus as strain Lister but IHD-J-infected cells released 100 times as much virus as Lister. Plaque size might be expected to be related to the quantity of virus released. Comparative measurements of plaque diameters 48 h p.i., however, did not fulfill this expectation. Eight strains, varying greatly in the amount of extracellular virus released, produced plaques that were within one standard deviation of IHD-J diameters. Venezuela vaccinia, for example, yielded plaques of the same size as IHD-J although IHD-J released 200 times as much extracellular virus as did Venezuela. On the other hand, the ability to spread virus to distant parts of a cell monolayer under liquid medium (comet formation) was very closely correlated to the amount of virus released. Only the three vaccinia strains...
Table 1. Relationship of in vitro virus release to in vitro and in vivo parameters of virus dissemination*

<table>
<thead>
<tr>
<th>Vaccinia strain</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; Virus production</th>
<th>Plaque size ± s.d. (mm) in</th>
<th>Comet formation in</th>
<th>Mouse mortality (%)§</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; Virus in brain (p.f.u./g)ǁ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RK-13</td>
<td>HeLa</td>
<td>RK-13†</td>
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<td></td>
<td>Extra-</td>
<td>Intra-</td>
<td>Extra-</td>
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<tr>
<td>South Africa</td>
<td>5.6</td>
<td>8.0</td>
<td>5.7</td>
<td>7.2</td>
<td>0.6±0.2</td>
</tr>
<tr>
<td>Capetown</td>
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<td>7.7</td>
<td>5.8</td>
<td>6.7</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>Venezuela</td>
<td>6.0</td>
<td>8.2</td>
<td>6.5</td>
<td>7.5</td>
<td>1.4±0.3</td>
</tr>
<tr>
<td>Lister</td>
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<td>8.8</td>
<td>6.7</td>
<td>8.4</td>
<td>1.2±0.3</td>
</tr>
<tr>
<td>Tashkent</td>
<td>6.6</td>
<td>8.8</td>
<td>7.1</td>
<td>8.3</td>
<td>1.4±0.3</td>
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<tr>
<td>WR</td>
<td>6.6</td>
<td>9.1</td>
<td>6.6</td>
<td>8.2</td>
<td>1.1±0.2</td>
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<tr>
<td>Lederle 7N</td>
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<td>6.9</td>
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<tr>
<td>Hall Institute</td>
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<td>8.5</td>
<td>7.1</td>
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<tr>
<td>White Dairen</td>
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<td>8.2</td>
<td>7.1</td>
<td>8.3</td>
<td>1.0±0.2</td>
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<tr>
<td>Lafontaine</td>
<td>7.6</td>
<td>9.2</td>
<td>6.9</td>
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<td>IHD-W</td>
<td>8.0</td>
<td>9.1</td>
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<td>1.2±0.2</td>
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<tr>
<td>Gallardo</td>
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<td>7.5</td>
<td>8.0</td>
<td>1.2±0.2</td>
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<tr>
<td>IHD-J</td>
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<td>9.1</td>
<td>7.5</td>
<td>8.7</td>
<td>1.4±0.2</td>
</tr>
</tbody>
</table>

* Three-day-old monolayers in 25 cm<sup>2</sup> plastic flasks were infected at 5 p.f.u./cell as described in Methods. Virus was harvested 48 h p.i. and plaque assayed.
† Plaque size was determined from three times enlarged photographs taken 48 h p.i. Each estimate represents measurements from at least 25 well-demarcated plaques.
‡ RK-13 cells in Petri dishes were infected as described in Methods and examined for comets 48 h p.i.
§ Groups of 20 mice were infected with 10<sup>6</sup> p.f.u. intranasally and observed for mortality.
ǁ Groups of five mice were infected with 10<sup>6</sup> p.f.u. intranasally and the brains titred on day 5 p.i. Virus titres of less than 100 p.f.u./g were not detectable and are denoted by −.
Fig. 2. The extracellular (○, IHD-J; △, WR; □, Lederle) and intracellular (●, IHD-J; ▲, WR; ■, Lederle) virus from 10^7 RK-13 cells infected with 150 p.f.u. of each vaccinia strain were assayed at intervals after infection by plaque titration.

(IHD-J, Gallardo and IHD-W) releasing the greatest quantity of virus from RK-13 cells were able to form well-defined comets.

The ability of the 13 vaccinia strains to cause mortality and to spread to and replicate in the brains of mice inoculated intranasally was investigated. The three comet-forming strains that released the most virus \textit{in vitro} caused a severe infection in mice ending in the death of 25% of the mice infected with Gallardo and IHD-W and 70% for IHD-J-infected mice. The remaining ten vaccinia strains did not result in the death of mice with the notable exception of an 85% mortality in mice infected with strain WR. The \textit{in vitro} release of virus correlated very well with mouse neurotropism. The five vaccinia strains (IHD-J, Gallardo, IHD-W, Lafontaine and Dairen) releasing the most virus from RK-13 cells spread to and replicated to a detectable level in the brains of mice. Strain WR was again a noteworthy exception to this correlation. It replicated in the mouse brain to the same level achieved by IHD-J although IHD-J released 50 times more extracellular virus from RK-13 cells than WR did.

These studies permit an ordering of the 13 vaccinia strains into one of three groups. One group typified by IHD-J is characterized by an \textit{in vitro} release of large amounts of extracellular virus and the ability to disseminate infection both \textit{in vitro} and \textit{in vivo}. A second group, containing strain WR, releases low amounts of virus \textit{in vitro} and consequently does not disseminate efficiently \textit{in vitro} but can, nevertheless, spread infection very effectively \textit{in vivo}. Lederle 7N was selected to represent the remaining virus strains placed in a third group. These strains have in common a low \textit{in vitro} release of extracellular virus and a poor dissemination both \textit{in vitro} and \textit{in vivo}. These three representative viruses were then further studied to determine the significance of enveloped virus for spread of infection.
In vitro and in vivo significance of EEV

Fig. 3. $^3$H-labelled extracellular virus from RK-13 cells harvested 72 h p.i. with 5 p.f.u./cell of IHD-J (●), WR (▲) and Lederle (■) was centrifuged in separate CsCl gradients as described in Methods. The density curves were close enough to permit representation of the results in the same figure. The gradient sample interface fractions are not included in the figure.

In vitro virus dissemination

From Table I it is apparent that RK-13 cells infected with IHD-J, Lederle and WR vaccinia synthesize approximately similar amounts of intracellular virus although IHD-J releases about 40 times more extracellular virus than Lederle or WR. These results were obtained under one-step growth conditions and therefore reveal little information on the three strains ability to disseminate infection. This biologically important problem was investigated by infecting cell cultures (approx. 10^7 cells) with only 150 p.f.u. and then following the appearance of intracellular and extracellular virus (Fig. 2). The quantity of intracellular virus at 6 h p.i. was, as expected, very nearly the same for the three vaccinia strains. At this time only primary-infected cells were synthesizing virus. By 24 h p.i., IHD-J and WR had produced similar quantities of intracellular virus which was 3 to 4 times as much virus as produced by Lederle. Intracellular virus production began to plateau for all three strains at 48 h p.i. when IHD-J-infected cells contained five times more virus than WR-infected cells and 20 times more than cells infected with Lederle. No great change in the relative intracellular virus concentrations occurred during the ensuing 72 h. The failure of WR and Lederle to attain the same levels of INV even after 5 days infection is probably due to restrictive culture conditions at such late times after infection.

The release of extracellular virus showed even greater strain differences than was evident for intracellular virus. Although IHD-J had released only about five times more virus by 24 h p.i. than WR or Lederle, the amount of virus released by IHD-J at 48 h p.i. was 300 and 600 times more than that released by WR or Lederle, respectively. It is not known why very little virus is released by WR and Lederle between 24 to 48 h p.i. despite the fact that there was a 10-fold increase in intracellular virus production during the same period. Nor is it known why there is a more than 10-fold increase in extracellular virus from 48 to 72 h p.i. without a significant increase in the amount of intracellular virus synthesized during this
period. Nevertheless, the data indicate that the magnitude of the infection as reflected by the production of intracellular virus is dependent on an intense early release of extracellular virus.

The preceding experiment, together with the difference in the ability of these three strains to release virus (Table 1) and to form comets (Fig. 1), clearly implicate extracellular virus as responsible for virus dissemination in vitro. It was, therefore, of interest to examine extracellular virus to determine the content of naked and enveloped virus. This was achieved by infecting cells with 5 p.f.u./cell and labelling with $^3$H-thymidine. All three strains caused a 100% c.p.e. by 24 h p.i.; however, the extracellular virus was not harvested until 72 h p.i. This was intended to maximize any eventual release of INV due to cell degradation. The extracellular virus was examined by centrifugation in CsCl gradients to effect a separation of naked and enveloped virus. Although the strains were analysed in separate gradients, there was very little variation in the density curves of the three gradients, permitting the presentation of the results on one graph (Fig. 3). To simplify interpretation, the sample-gradient interface is not included. Extracellular virus entering the gradient from IHD-J-infected cells gave a sharp peak at an EEV density of 1.24 g/ml. Extracellular virus from WR and Lederle also banded at an EEV density but displayed a considerable skewing of the peak to lighter densities. Eighty-eight percent of IHD-J extracellular virus banded as enveloped virus while only 2% banded as naked virus (1.27 g/ml). The corresponding figures for enveloped and naked virus were 50% and 8% for WR and 57% and 11% for Lederle. The extracellular virus that accumulated 48 h after complete c.p.e. is thus very clearly dominated by enveloped virus.

### Table 2. Kinetics of in vivo virus dissemination*

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Organ</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tbody>
<tr>
<td>Lederle</td>
<td>Lung</td>
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<tr>
<td></td>
<td>Liver</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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<td>Lung</td>
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<td>8.4</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.0</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>-</td>
<td>-</td>
<td>2.3</td>
<td>2.7</td>
<td>3.8</td>
<td>4.8</td>
</tr>
<tr>
<td>WR</td>
<td>Lung</td>
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<tr>
<td></td>
<td>Liver</td>
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<td>-</td>
<td>-</td>
<td>2.8</td>
<td>4.2</td>
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</tr>
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</table>

* Mice in groups of five were infected intranasally with $10^6$ p.f.u. Organs were harvested at intervals and processed for infectivity titration as described in Methods. The p.f.u. infectivity titres are expressed in log_{10}. Virus titres of less than $100$ p.f.u./g were not detectable and are denoted by -.

In vivo virus dissemination

The data in Table 1 suggest that only vaccinia strains able to replicate in the brain were able to produce a lethal infection by the respiratory route. Groups of mice infected with vaccinia strains Lederle, IHD-J and WR were sacrificed at intervals after intranasal inoculation in order to examine the kinetics of virus spread by these three strains (Table 2). Virus was detected in lungs on day 1 p.i. for all three vaccinia strains and rose to peak titres by day 4. The extent of replication was about the same for strains WR and IHD-J which was approx. 2 to 3 logs higher at all times p.i. than seen for strain Lederle. Dissemination to and replication in the livers and brains of mice infected with strain Lederle were not detectable...
at any time. Infectious virus was demonstrable in the brains of mice infected with WR and IHD-J on day 3 to 4 and rose to about the same titres. The livers of these mice contained virus on day 4 to 5. Strain WR produced approx. 2 logs more virus in the liver by day 6 than strain IHD-J. Mice infected with WR and IHD-J began to die between days 7 and 9.

Large volumes of blood are required in order to investigate the blood-borne extracellular virus as to the quantity of naked and enveloped virus forms. Adult rabbits instead of mice were used in order to examine this problem. Rabbits were injected intraperitoneally with 150 mg/kg cyclophosphamide 24 h before an intravenous administration of 10⁷ p.f.u. of IHD-J vaccinia. Cyclophosphamide depressed the rabbits immune defences and resulted in a much more severe infection. CsCl centrifugation of virus concentration from the plasma of titrated blood taken 72 h p.i. revealed that virus banded at both an INV density (1.27 g/ml) and an EEV density (1.23 to 1.24 g/ml) (Fig. 4). Virus banding as INV constituted 11% of the virus present in the gradient, whereas virus banding as EEV composed 64% of the total. This quantitative difference was consistently observed.

In vitro and in vivo protective effect of vaccinia antisera

The preceding experiments investigated virus dissemination by examining the virus per se. An alternative to this approach is to study the effect of vaccinia antisera on parameters of in vitro and in vivo virus spread (Table 3). Interpretation of such experiments is dependent on knowing the anti-INN and anti-EEV content of each antisera. Rabbits immunized with live INV produced high titres of neutralizing antibody to both INV and EEV since the virus was able to replicate in the rabbits. Immunization with inactivated INV, on the other hand, elicited high titres of antibody against INV but not EEV. Inactivated EEV evoked the production of antibody to both INV and EEV, whereas purified envelopes produced antibody that after INV adsorption neutralized only EEV. Antisera containing neutralizing antibody to both INV and EEV inhibited comet formation and passively protected mice against death from IHD-J vaccinia, whereas antisera neutralizing only INV did not.
Table 3. Comparison of anti-vaccinia sera for in vitro and in vivo protection*

<table>
<thead>
<tr>
<th>Rabbit antisera specificity</th>
<th>Neutralization enhancement titre</th>
<th>Anti-comet activity</th>
<th>Mouse protection (deaths/10 mice)</th>
</tr>
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<tr>
<td>Live INV</td>
<td>320,000</td>
<td>80,000</td>
<td>160</td>
</tr>
<tr>
<td>Inactivated INV</td>
<td>100,000</td>
<td>&lt; 100</td>
<td>10</td>
</tr>
<tr>
<td>Inactivated EEV</td>
<td>80,000</td>
<td>160,000</td>
<td>1280</td>
</tr>
<tr>
<td>Envelope</td>
<td>&lt; 100</td>
<td>20,000</td>
<td>640</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>&lt; 100</td>
<td>&lt; 100</td>
<td>ND†</td>
</tr>
</tbody>
</table>

* Rabbit antisera were raised and titrated by neutralization enhancement and for anti-comet activity as described in Methods. Anti-envelope sera had an anti-INV and EEV titre of 20,000 and 40,000, respectively, before adsorption of 2 ml of sera with 3 mg of purified INV. The post-adsorption anti-envelope titres are provided in the table. Passive transfer of antisera and challenge with IHD-J vaccinia of groups of 10 mice were performed as described in Methods.

† ND, Not done.

Antisera directed at the envelope and neutralizing only EEV also prevented comet formation and passively protected mice. Mice infected with WR vaccinia were also protected by antisera directed against live vaccinia, inactivated EEV and purified envelope but were not protected by antisera against inactivated INV (data not shown).

DISCUSSION

A rapid in vitro virus dissemination to cells distant from primary infected cells must be mediated by extracellular virus (Boulter, 1969). The demonstration that extracellular vaccinia virus was surrounded by an envelope not present on intracellular vaccinia virus (Appleyard et al. 1971; Payne & Norrby, 1976) provided circumstantial evidence that spread of infection might be mediated by the enveloped form of vaccinia. This was supported by in vitro studies of the effect of antisera on virus spread as measured in the very simple comet test (Appleyard et al. 1970). In this test, cell monolayers infected at very low multiplicities of infection developed primary plaques or comet heads while released virus spread through the medium producing a series of secondary plaques or comet tails. Antisera neutralizing both INV and EEV prevented comet formation while antisera neutralizing only INV did not (Appleyard et al. 1971; Turner & Squires, 1971; Boulter & Appleyard, 1973; Appleyard & Andrews, 1974). EEV was thereby clearly implicated as the vaccinia form charged with spreading virus infection. Recent evidence further supports the role of EEV in dissemination. The addition of IMCBH to the medium of low multiplicity infected cell prevented the release of EEV and completely inhibited comet formation but did not affect the production of INV (Payne & Kristensson, 1979).

The data presented here lend further support to the significance of EEV for virus dissemination in vitro. Three vaccinia strains were able to form comets. These three strains released more extracellular virus than ten other strains that were not able to form comets. After low multiplicity infection, the evolution of the infection is reflected in the amount of INV produced, such that the greater the INV production in the cell culture, the greater the dissemination of the infection. Vaccinia strains that synthesize equal amounts of INV in primary infected cells but differ in the quantity of released virus from the primary infected cells should show differences in the kinetics of INV production in the cell culture. Indeed, this is what was observed after low multiplicity infection with vaccinia strains differing 40-fold in virus release but not differing in the capacity to synthesize INV. CsCl density
gradient centrifugation revealed that extracellular virus harvested even 48 h after complete c.p.e. was mainly composed of EEV. Naked virus was not only quantitatively the minor infectious component in the extracellular environment but apparently contributed little or nothing to virus dissemination as shown by the effect of antisera on comet formation. Antisera to purified envelopes prevented comet formation while antisera to INV could not. The accumulated data thus established that in vitro dissemination is mediated by EEV and the rapidity of virus spread is dependent on the magnitude of EEV release.

A significant correlation was observed between in vitro EEV yields from 12 of 13 vaccinia strains and in vivo pathogenesis. Strains that released large amounts of EEV from RK-13 cells in vitro were able to disseminate from the respiratory tract to the brains of mice and to cause the death of mice. This clearly suggests that the amount of virus released in vitro in RK-13 cells is correlated with in vivo virulence. It does not, however, permit the interpretation that the amount of in vitro and in vivo released EEV is related. Virulence is undoubtedly the result of a more complicated interplay of several host- and virus-specified factors. The exceptional behaviour of strain WR is interesting in this regard. Although WR released rather low amounts of EEV, it was nevertheless able to spread in infected mice and cause a high rate of mortality. WR differs from the other vaccinia strains tested in that it has been adapted to mouse brain passage. Further work is required to discover the basis of WR's deviation from the norm.

Antisera containing antibodies to both INV and EEV can passively protect rabbits against an otherwise lethal rabbitpox infection while antisera containing only anti-INV specificities cannot (Appleyard et al. 1971; Boulter et al. 1971; Boulter & Appleyard, 1973). We have shown here that antibodies directed only against the envelopes are sufficient for protection of mice. Furthermore, extracellular virus found in the blood of infected mice was predominantly in the enveloped form. This is a significant finding since it means that the in vitro studied EEV is not simply an artefact of vaccinia virus propagation in cell cultures. Naked virus is found free in the circulatory system in only minor quantities compared to EEV. Taken together the data relegate free naked virus to a very minor role in the spread of virus in infected animals. Conversely EEV would seem to be of significance for in vivo virus dissemination. These in vivo results are clearly reminiscent of the in vitro results obtained on the relative significance of naked and enveloped forms for virus spread. The in vitro significance of EEV seems very clear but its role in vivo is not certain. Interpretation of the in vivo data must be more restrained than for the in vitro results since the two systems are not strictly analogous. It must be remembered, for example, that the viraemia of other poxviruses, such as mousepox (Blanden, 1970) and rabbitpox (Bedson & Duckworth, 1963) is largely cell associated. It should also be realized that the passively transferred antibody does not act in an immunological vacuum as it does in vitro. Antibodies directed at the envelope may not alone be sufficient to cause the regression of the infection. These antibodies may simply slow down virus dissemination long enough to permit the immunologically intact recipient to mobilize its cell-mediated immune defences. Further work is needed to obtain clarification on this point.

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REFERENCES


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