A Rabies Virus Persistent Infection in BHK21 Cells

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SUMMARY

A rabies virus persistent infection in BHK21 S13 cells was established and maintained in culture for more than 4 years. Initially, the cultures produced a large plaque virus similar to that produced by the original virus, but between the 10th and 20th passage, this was replaced by a small plaque variant. By the 200th passage, infectious virus could no longer be detected in the medium. After further cell passages (≥ 300) no infectious particles could be detected in the medium. At various passage levels, the persistently infected cells were labelled with [35S]methionine and the virus antigens immunoprecipitated and analysed by polyacrylamide gel electrophoresis. No changes in the virus polypeptides were observed in the establishment of the persistent state. However, after the 20th passage (predominance of small plaque variant) there was an increase in the size of the glycoprotein. This was followed (164th passage) by a change in the M1 polypeptide which was subsequently further modified in the defective state (≥ 300 passages). Virus isolated from the 400th passage by treatment of the cells with DEAE-dextran, was also modified in the glycoprotein and M1 polypeptides and contained less L polypeptide than the original virus. This virus grew more slowly, to a lower titre and was no longer pathogenic in suckling mice.

INTRODUCTION

Several animal viruses which normally produce cytocidal infections can, under certain circumstances, establish a long-term persistent infection. Several hypotheses have been advanced to account for such infections and these include the induction of defective-interfering (DI) particles (Huang & Baltimore, 1970), the synthesis of interferon (Nishiyama, 1977; Ramseur & Friedman, 1977) or the appearance of temperature-sensitive (ts) mutants (Preble & Youngner, 1975). The importance of these factors probably varies in different virus–host systems. Once a persistent state is established, the virus may undergo mutations leading to a virus with a more restricted biological activity.

Rabies virus, a member of the rhabdovirus group, causes infection of the central nervous system in all warm-blooded animals including man and readily gives rise to persistent infections in vitro (Holland et al., 1976). The virus contains five structural polypeptides: L, associated with the polymerase activity (Kawai, 1977), a glycoprotein (G), a nucleoprotein (N) and two membrane-associated polypeptides (M1 and M2) (Sokol et al., 1971). In virus-infected cells, non-structural proteins have not been identified, but the glycoprotein is found in two forms, G1 and G2, of which only G2 represents the fully glycosylated molecule found in the virus (Neurath et al., 1972). In the present study, we have attempted to correlate

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the modifications of biological properties of rabies virus and the variations in virus polypeptide structure during a long-term infection of BHK cells.

METHODS

Cells and viruses. BHK21 S13 cells and a line of the same cells persistently infected with rabies virus were grown in Eagle's modified medium supplemented with 10% tryptose-phosphate broth, 10% foetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). The cells were subcultured every 3 to 4 days at a split of 1:6 for the non-infected and 1:4 for the persistently infected cells.

The HEP-Flury vaccine strain of rabies virus was obtained from the World Health Organization, Geneva. A large plaque variant (LP) was selected and it was subsequently recloned three times by the plaque method of Bijlenga & Joubert (1980). For routine virus production, infected cells were cultivated in Eagle's medium containing 2% foetal calf serum (maintenance medium). Virus was titrated by the method of Bijlenga & Joubert (1980).

Immunofluorescence. Immunofluorescence was performed on formalin-fixed cells either by the direct method using a fluorescein-conjugated anti-rabies virus nucleocapsid serum (Pasteur Institute, Paris), or by the indirect method with an anti-rabies virus hyperimmune guinea-pig serum and fluorescein-conjugated anti-guinea-pig γ-globulin (Behringwerke, Marburg, F.R.G.).

Virus purification. The supernatant from the virus-infected cells was centrifuged at 10000 g for 30 min. An equal volume of cold saturated ammonium sulphate (adjusted to pH 7.6) was added and the precipitate collected by centrifugation for 30 min at 3000 g. The precipitate was resuspended in phosphate-buffered saline (PBS) and clarified at 10000 g for 10 min. The virus was centrifuged through a 30 to 60% (w/v) discontinuous sucrose gradient at 25000 rev/min for 2 h in a Beckman SW27.1 rotor. The virus band at the interphase was removed from the side with a syringe, diluted threefold in PBS and pelleted at 30000 rev/min for 1 h in a Beckman SW41 rotor. The virus was resuspended in PBS. Virus labelled with [35S]methionine was purified by the same method except that the ammonium sulphate step was omitted.

Antiserum. Purified HEP-Flury virus was inactivated with β-propiolactone and mixed with an equal volume of Freund's complete adjuvant. The mixture was inoculated subcutaneously into guinea-pigs at 10 to 15 sites/animal. The animals were re-inoculated with a similar mixture 4 weeks later and bled after a further 3 weeks.

Radiolabelling of cells and virus. Virus-induced proteins: 3 to 5 days after infection with rabies virus at an m.o.i. of 6, 5 × 10⁶ BHK cells were incubated for 30 min in Eagle's medium without methionine. The cells were then labelled in 2 ml of the same medium containing 50 μCi/ml [35S]methionine (800 mCi/mmol, The Radiochemical Centre, Amersham) for 2 h. The proteins were extracted with 2 ml 1% Triton in 0.15 M-NaCl, 10⁻⁵ M-EDTA and 0.02 M-tris pH 7.6, containing 0-28 trypsin-inhibiting (T.I.) unit/ml aprotinin. The extract was centrifuged at 3000 g for 10 min and the supernatant stored at −20 °C. Plasma membrane proteins of confluent cultures were labelled with 125I as described previously (Wild & Greenland, 1979).

Immunoprecipitation. Immunoprecipitation was performed as previously described (Giraudon & Wild, 1981).

Polyacrylamide gel electrophoresis (PAGE). PAGE was performed according to Laemmli (1970). ¹⁴C-labelled marker proteins: myosin (200K), phosphorylase b (92.5K), BSA (69K), ovalbumin (46K) and carbonic anhydrase (30K) were obtained from The Radiochemical Centre, Amersham. Fluorography was performed according to the method of Bonner & Laskey (1974).
Table 1. Growth of VSV and rabies (LP) virus in BHK cells persistently infected with rabies (passage 397)*

<table>
<thead>
<tr>
<th>Challenge virus</th>
<th>BHK-rabies passage 397 (p.f.u./ml)</th>
<th>Cloned BHK-rabies cells (p.f.u./ml)†</th>
<th>BHK non-infected (p.f.u./ml)</th>
</tr>
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<tbody>
<tr>
<td>VSV</td>
<td>$3 \times 10^7$</td>
<td>$2 \times 10^7$</td>
<td>$5 \times 10^7$</td>
</tr>
<tr>
<td>Rabies LP (1):</td>
<td>$10^2$</td>
<td>$2 \times 10^2$</td>
<td>$10^6$</td>
</tr>
<tr>
<td>(2)</td>
<td>$2 \times 10^3$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Cells were infected at an m.o.i. of 5 with either rabies LP virus or VSV. Virus was harvested at maximum cytopathic effect which corresponded to 24 h for VSV and 5 days for rabies. The persistently infected cells after superinfection were harvested at the corresponding time.
† The clone of cells isolated from the rabies persistent infection at the 200th passage level. These cells were subsequently found to be negative by immunofluorescence and virus assay for rabies virus.
‡ The persistently infected cells were superinfected either at the 2nd (1) or 5th (2) day after subculture. On parallel cultures, it was determined by immunofluorescence that 50% (2) and 80% (1) of the cells contained rabies virus antigens.

RESULTS

Establishment and maintenance of the persistent infection

Monolayer cultures of BHK21 S13 cells infected with LP virus at an m.o.i. of 6 were cultivated in maintenance medium for 5 days. The cells surviving the infection were trypsinized and cultivated in growth medium. After a further 5 days the cell culture reached confluency and was subsequently subcultured every 3 to 4 days. The cells have now been maintained in culture for more than 4 years (450 passages). The original LP virus produced large plaques (3 to 4 mm diam.). The persistently infected cultures continued to secrete virus of a similar plaque size during the initial passages, but after the 10th passage a small plaque variant (1 mm diam.) appeared, which by the 20th passage was the only virus excreted into the medium. Further cell passages ($\geq$200) led to a state where infectious virus was no longer detectable in the medium, although non-infectious particles were observed. After the 300th cell passage, virus particles could not be detected in the medium either by biological or radiolabelling techniques.

Throughout the evolution of the chronic infection, the appearance of virus antigens in the cells and the presence of infectious virus in the medium followed a cyclic pattern similar to that described by Kawai et al. (1975). The number of cells containing virus antigen, as measured by immunofluorescence, varied between 5 and 80%. In the early passages, where the large plaque virus was produced, titres ranged from $10^4$ to $10^8$ p.f.u./ml, but by the 20th passage (small plaques only), virus titres ranged between $10^4$ and $10^7$ p.f.u./ml.

In agreement with the results of Kawai et al. (1975), but in contrast to the findings of Wiktor & Clark (1972), the persistently infected cells supported the growth of VSV to the same levels as uninfected cells, but superinfection with the original rabies virus strain gave varying results depending on the number of cells expressing virus antigens (Table 1). When the persistently infected cells were cloned, both infected and virus-free clones were obtained. Superinfection with LP virus of the infected clones gave results similar to the uncloned cultures, but the uninfected clones on superinfection gave rise to virus titres almost as high as the control BHK cells (Table 1).

Between the 300th and 400th cell passages, several attempts were made to isolate infectious virus from the cultures. Co-cultivation or fusion with polyethylene glycol
Titration of LP and 400P rabies viruses in 5-day-old suckling mice*

<table>
<thead>
<tr>
<th>Virus dilution</th>
<th>LP virus</th>
<th>400P virus</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Inoculation (p.f.u./mouse)</td>
<td>Mortality</td>
</tr>
<tr>
<td>10^-6</td>
<td>2 x 10^6</td>
<td>8/8</td>
</tr>
<tr>
<td>10^-5</td>
<td>2 x 10^5</td>
<td>10/10</td>
</tr>
<tr>
<td>10^-4</td>
<td>2 x 10^4</td>
<td>10/10</td>
</tr>
<tr>
<td>10^-3</td>
<td>2 x 10^3</td>
<td>9/9</td>
</tr>
<tr>
<td>10^-2</td>
<td>2 x 10^2</td>
<td>10/10</td>
</tr>
<tr>
<td>10^-1</td>
<td>2 x 10^1</td>
<td>8/9</td>
</tr>
<tr>
<td>10^-0</td>
<td>2</td>
<td>5/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1/9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0/10</td>
</tr>
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* Five-day-old suckling mice were inoculated intracerebrally with 0.02 ml of virus. The results are recorded as the number of mice killed over the number inoculated.

(Pontecorvo, 1975) with either Vero or fresh BHK cells failed to lead to the isolation of a lytic-type virus. Despite the inability to rescue a virus by the classical methods, we were able to isolate virus by treatment of the cells with DEAE-dextran. Persistently infected cells at the 400th passage were inoculated into maintenance medium containing 100 μg/ml DEAE-dextran. After 48 h most of the cells were in suspension due to the toxicity of the DEAE-dextran. The supernatant was clarified by low-speed centrifugation and subsequently passaged in BHK cells. Although no virus could be detected in the DEAE-dextran supernatant by the plaque assay method, cells were found to be infected when examined by immunofluorescence (10 days after infection). Titration of the supernatant of the first passage of the DEAE-dextran material revealed the presence of small plaques (0.5 mm) at the 10^-2 dilution. One of these plaques was isolated and subsequently passaged in fresh BHK cells. This isolate was designated 400P.

Comparison of the original rabies (LP) virus and the 400th passage isolate (400P)

In comparative growth cycles of the two viruses in which BHK cells were infected at an m.o.i. of 3, the LP strain gave maximum titres of 500 p.f.u./cell 4 to 5 days after infection. The 400P isolate gave maximum titres of 4 p.f.u./cell 7 days after infection. In the plaque assay, the LP virus gave plaques of 3 to 4 mm diam. after 5 days, whereas the 400P virus gave 1 mm plaques after 7 days.

Under the conditions of the experiment, both viruses produced a cytocidal infection. The 400P virus did not have a greater tendency to initiate persistent infections than the LP strain. The pathogenicity of the two viruses was compared in suckling mice. Tenfold dilutions of the viruses were inoculated intracerebrally into suckling mice (Table 2). Mice inoculated with the LP strain died between the 5th and 14th day depending upon the amount of virus inoculated. There was a direct correlation between the virus titre obtained in mice and by the plaque assay on BHK cells. In contrast, the 400P virus did not kill suckling mice even with inoculations as high as 2 x 10^4 p.f.u./mouse.

In order to compare the polypeptide composition of the two viruses, cells infected with either of the viruses were labelled with [35S]methionine and the viruses subsequently purified from the supernatant. The 35S-labelled viruses were analysed by PAGE (Fig. 1). Two of the 400P virus polypeptides, the M1 and the glycoprotein, migrated more slowly in the gels than those of the LP virus. There was also a reduced level of L-polypeptide in the 400P virus. It was estimated that the latter virus contained less than 5% of the level of L polypeptide of the LP strain.
Fig. 1. PAGE analysis of 35S-labelled rabies virus and rabies virus-induced polypeptides. (a) Purified 400P virus; (b) purified LP virus; (c) BHK cells infected with LP virus; (d) BHK cells infected with 400P virus; (e) non-infected cells. The rabies virus antigens were immunoprecipitated from the 35S-labelled samples shown in (c to e) and analysed by PAGE. (f) 400P virus-induced protein; (g) LP virus-induced proteins; (h) uninfected cells.

Fig. 2. Comparison of the 400P virus-induced proteins with those synthesized by the rabies persistently infected cells (420th passage). 400P virus-infected BHK cells and persistently infected BHK cells were labelled with [35S]methionine and the virus proteins extracted, immunoprecipitated and analysed by PAGE. (a) Purified LP virus; (b) purified 400P virus; (c, d) rabies virus persistently infected BHK cells 420th passage; (e) 400P virus-infected cells; (f) LP virus-infected cells.

To confirm these observations, we also examined the virus-induced proteins of the two virus strains. BHK cells were infected with the two viruses at an m.o.i. of 6. Four days later the cells were labelled with [35S]methionine for 2 h and cytoplasmic extracts prepared. The
Fig. 3. Comparison of rabies virus-induced proteins during the evolution of a persistent infection in BHK cells. The BHK-rabies persistently infected cells were labelled with $^{35}$S-methionine at various passage levels. The virus antigens were extracted, immunoprecipitated and analysed by PAGE. (a) LP virus-infected BHK cells. (b to g) Persistently infected cells at passage level 2(b), 4(c), 6(d), 58(e), 164(f) and 450(g). The tract (m) contains the $^{14}$C-marker proteins at 200K, 92.5K, 69K, 46K and 30K.

$^{35}$S-labelled virus antigens were immunoprecipitated from the extracts with an antiserum (guinea-pig) prepared against the purified LP virus. The precipitates were analysed by PAGE (Fig. 1). The modifications of the M1 polypeptide in the purified viruses were also found in the virus-induced proteins. The rabies virus glycoprotein from the cells can be separated into the two proteins designated G1 and G2. Comparison of the two strains revealed that the 400P G1 and G2 polypeptides both had a corresponding increase in mol. wt. in comparison with those of the LP virus. This would suggest that the apparent increase in mol. wt. is not due to
glycosylation. We were unable to measure the levels of L polypeptide in the infected cells as none of our sera precipitated the L polypeptide.

**Comparison of 400P virus and persistently infected cells**

The differences observed in the 400P virus isolate may represent the evolution of the virus population in the persistent infection or, alternatively, we may have isolated a variant not related to the persistent state. To investigate this aspect, rabies virus-induced proteins labelled with $[^{35}\text{S}]$methionine were extracted from BHK cells infected with either the LP or 400P strains or from persistently infected cells at the 420th passage level. The virus proteins were immunoprecipitated and analysed by PAGE (Fig. 2). The virus-induced polypeptides present in the high passage persistently infected cells (420th passage) migrated to the same position as those from the 400P isolate.

**Polypeptide modifications leading to the defective state**

Our observations have shown that during the establishment and maintenance of the persistent state, the rabies virus underwent modifications in at least two proteins, the glycoprotein and M1. It has been observed in persistent infection of hamster cells by measles virus, that mutations can arise in the glycoprotein which affect its integration into the plasma membrane (Fisher & Rapp, 1979). Iodination (with $^{125}\text{I}$) of the surface of the persistently infected cells radiolabelled the virus glycoprotein (not shown) and thus the latter is not defective in its ability to integrate into the membrane.

In the evolution of the persistent infection, the sequence observed was the establishment of persistence, the change from large to small plaque virus and eventually a defective state. As we had found biochemical modifications (in the G and M1 proteins) we attempted to correlate these changes with the biological phenomenon. Cells acutely infected with LP virus or persistently infected cells at passage levels 2, 4, 6, 58, 164 and 450 were labelled with $[^{35}\text{S}]$methionine. Cytoplasmic extracts were prepared and the virus proteins immunoprecipitated and analysed by PAGE (Fig. 3). During the establishment and initial passages of the persistent infection, no changes were observed in the migration of the virus polypeptides. At the 58th passage, i.e. when only small plaque virus was being produced, there was a change in the glycoprotein similar to that found in the 400P virus. By the 164th passage, there was also a change in the M1 polypeptide. This was modified yet again in the defective (450th passage) persistent infection.

**DISCUSSION**

The outcome of a virus–cell interaction is governed by both the virus and its host. Viruses which inhibit host cell metabolism generally produce cytocidal infections, whereas viruses which do not inhibit host cell metabolism more readily give rise to persistent infections. In order to attenuate an otherwise cytocidal infection, the host cell may synthesize interferon or stimulate DI particle production. Certain lines such as L cells readily produce high levels of interferon in response to virus infection (Ramseur & Friedman, 1977) whereas others, such as BHK and Vero cells, synthesize little or none (Kawai et al., 1975; Wild & Dugrè, 1978). The synthesis of DI particles is controlled at both the host and virus level (Holland et al., 1976). Although plaque-purified VSV gives rise to DI particles in most cell lines after several undiluted passages, none is produced in HeLa cells (Holland et al., 1976). In contrast, plaque-purified rabies virus produced DI particles after a single undiluted passage.

Once the persistent state is established, the evolution of the culture probably depends upon whether the state is regulated by interferon or DI particles. Interferon may limit the infection to a small percentage of cells (Meinkoth & Kennedy, 1980), whereas DI particles can give a
cyclic phenomenon (Kawai et al., 1975) or not (Holland & Villarreal, 1974). In our BHK cells persistently infected with rabies, the number of virus antigen-containing cells fluctuated between 5 and 80%. Resistance to superinfection with the homologous virus was dependent on the state of expression of virus antigens in the culture. This would suggest that an equilibrium exists between infected and non-infected cells. This is borne out by the fact that non-infected clones can be isolated from these cultures (Andzhaparidze et al., 1981).

It has been shown for both the VSV–BHK and Semliki Forest virus–L cell systems, that the virus undergoes mutations during the long-term culture in the persistent state (Holland et al., 1979; Meinkoth & Kennedy, 1980). In the case of measles virus, such mutations may or may not lead to changes in the apparent mol. wt. of the structural polypeptides (Wechsler et al., 1979; Wild et al., 1981).

In the present study, we observed that in the establishment of the rabies-BHK persistent infection, there was no modification of the size of the polypeptides. However, with the appearance of the small plaque variant, a change in the migration of the glycoprotein was observed. Further passage led to an alteration in the size of the M1 protein but this was superseded by a further mutation in the same gene on changing to the defective state. The LP virus contained an additional minor band at the M1 level and double bands were consistently found at this level of the acrylamide gels for proteins immunoprecipitated from the persistently infected cells. Cloning of the viruses failed to change the profiles and so the mutations occurring in these viruses may be indicative of altered post-translational modifications. It is evident that the observed changes are not necessarily those responsible for the biological observations, as mutations in the same and other genes are probably occurring simultaneously. However, they do act as markers of the biological state of the persistent infection.

Viruses isolated from long-term persistent cultures grow more slowly and to lower titres than the parent virus (Rowlands et al., 1980). This probably reflects the introduction of less favourable mutations which because of the non-lytic nature of the persistent state, are not eliminated. The present study was no exception, but it was particularly interesting that the virus isolated from the long-term persistent infection had lost its pathogenicity for suckling mice. Andzhaparidze et al. (1981) have recently reported that a Pasteur strain of rabies virus after 50 passages in a persistent culture lost its neurovirulence for adult mice but not suckling mice. However, Fernandes et al. (1964) showed that extracts of CVS (rabies) persistently infected rabbit endothelium cells lost neurovirulence for both newborn and adult mice by the 42nd passage.

The appearance of mutations in an in vitro persistent infection suggests that such a mechanism may occur in nature. This or a related mechanism of persistence may be responsible for the presence of rabies-related and rabies-like viruses in nature.

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REFERENCES


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