Isolation and Characterization of Acyclovir-resistant Mutants of Herpes Simplex Virus

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SUMMARY

Mutants of HSV which are resistant to acyclovir (acycloguanosine) have been isolated following serial passages of several herpes simplex virus (HSV) strains in the presence of the drug. The majority of the mutants isolated are defective in induction of thymidine kinase (TK) and this is consistent with the observation that independently isolated TK− viruses are naturally resistant to ACV. One mutant is described (SC16 R6C9) which is resistant in biochemically transformed cells which express HSV TK. This suggests that its resistance resides at a level other than TK. It is also resistant to phosphonoacetic acid, suggesting that the DNA polymerase locus may be involved. A further mutant is described [Cl (101) P3C9] which induces normal levels of TK, although the nature of resistance of this virus is not yet elucidated.

INTRODUCTION

Acyclovir or acycloguanosine (ACV) is a nucleoside analogue [9-(2-hydroxyethoxy-methyl)-guanine] which is an extremely effective inhibitor of the replication of herpes simplex virus (HSV) in tissue culture cells (Elion et al. 1977; Crumpacker et al. 1979) but which has very low toxicity for uninfected cells (Elion et al. 1977). It has a complex mode of action. The drug is first phosphorylated by virus-specified thymidine kinase (TK) and it is the triphosphate form of the drug which is inhibitory to HSV-specified DNA polymerase (Elion et al. 1977; Fyfe et al. 1978).

Recent studies in animal models (Kaufman et al. 1978; Field et al. 1979; Klein et al. 1979; Park et al. 1979) have suggested that this drug may have considerable therapeutic value for the treatment of HSV infections, and clinical trials with the compound are now underway. One possible problem that may be anticipated is that prolonged use of the drug will result in the generation of resistant virus strains. It is hoped that a satisfactory understanding of the underlying mechanisms of resistance may permit the design of effective alternative chemotherapy.

In this study we have isolated and characterized ACV-resistant viruses following passage of several HSV-t and HSV-2 strains in tissue culture in the presence of the drug. The majority of resistant strains are defective in expression of the TK gene. However, the virulence of such TK− viruses for mice appears to be attenuated (Field & Darby, 1980) and they are therefore at a disadvantage in vivo. We have shown that resistance may develop at other levels in the virus genome and one mutant is described which expresses normal levels of TK. Such TK− resistant viruses would be expected to be more successful in vivo and therefore deserve particular attention.
METHODS

Cells and tissue culture. Several continuous cell lines were used: baby hamster kidney (BHK21); African green monkey kidney (VERO), BrdUrd-resistant BHK (BU-BHK) which is a line derived from BHK21 by passage in the presence of BrdUrd and which fails to express TK activity; LMTK- and D21 cells, a line derived from LMTK- cells by biochemical transformation with fragments of HSV-2 DNA (Minson et al. 1978). These cells express the HSV-2 TK gene. Both TK- cell lines were obtained from Dr M. Thouless.

Cell lines were maintained in Glasgow-modified Eagle's medium supplemented with 10% calf serum and 10% tryptose phosphate broth (ETC). Selective medium containing methotrexate (Minson et al. 1978) was used for passage of D21 cells.

Mouse embryo cells were prepared from BALB/c embryos taken in the second week of gestation. These were minced, washed in buffered saline and trypsinized in conventional fashion. They were cultured in ETC (using foetal calf serum). After 3 days they were passaged and after a further 3 days removed from the culture vessels and plated in 5 cm diam. plastic Petri dishes.

Human amnion cells were a gift from Dr J. Nagington (Public Health Laboratory Service, Cambridge). They were derived from a normal amnion obtained at delivery. The cells were trypsinized and were used at the second passage.

Virus strains. The HSV-I strains used were SCI6 (Hill et al. 1975), CI (101) (Dubbs & Kit, 1964) and the HSV-2 strain was Bry (obtained from Dr G. R. B. Skinner, Department of Medical Microbiology, Birmingham). CI (101) TK- and Bry TK- were derived from their respective parents by passage in the presence of BrdUrd (Dubbs & Kit, 1964; Thouless, 1972) and neither expresses TK activity. Virus stocks were prepared in BHK21 cells using low multiplicity infections.

Isolation of resistant mutants. Viruses resistant to ACV were isolated by passage of the parental viruses at low multiplicity in BHK21 cells in the presence of the drug. The medium used was Glasgow-modified Eagle's medium supplemented with 2% calf serum (EC2) with the required concentration of ACV added.

Following serial passage of virus in the presence of ACV, clones were isolated from the resistant population. Virus was inoculated on to confluent monolayers of BHK21 cells in 96-place multiwell trays at an input multiplicity of approx. 0.1 p.f.u./well. After 2 days, each well was examined microscopically and any containing a single well-defined plaque was marked. After a further 2 days' incubation the contents of each marked well were used to inoculate a monolayer of BHK cells in a 25 cm² plastic tissue culture flask. The medium used for the cloning was EC2 with 1 µg/ml ACV added.

Measurement of virus sensitivity to ACV.

Plaque reduction assay. Monolayers of cells in 5 cm plastic Petri dishes were inoculated with 100 p.f.u./dish of virus in 0.2 ml EC2. After 1 h to permit adsorption, 5 ml of EC2 (or EC10) containing 10% carboxymethylcellulose and the required concentration of ACV were added. Plaques were allowed to develop for 2 to 3 days, depending upon cell type, and the cells were then fixed and stained. To obtain the ED50 dose the reduction in plaque number was plotted against log (drug concentration in µg/ml) and the dose required for a 50% plaque reduction was read directly from the graph.

Assay by reduction in virus yield. Monolayers of cells in 5 cm plastic Petri dishes were inoculated with 5 p.f.u./cell of virus in 0.2 ml EC2 with the required amount of ACV added. After 1 h adsorption the inoculum was removed and 5 ml EC2 (or EC10) + ACV was added. Twenty-four hours later the cells were harvested into the overlying medium, the cells were disrupted by ultrasonic vibration and the total virus yield was measured. The
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ED$_{50}$ dose was determined by plotting the reduction in virus yield against log(drug concentration, $\mu$g/ml) and the dose required to give a 50% reduction yield was read from the graph.

*Induction of thymidine kinase activity.* To test the ability of viruses to induce TK, confluent monolayers of BU-BHK cells in 5 cm plastic Petri dishes were infected at a multiplicity of 5 p.f.u./cell. After 18 h the cells were washed once with phosphate-buffered saline and then 0.01 M-tris buffer, pH 7.4 (1 ml), was added. The cells were harvested, disrupted by ultrasonic vibration and then assayed as described by Klemperer et al. (1967).

*Sensitivity of viruses to phosphonoacetic acid.* The sensitivity of a virus to phosphonoacetic acid (PAA) was assessed by measuring the reduction in virus yield in the presence of 100 $\mu$g/ml of the drug. BHK$_{21}$ cells were infected with 10 p.f.u./cell of virus in EC$_{10}$. After 1 h the cells were washed thoroughly with PBS three times and then EC$_{10}$+ 100 $\mu$g/ml PAA was added. After 24 h the cells and overlying medium were frozen at $-20^\circ$C. The samples were thawed, the cells disrupted by ultrasonic vibration and the total virus yield was assayed. Controls were identical except that PAA was omitted from the medium.

An alternative procedure was to use the plaque reduction technique described above for measurement of ACV sensitivity. For this assay the medium used was ETC with the required concentration of PAA added.

*Sensitivity of cells to ACV.* Cells were tested for sensitivity to ACV in 3-day growth experiments. Cells ($10^5$) were seeded into 5 cm dishes in ETC and incubated overnight at 37 $^\circ$C. Sample dishes were harvested and the cells were counted at that time. The medium on the remaining dishes was changed to EC$_{6}$ containing ACV. The yields of cells after 3 days' incubation were measured and the ED$_{50}$ dose was the quantity of drug which reduced the yield to 50% of the control with no drug added.

**RESULTS**

*Isolation of resistant viruses*

Following several independent series of passages of SC16 at low multiplicity in the presence of ACV, three resistant SC16 clones were obtained. SC16 RsC$_1$ was obtained after a single passage in the presence of 5 $\mu$g/ml ACV, SC16 RsC$_4$ after passage three times in 7 $\mu$g/ml and once in 10 $\mu$g/ml ACV, and finally SC16 RsC$_8$ was obtained after a more extended passage series, twice in 1 $\mu$g/ml, twice in 5 $\mu$g/ml and six times in 10 $\mu$g/ml ACV.

Cl (101) and Bry were each passaged twice in the presence of 10 $\mu$g/ml ACV and resistant clones were obtained from each yield [Cl (101) P$_3$C$_6$, Bry P$_3$C$_1$ and Bry P$_9$C$_2$].

Cl (101) TK$^-$ was passaged seven times in 10 $\mu$g/ml ACV. The resulting isolate, Cl (101) TK$^-$ p7, was not cloned.

*Sensitivity of viruses to ACV*

BHK$_{21}$, VERO, BU-BHK, LMTK$^-$, mouse embryo cells and human amnion cells were used to examine the sensitivities of the parent viruses to ACV, and also the resistances of the derived clones and independently isolated TK$^-$ viruses. The data obtained using the plaque reduction method are summarized in Table 1. Several important points emerged from these experiments.

Obviously valid comparisons can only be made when viruses are evaluated in the same cell line. Although overall patterns of resistance are similar, the absolute ED$_{50}$ doses vary from one line to another. For example, the ED$_{50}$ dose for Cl (101) TK$^-$ in LMTK$^-$ cells is 1.3 $\mu$g/ml, but in VERO cells it is 20-fold greater and in human amnion cells at least 70-fold greater. One possible explanation for this observation is that uninfected cells
Table 1. ED\textsubscript{50} doses of ACV\textsuperscript{*}

<table>
<thead>
<tr>
<th>Cell type</th>
<th>ED\textsubscript{50} ((\mu)g/ml)</th>
<th>Cell type</th>
<th>ED\textsubscript{50} ((\mu)g/ml)</th>
<th>Cell type</th>
<th>ED\textsubscript{50} ((\mu)g/ml)</th>
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<th>ED\textsubscript{50} ((\mu)g/ml)</th>
<th>Cell type</th>
<th>ED\textsubscript{50} ((\mu)g/ml)</th>
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<tbody>
<tr>
<td>VERO</td>
<td>50</td>
<td>N.D.</td>
<td>&lt; 0.05</td>
<td>N.D.</td>
<td>0.05</td>
<td>N.D.</td>
<td>0.05</td>
<td>N.D.</td>
<td>0.05</td>
</tr>
<tr>
<td>BHK</td>
<td>10</td>
<td>N.D.</td>
<td>2.3</td>
<td>N.D.</td>
<td>1.4</td>
<td>N.D.</td>
<td>1.4</td>
<td>N.D.</td>
<td>1.4</td>
</tr>
<tr>
<td>BU-BHK</td>
<td>N.D.</td>
<td>0.05</td>
<td>3.0</td>
<td>N.D.</td>
<td>1.5</td>
<td>N.D.</td>
<td>1.5</td>
<td>N.D.</td>
<td>1.5</td>
</tr>
<tr>
<td>LMTK\textsuperscript{-}</td>
<td>10</td>
<td>N.D.</td>
<td>&gt; 40</td>
<td>N.D.</td>
<td>&gt; 40</td>
<td>N.D.</td>
<td>&gt; 40</td>
<td>N.D.</td>
<td>&gt; 40</td>
</tr>
<tr>
<td>Mouse embryo</td>
<td>&gt; 40</td>
<td>N.D.</td>
<td>&gt; 40</td>
<td>N.D.</td>
<td>&gt; 40</td>
<td>N.D.</td>
<td>&gt; 40</td>
<td>N.D.</td>
<td>&gt; 40</td>
</tr>
<tr>
<td>Human amnion</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* Sensitivities of HSV strains to ACV were measured by plaque reduction assay in six cell types. The resistances of the cells, measured by a growth inhibition test, are shown in the second column (Cell ED\textsubscript{50}). All values are the means of at least three determinations. For any virus cell combination the variation in individual values was never more than twofold.

\textsuperscript{†} Not done.
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are able to phosphorylate small quantities of ACV and the level of resistance of the TK− viruses reflects the level of phosphorylated ACV in the cells. This idea was supported to some extent by an examination of the sensitivity of various cell lines (also shown in Table 1) which indicated that BHK21 cells are correspondingly more sensitive to ACV (ED50 10 µg/ml) than VERO cells (ED50 50 µg/ml) and hence may phosphorylate the drug more efficiently. If this explanation is correct, it is unlikely to be the cellular TK that is responsible for phosphorylating the drug, since TK− virus strains are of similar resistance to ACV in BHK21 and in BU-BHK cells which contain no detectable TK activity.

The data in Table 1 conform with the observation of Crumpacker et al. (1979) that independent virus isolates show marked differences in their sensitivities to ACV. Thus the assessment of whether a virus has acquired resistance to the drug involves comparison with its parent strain.

One difficulty in the measurement of ED50 values by plaque reduction was that, as the drug concentration was increased, the size of plaque diminished, and this made plaque titrations more difficult. Therefore the effect of drug concentration on virus replication was also evaluated by the growth inhibition assay. The strains CI (101) and CI (101) TK− were tested in both BHK21 and VERO cells, and the results showed that the drug doses required to produce 50% decreases in yield were similar to the ED50 values measured by plaque reduction.

Induction of thymidine kinase

The ACV-resistant clones were tested for their ability to induce TK activity following infection of BU-BHK cells. These cells were chosen because they express extremely low levels of TK activity themselves, thus permitting the detection of quite small amounts of induced enzyme. The results are shown in Fig. 1. Parent viruses and the independently derived TK− viruses [CI (101) TK− and Bry TK−] which have been shown by others to express no detectable TK (Dubbs & Kit, 1964; Summers et al. 1975; Thouless & Wildy, 1975), were included as controls. There was a suggestion that CI (101) and Bry induced somewhat lower levels of activity than SC16 and, as expected, CI (101) TK− and Bry TK− showed no detectable increase in kinase activity.

Several of the ACV-resistant clones had properties similar to independently isolated TK− viruses in that there was no significant increase in kinase activity following infection. These were the SC16-derived strain R9C4, and the Bry-derived strains P9C1 and P9C8. However SC16 R9C1 and SC16 R9C2 expressed low but significant kinase levels (see Fig. 1b), although there was no direct evidence that the enzymes involved were specified by the viruses. CI (101) P9C6 was remarkable in that it induced normal levels of TK (Fig. 1d).

Our conclusion from these data was that the majority of ACV-resistant isolates derived by passage in the presence of the drug were severely handicapped as far as expression of TK was concerned and hence their resistance reflected, at least in part, their failure to phosphorylate the drug in the infected cell. However, the results with CI (101) P9C6 suggested the possibility that resistance might arise at an alternative locus.

When the data on the induction of thymidine kinase activity are considered along with the data in Table 1 there are several other indications that resistance to ACV may develop at loci distinct from those involved in expression of TK. Firstly, the clone derived from SC16, SC16 R9C2, is more resistant in all cell lines than the TK− clone, SC16 R9C1, derived from the same parental virus. This was in spite of the observation that SC16 R9C2 induced a low but significant level of TK activity. Secondly, perhaps the strongest indication was that CI (101) TK−, which was itself naturally resistant to ACV, developed increased
Fig. 1. Induction of TK. The viruses were tested for their ability to induce thymidine kinase activity in BU-BHK cells. The assays were performed as described in Methods. (a) The activity induced by SC16 (●) was compared with that induced by the mutants derived from it (SC16 R9C2, SC16 RsC1 and SC16 R1C0). The data for all three mutants are contained within the cross-hatched area. These data are shown in expanded form in (b) where the viruses are SC16 R9C2 (□), SC16 RsC1 (○) and SC16 R1C0 (■). (c) BryTK- (●) was compared with three mutants derived from it: Bry TK-, BryP9C1 and Bry P9C2. The data for the mutants (■) were indistinguishable. (d) The parent virus, C1 (101) (●) was compared with C1 (101)TK- (■) and the ACV-resistant mutant C1 (101) P9C3 (○).

resistance to the drug when passaged extensively in its presence. As C1 (101) TK- induces no TK activity it is reasonable to assume that the increased resistance seen in C1 (101) TK- p7 had arisen at a different locus.

Sensitivity to ACV in D21 cells

We have previously used the biochemically transformed cell line D21, in which HSV-specific TK is expressed, to decide whether viruses induce DNA polymerases which are sensitive to phosphorylated ACV (Darby et al. 1980). This technique may also be applied to TK- resistant virus isolates since there is no requirement in this system for TK induction. The phosphorylation of the drug by the virus enzyme present in the cells ensures that a virus is resistant in these cells only if it has a resistant polymerase.

The value of D21 cells in revealing resistance at loci other than TK is illustrated by comparison of the sensitivities of C1 (101), C1 (101) TK- and C1 (101) TK- p7 (see Fig. 2a). Whereas in all other cells used C1 (101) TK- showed increased resistance to ACV relative to the parental strain C1 (101) (see Table 1), in D21 cells the viruses were equally sensitive. This confirmed that the virus-specific TK gene in D21 cells can complement defects in
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Fig. 2. Virus sensitivities to ACV in D21 cells. The sensitivities of several viruses to ACV were examined in D21 cells by a plaque reduction assay. (a) C1 (101) (●) was compared with C1 (101) TK− (○), C1 (101)P2C5 (□) and a mutant derived from C1 (101)TK−, C1 (101)TK−p7 (■). (b) SC16 (●) was compared with the ACV-resistant mutants SC16 R1C1 (○), SC16 R5C1 (□) and SC16 R6C6 (■). The percentage reduction in plaque number is plotted against the drug concentration on a log scale.

kinase expression in an infecting virus, conferring on that virus sensitivity to the drug. Furthermore, C1 (101) TK− p7 was less sensitive to the drug in D21 cells than C1 (101) TK− suggesting resistance at a locus which could not be complemented by the TK expressed in D21.

Similar experiments on SC16 and clones derived from it showed that SC16 R1C1 and SC16 R6C1 were as sensitive as the parental virus from which they were derived, but SC16 R6C6 had increased resistance (see Fig. 2b). The interpretation of these data is that SC16 R1C1 and SC16 R6C1 are resistant to ACV solely at the level of TK expression but that SC16 R6C6 has developed resistance at another locus not involved in expression of TK, probably DNA polymerase.

The data with C1 (101) P2C5, which induces apparently normal TK levels, are less clear. It has slightly increased resistance in comparison with the parent in D21 cells, suggesting some involvement of the polymerase locus, but this must be considered in the light
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Table 2. Resistance of viruses to PAA*

<table>
<thead>
<tr>
<th>Virus</th>
<th>% Yield in 100 μg/ml PAA</th>
<th>ED₅₀ (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
<td>Expt. 2</td>
</tr>
<tr>
<td>SCI6</td>
<td>1.5</td>
<td>6.0</td>
</tr>
<tr>
<td>SCI6 R₃C₁</td>
<td>3.8</td>
<td>6.0</td>
</tr>
<tr>
<td>SCI6 R₅C₁</td>
<td>0.6</td>
<td>5.3</td>
</tr>
<tr>
<td>SCI6 R₅C₅</td>
<td>50.0</td>
<td>100.0</td>
</tr>
<tr>
<td>CI (101)</td>
<td>4.7</td>
<td>7.0</td>
</tr>
<tr>
<td>CI (101) TK⁻</td>
<td>0.3</td>
<td>2.0</td>
</tr>
<tr>
<td>CI (101) TK⁻₇</td>
<td>N.D.†</td>
<td>67.0</td>
</tr>
<tr>
<td>CI (101) P₅C₅</td>
<td>0.2</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* Sensitivities of HSV strains to PAA were measured either by examining the reduction of virus yield in the presence of 100 μg/ml PAA (Expt. 1 and 2), or by plaque reduction assay (ED₅₀, μg/ml). All experiments were in BHK₂₁ cells.
† Not done.

of its high resistance in other cell lines (Table 1). It is conceivable that the TK induced by this virus has a modified substrate specificity such that it is unable to phosphorylate ACV although it retains the capacity to phosphorylate thymidine.

One further approach which proved useful in revealing alterations in polymerase substrate specificity was suggested by the observations of ourselves and others (D. M. Coen and P. A. Schaffer, personal communication) that viruses which have been isolated in the presence of PAA and are resistant to that drug, are also frequently resistant to ACV. The simplest interpretation of these observations is that PAA permits selection of mutants with altered DNA polymerase specificity and that such an alteration may at the same time confer ACV-resistance. It was considered possible that the converse might also occur, where alterations in polymerase under pressure from ACV would confer resistance to PAA. We therefore examined ACV-resistant viruses for their PAA-sensitivity.

Resistance of viruses to phosphonoacetic acid

Experiments on the sensitivity of the viruses to PAA are shown in Table 2. The sensitivities were assessed both by plaque reduction and by growth inhibition. Two of the viruses examined were resistant to PAA. These were SCI6 R₅C₅ and CI (101) TK⁻₇. Both were suspected of having altered polymerase specificity on the basis of their resistances to ACV in D₂₁ cells and these results added confirmation. The parental viruses [SCI6 and CI (101)], the independently derived TK⁻ viruses [CI (101) TK⁻ and Bry TK⁻] and the resistant viruses which were believed to have lesions only in TK (SCI6 R₅C₁, SCI6 R₅C₅, Bry P₅C₁ and Bry P₅C₅) were all sensitive to PAA. The TK⁺, ACV-resistant isolate CI (101) P₅C₅ was also sensitive to PAA in both assays.

DISCUSSION

Since ACV is a drug which is already in use for the treatment of herpes simplex-mediated diseases of humans, it is clearly important to characterize resistant virus variants which may arise under the influence of the drug. Studies on the mode of action of the drug (Elion et al. 1977) have pointed to two important loci at which resistance might develop. As the drug must be phosphorylated by the virus-induced TK, loss or alteration of this function could result in resistance. In addition, the phosphorylated compound inhibits the action of the virus-specified DNA polymerase and so mutation at that locus might also result in resistance.
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In this study we have described the isolation of resistant viruses following serial passage in the presence of the drug in tissue culture. However, with the single exception of Cl (101) P₄C₅, all isolates were defective in TK expression. In retrospect this is probably the result that we would have expected from a consideration of the loci involved in ACV-sensitivity. In actively dividing tissue culture cells TK is not essential for virus replication (Dubbs & Kit, 1964) and so mutations which result in complete loss of gene function would confer resistance. In contrast, DNA polymerase is essential for virus replication under all circumstances and this places considerable restraint on the mutations which can occur in this function.

In view of these considerations it was perhaps not surprising that viruses which had acquired resistance at the level of polymerase were isolated comparatively rarely relative to those with lesions in TK.

One virus isolate (SCI6 R₉C₂) which was defective in TK expression had acquired resistance to PAA and showed increased resistance relative to its parent in D₂₁ cells. Similarly, the independently isolated TK⁻ virus [Cl (101) TK⁻], when passaged extensively in the presence of the drug, acquired enhanced resistance in all cells (including D₂₁) and also developed resistance to PAA. Resistance in D₂₁ cells, which can complement defects in TK expression, is a clear indication of involvement of a locus not involved in the induction of TK, and the acquisition of PAA-resistance points to modification at the DNA polymerase locus (Honess & Watson, 1977).

One problem which has not been resolved satisfactorily is the nature of the resistance acquired by Cl (101) P₄C₅. This virus induces normal TK levels but is highly resistant to ACV. It has slightly enhanced resistance in D₂₁ cells but it is sensitive to PAA. It is possible that there has been a modification of the polymerase substrate-specificity which has conferred resistance to phosphorylated ACV but which has not altered its PAA sensitivity. However, a further possibility is that there has been a change in TK specificity such that the enzyme no longer recognizes ACV as a substrate. This would account for the considerable resistance observed in BHK2₁ and VERO cells.

There are reasons to suppose that the isolation of resistant viruses which are defective in TK expression may be an artifact of the tissue culture systems employed and such viruses may not readily emerge in vivo during treatment with the drug. We have previously established (Field & Wildy, 1978) that BrdUrd-derived TK-deficient mutants have considerably reduced pathogenicity for mice and replicate poorly in comparison with their parents both in skin and in neurological tissues. Therefore there appears to be a natural selection system operating against the establishment of such viruses in vivo. The situation with viruses which express low levels of TK (such as SCI6 R₉C₂) is less clear-cut since although they appear to replicate poorly in cells of the nervous system, they multiply rather better than the TK⁻ viruses in the skin (Field & Darby, 1980). So far, our attempts to isolate resistant viruses from laboratory inoculated animals which have been treated with ACV have been unsuccessful. Low levels of virus replication have been observed in the tissues of mice infected with several of the parent isolates and undergoing treatment with ACV. However, when yields of virus obtained from the tissues of these mice were examined for in vitro sensitivity to ACV there was no evidence of increased resistance to the drug (Field et al. 1979; Field & Darby, 1980). Studies on the pathogenicity of the mutants described here have shown that the ACV-resistant viruses that are TK-deficient have reduced neurovirulence in mice, thus supporting the arguments above. The most virulent of our isolates is Cl (101) P₄C₅, which expresses normal TK levels.

Recently we have used serum starved cells in which there is a positive selection pressure on viruses to express TK activity (Jamieson et al. 1974) and we have isolated resistant
mutants from these cells following passage in ACV. Early indications show that the majority, but not all, of the resistant isolates induce TK activity. We therefore hope that this system parallels more closely the situation in natural infections and we are now attempting to define the nature of the resistance to ACV of these viruses. Their pathogenicity in mice is also under investigation.

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