Characterization of a neurovirulent aciclovir-resistant variant of herpes simplex virus

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A clinical isolate of herpes simplex virus type 1 that is aciclovir resistant but neurovirulent in mice was described previously. The mutation in this virus is a double G insertion in a run of seven G residues that has been shown previously to be a mutational hotspot. Using a sensitive assay, it has been demonstrated that preparations of this virus are able to induce low but consistent levels of thymidine kinase (TK) activity. However, this activity results from a high frequency mutational event that inserts a further G into the ‘G-string’ motif and thus restores the TK open reading frame. Passage of this virus through the nervous system of mice results in the rapid selection of the TK-positive variant. Thus, this variant is the major component in virus reactivated from latently infected ganglia. Mutation frequency appears to be influenced by the genetic background of the virus.

INTRODUCTION

Aciclovir (ACV) is widely used for the treatment and prophylaxis of herpes simplex virus (HSV) infections in immunocompromised patients (O’Brian & Campoli-Richards, 1989). Such patients frequently experience more severe and prolonged HSV disease than immunocompetent individuals and the extended treatment required to manage the disease may, in some cases, lead to the appearance of ACV-resistant variants (Sibrack et al., 1982a; Wade et al., 1982; Erlich et al., 1989; Nugier et al., 1992; Christophers et al., 1998; E. Kern, International Task Force on Herpesvirus Resistance, personal communication). Resistance can result from mutation in either the thymidine kinase (TK) gene or the DNA polymerase gene. However, it results most commonly from a failure to express (or expression of low levels of) TK (TK deficiency), a consequence of mutation in the TK gene (Coen & Schaffer, 1980; Collins & Darby, 1991; Hill et al., 1991).

It is generally accepted that TK-deficient viruses have low neurovirulence (Field & Darby, 1980; Field & Wildy, 1978; Tenser et al., 1981; Sibrack et al., 1982b) and are unable to reactivate from latency (Tenser & Dunstan, 1979; Coen et al., 1989; Efthathiou et al., 1989; Jacobson et al., 1993). Periodically, however, reports have emerged that describe individual TK-deficient isolates which appear to challenge these principles, i.e. they can be recovered, albeit somewhat inefficiently, from latently infected animals (Sakuma et al., 1988; Erlich et al., 1989; Kost et al., 1993; Sasadeusz et al., 1997; Horsburgh et al., 1998). A possible explanation is that these isolates induce low levels of TK, which accounts for their neurovirulence, either because they are heterogeneous and contain a small proportion of wild-type virus or because the mutant gene permits expression of limited TK activity (Hwang et al., 1994). A more intriguing possibility is that, in some cases, other gene products have the potential to complement TK function and influence the ability of HSV to replicate in the nervous system and to reactivate from latency (Horsburgh et al., 1998).

In a study of ACV-resistant clinical isolates, collected in Europe between 1980 and 1995 (Harris et al., 2003), we confirmed that viruses with mutations that disrupt the TK region are generally attenuated with respect to their ability to replicate in the nervous system, with no evidence of zosteriform spread in infected animals. Furthermore, consistent with previous observations (Horsburgh et al., 1998; Hwang et al., 1994; Sasadeusz et al., 1997), it was shown that variants with the potential to express minimal levels of TK can be recovered, albeit inefficiently, from cultured ganglia. These include isolates with single G insertions in the ‘G-string’ (Fig. 1), in which translational frame-shifts (+1) during protein synthesis are thought to result in the expression of a low level of wild-type polypeptide (Hwang et al., 1994), and those with amino acid substitutions, where the mutated polypeptides retain minimal activity.

Data from Harris et al. (2003) show that there are degrees of

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The wild-type HSV-1 TK polypeptide of 376 aa is shown as a reference. The characteristic of seven G residues, which has been identified previously as a mutational hotspot (G-string), is also indicated. Numbers in brackets indicate the amino acid residue position relative to N terminus of the polypeptide. Beneath this schematic representation are representations of the theoretical TK polypeptides that would be produced from the ACV-resistant viruses C4a, C4b, an HSV-1 SC16 double G insertion mutant and the TK deletion mutant DM21. The representations are based on the sequence analysis of the TK gene and in each case the possible effects caused by the mutation are indicated. Open boxes indicate frame-shifts, and premature stop codons are indicated by the position at which the TK polypeptide is truncated.

METHODS

Cells. Vero (African green monkey kidney) cells were grown in GMEM supplemented with 10% foetal calf serum (FCS), 100 units penicillin G ml\(^{-1}\), 100 μg streptomycin ml\(^{-1}\) and 0-01 mM glutamine.

BuBHK cells are derived from baby hamster kidney cells (BHK) and are devoid of cellular TK activity. They were generated by passage in the presence of 5-bromo-2'-deoxyuridine and were grown under the conditions stated above.

All cells were grown, and assays performed, at 37 °C in 5% CO\(_2\).

HSV clinical isolates and reference laboratory strains. The ACV-resistant, TK-deficient HSV-1 clinical isolate strain 4 was obtained from an immunocompromised patient who failed to respond to ACV treatment. The isolate was plaque-purified twice by limiting dilution in Vero cells. Two clones, C4a and C4b, were selected for further analysis. Sequence analysis (Harris et al., 2003) had shown that the clones carried identical TK genes, each with a GG insertion in the G-string motif. One of these clones, C4b, was used in the studies described in this paper.

The recombinant virus, SC16 GG, was constructed by inserting two G residues (GG) into the G-string motif of the SC16 TK gene, which was cloned into the HindIII and Xhol sites of the plasmid pcDNA3 (Invitrogen) using the Exsite Mutagenesis kit (Promega). The integrity of recombinant plasmids was confirmed by appropriate restriction enzyme digestion and by sequencing. Plasmids were co-transfected into Vero cells with infectious SC16 DNA and overlaid with medium containing 2-5 mM ACV. Resistant plaques carrying the GG insertion in TK were plaque-purified twice and the presence of the mutation was then confirmed by sequencing.

To construct the TK-disrupted recombinant virus C4bgfp, the plasmid pAR29 (a gift from C. Preston, MRC Institute of Virology, Glasgow, UK) was used. This plasmid comprises the BamHI P fragment of HSV-1 (nt 45 055–48 634) containing the human cytomegalovirus (HCMV) immediate-early promoter driving expression of the green fluorescent protein (GFP) gene inserted in the Srd site of the TK gene (nt 47 359). GFP is transcribed anti-sense in relation to the TK gene. Recombinant virus was generated by co-transfection into Vero cells of 10 μg clone C4b virus-infected cell DNA with 3 μg of linearized plasmid DNA by the CaCl\(_2\)–DMSO boost method (Stow & Wilkie, 1976). Progeny virus was recovered by limiting dilution and selection of GFP-positive recombinant viruses. The expected genomic structure was verified by Southern hybridization analysis (data not shown).

HSV-1 strain SC16 (Hill et al., 1975) was used as the reference type 1 strain. The deletion mutant, DM21, derived from it (Efstathiou et al., 1989), which carries an 816 bp deletion in TK, was used as the control TK-negative strain.

The characteristics of these viruses are outlined in Table 1.

Assessment of TK activity.

Preparation of enzyme extracts. TK-negative BuBHK cells (4 × 10\(^7\)) were infected at an m.o.i. of 10 p.f.u. per cell and harvested 18 h post-infection (p.i.). Pelleted cells were washed twice in 0-01 M Tris
Table 1. TK Characteristics of clinical isolates, clones and control viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>TK mutation</th>
<th>TK polypeptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC16</td>
<td>Wild-type</td>
<td>Wild-type (364 aa)</td>
</tr>
<tr>
<td>Clone C4b</td>
<td>GG insertion in G-string</td>
<td>-1 Frame-shift and truncation (182 aa)</td>
</tr>
<tr>
<td>SC16 GG</td>
<td>GG insertion in G-string</td>
<td>-1 frame-shift and truncation (182 aa)</td>
</tr>
<tr>
<td>SC16 DM21</td>
<td>816 bp deletion</td>
<td></td>
</tr>
</tbody>
</table>

(pH 7.5) and resuspended in 3 ml 0·01 M Tris (pH 7.5). Cells were then sonicated for 2 min on ice. Supernatants were obtained by centrifuging at 100 000 g for 30 min at 4 °C and aliquots stored at −70 °C.

Enzyme assays. A 50 μl sample of protein extract was added to 200 μl of reaction mixture containing 6 mM ATP, 2 mM disodium orthophosphate, 18 mM sodium orthophosphate, 6 mM MgCl₂ and 0·4 μCi [¹⁴C]thymidine (sp. act. 40–60 mCi per mmol; 1·48–2·22 GBq per mmol), and incubated at 37 °C. Samples were taken at 10 and 20 min and heated at 100 °C for 2 min to stop the reaction. Samples were spun in a Heraeus Biofuge at 13 000 r.p.m. for 2 min, then duplicates of 50 μl samples were spotted onto DEAE filter paper and allowed to dry. Filter papers were washed three times for 20 min with 1 mM ammonium formate. The incorporation of [¹⁴C]thymidine was then quantified by liquid scintillation counting.

Genotype analysis. Sequencing was performed by the cycle sequencing of PCR-generated products from infected cell DNA using 12 overlapping sense and anti-sense internal primers to encompass the entire TK open reading frame. Sequence reactions were carried out using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit and electrophoresis on an ABI 377 sequencer, according to the manufacturer’s instructions. The resulting sequence was analysed using Lasergene software (DNASTAR).

In vivo analysis. The left ear pinnae of anaesthetized 4-week-old BALB/c mice were infected by inoculating 10 μl of virus suspension (containing 5 × 10⁶ p.f.u.) onto an area scarified previously with a 27 gauge needle. Mice were sacrificed at days 3 and 5 p.i. and the inoculated ear and dorsal root ganglia C2, C3 and C4 were removed and homogenized in 500 μl GMEM supplemented with 10% FCS, 100 units penicillin G ml⁻¹, 100 μg streptomycin ml⁻¹ and 0·01 mM glutamine. For latency reactivation assays, mice were sacrificed at least 30 days p.i. and the dorsal root ganglia C2, C3 and C4 were removed into GMEM supplemented with 10% FCS, 100 units penicillin G ml⁻¹, 100 μg streptomycin ml⁻¹ and 0·01 mM glutamine. Ganglia were incubated at 37 °C in a 5% CO₂ humidified atmosphere for 72 h before being homogenized and assayed for infectious virus on Vero cells.

RESULTS

Expression of TK
Initially, the neurovirulent clone C4b was assessed using a TK assay to determine whether the expression of functional TK activity could be detected. The results in Fig. 2(a) show high levels of TK activity in extracts of cells infected with SC16. Extracts from mock-infected cells or cells infected with either clone C4b or DM21 appear to lack significant levels of TK activity. However, when the results of clone C4b, DM21 and mock-infected cell extracts are examined in

Fig. 2. TK assays. (a) Comparison of TK-positive SC16, TK-negative DM21, C4b and mock-infected cell extracts. (b) Comparison of TK-deficient viruses showing higher levels of TK activity in extracts of cells infected with C4b in comparison to DM21-infected cell extracts. (c) Comparison of TK activity of the recombinant virus C4bgfp, DM21, C4b and mock-infected cell extracts. These data show that insertion of the GFP cassette into the TK region of C4b disrupted the low level of TK activity associated normally with this clinical isolate.
more detail, clone C4b is found to express a low level of TK activity (\(<1\%\) of the activity of the wild-type control, SC16) (Fig. 2b). This result has been shown to be reproducible in several independent assays (data not shown).

To determine whether the TK open reading frame was required for low level expression of TK or whether this functionality was provided by a compensating gene function (Horsburgh et al., 1998), the TK open reading frame was interrupted by insertion of an HCMV GFP gene cassette. The data in Fig. 2(c) show clearly that disruption of the TK gene leads to a loss of the low level of TK activity observed consistently with extracts from cells infected with clone C4b.

**In vivo characteristics of clone C4b**

To characterize further the phenotype of clone C4b, a detailed *in vivo* analysis was carried out. The *in vivo* characteristics of clone C4b were compared with SC16 and the TK deletion mutant DM21. Mice were infected with \(5 \times 10^6\) p.f.u. of virus via scarification of the ear pinnae. At days 3 and 5 p.i., the ears and ganglia of infected mice were taken and assayed for the presence of infectious virus. Infected ganglia were also explanted 34 days p.i. to examine virus reactivation from latency (Fig. 3a). Virus titres in the ear at days 3 and 5 indicated efficient replication of all three viruses, although there may be a slight attenuation of DM21 at day 3 and both clone C4b and DM21 at day 5 compared with SC16. The attenuation of DM21 is far more pronounced in the ganglia of infected mice at both days 3 and 5. Clone C4b is also attenuated in its ability to replicate in the ganglia at both days when compared with SC16. However, clone C4b was able to replicate with greater efficiency than DM21 on both days and was able to reactivate from a latent state with an efficiency equivalent to that of SC16. In contrast, no infectious virus was detected in explanted ganglia from mice infected with DM21.

The ability of the virus to establish a latent infection in the ganglia of mice was also investigated by *in situ* hybridization. Ganglia were taken 34 days p.i. and then fixed and sectioned. Sections were then probed for the presence of the major latency-associated transcript (LAT); the LAT transcript is found in neuronal cells that harbour a latent infection. The results of *in situ* hybridization, as presented in Table 2, show that although clone C4b is less efficient than SC16 in establishing a latent infection, it is still more efficient than DM21.

It has been suggested previously that low levels of TK activity and the ability to replicate in the peripheral nervous system can be due to heterogeneity caused by low levels of parental wild-type virus being present in the sample. To rule out this possibility, clone C4b was subcloned further. Two subclones of C4b, PP1 and PP2, were analysed to assess their *in vivo* characteristics (Fig. 3b). Results showed that both subclones behaved in a manner similar to clone C4b, both showing an ability to replicate in the peripheral nervous system and...
to reactivate from latency, suggesting that the in vivo phenotype is not due simply to low levels of wild-type virus contamination.

To address the question of whether the TK gene of clone C4b and its low level of TK activity is playing a role in its unusual in vivo phenotype, we disrupted the TK gene by insertion of the GFP reporter gene. The in vivo characteristics of the recombinant virus, C4bgfp, were then compared to SC16 and clone C4b. The results showed that this modified virus lost its ability to replicate in the peripheral nervous system of mice and its ability to reactivate from latency (Fig. 3c). Furthermore, in situ data showed a reduced level of latency establishment of the recombinant clone C4bgfp relative to clone C4b and SC16 (Table 2).

### Can translational frame-shifting explain the low level of TK activity in clone C4b?

One possible explanation for the low level of TK activity induced by clone C4b was that frame-shifting in the G-string during translation could result in the synthesis of a small quantity of functional product. Data to support this mechanism had been obtained in the case of a single G insertion mutant (Hwang et al., 1994), although, in this case, the frame-shift invoked was +1 (Fig. 1). Clone C4b has a double G insertion and so the frame-shift required in this case to move translation back to the correct reading frame would be +2. Alternatively, a −1 frame-shift would also shift translation back to the correct reading frame but with the insertion of an additional glycine residue. If this is the explanation, then it is likely that any virus having a −2 (or +1) frame-shift in the G-string would exhibit a similar high level of neurovirulence and that virus recovered from the nervous system of animals would be genotypically and phenotypically identical to the infecting virus. SC16 GG was, therefore, constructed. SC16 GG contains in the SC16 genetic background a double G insertion in the G-string and, therefore, has a run of nine G residues. However, this virus was shown to be TK negative in all assays (data not shown) and reactivated at low levels from only 2 of 10 explanted ganglia cultures (2.3 × 10^5 and 1 × 10^5 p.f.u., respectively). As translational frame-shifting could not explain the low level of TK activity or the ability of the virus to replicate in the peripheral nervous system, further phenotypic analyses of the virus recovered from the peripheral nervous system of mice infected with clone C4b were undertaken.

### TK phenotype of virus recovered from the nervous system

Virus samples were recovered from animals infected with clone C4b from local sensory ganglia during the acute phase of the infection and following reactivation from latency. Virus stocks were grown on Vero cells from the original ganglionic homogenates derived from individual mice. These were tested first for their ability to express TK activity (Fig. 4). All were found to be positive for TK expression. Viruses isolated from acutely infected ganglia expressed variable levels of TK activity, with one clonal isolate expressing levels as high as SC16 (Fig. 4a). All viruses recovered from latently infected ganglia expressed levels similar to those expressed by SC16 (Fig. 4b). Therefore, it appears that passage through the peripheral nervous system exposes the virus to positive selection for TK expression.

### Table 2. In situ data

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. positive neurones</th>
<th>No. sections counted</th>
<th>No. positive per section</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC16</td>
<td>4430</td>
<td>370</td>
<td>12.0</td>
</tr>
<tr>
<td>DM21</td>
<td>359</td>
<td>287</td>
<td>1.2</td>
</tr>
<tr>
<td>Clone C4b</td>
<td>1684</td>
<td>532</td>
<td>3.2</td>
</tr>
<tr>
<td>C4bgfp</td>
<td>526</td>
<td>718</td>
<td>0.7</td>
</tr>
</tbody>
</table>

**Fig. 4.** TK activity of cells infected with virus isolates recovered from the peripheral nervous system of mice infected with C4b was assayed. Virus was isolated from dorsal root ganglia either 5 days p.i. (acute phase) or after reactivation from latently infected ganglia. (a) TK activity of acute phase virus isolates acu1 to 5 was compared with SC16. (b) TK activity of reactivated virus isolates exp1 to 4 were compared with SC16 and DM21. Each virus isolate originated from a separate mouse.
**Genotypic analysis of virus recovered from the peripheral nervous system**

To identify the genetic change responsible for the reversion of the mutant virus phenotype, clonal isolates of reactivated clone C4b were obtained by limiting dilution and the TK gene of one of these cloned isolates was sequenced. Rather than the virus losing the original two G nucleotide insertions, it had instead gained an additional G nucleotide, shifting the remainder of the gene back into frame and encoding for an extra glycine residue. This has no apparent effect on the activity of the enzyme (Fig. 5). To determine whether this cloned isolate represented the major genotype in what may be a mixed population, the TK gene of the original reactivated virus population was sequenced. The result also indicated that an additional G nucleotide had been gained, suggesting that the majority of reactivated clone C4b had the same genotype as the cloned isolate.

The virus recovered from the peripheral nervous system was, therefore, distinct from the input virus in its TK phenotype. Furthermore, virus recovered from ganglia latently infected with clone C4b was also genotypically distinct.

**DISCUSSION**

Clinical isolate 4 was chosen from a panel of drug-resistant TK-deficient variants for further study because of its unusual pattern of neurovirulence. It appeared to be unique amongst our collection of such isolates in that it was capable of efficient replication in the peripheral nervous system during acute primary infection and of zosteriform spread to secondary peripheral sites in the skin. Furthermore, it was shown in latency studies that this variant alone appeared to reactivate efficiently (Harris et al., 2003). Following initial cloning of isolate 4, 10 individual clones were sequenced and all showed the same mutation in TK, a double G insertion in the G-string motif (data not presented). One of these clones (clone C4b) was used for the work described in this paper.

The first task was to establish whether clone C4b induced any measurable TK activity. Although levels were extremely low (<1% of the level of the wild-type virus SC16), there appeared to be consistent TK activity. The nature of the genetic lesion in clone C4b (a double G insertion inducing a −2 frame-shift) appeared to preclude any induction of TK activity by authentic translation of the gene since many functionally critical regions are disrupted by the frame-shift (Brown et al., 1995). Amino acids with direct involvement in the active site of the enzyme [e.g. residues 161–192 involved in nucleoside binding and other conserved residues in the active site, such as Arg222 and Glu225 and Asp162, which is believed to be responsible for Mg2+ co-ordination (Brown et al., 1995)] would be lost following the frame-shift.

Two alternative explanations were considered for the low level of TK expression. Hwang et al. (1994) produced evidence that a compensating +1 translational frame-shift could result in the induction of a small amount of wild-type polypeptide from a TK gene with a single G insertion in the G-string, and so frame-shifting had to be considered as a possible explanation. An alternative explanation was suggested by the work of Horsburgh et al. (1998) who showed that other gene products could compensate for the loss of TK function in interactions of the virus with the nervous system. Furthermore, work with the drug ganciclovir in HCMV, which has no TK gene, has demonstrated that phosphorylation of this nucleoside analogue is carried out by the UL97 gene product, a product believed to be a protein kinase (Biron et al., 1986; Littler et al., 1992). The HCMV UL97 gene is conserved across the herpesviruses and is homologous to the UL13 product of HSV. Therefore, another gene product compensating for the loss of TK was considered also.

A simple way of assessing whether the TK gene had a role in neurovirulence was to introduce an irreversible lesion into the TK gene of clone C4b. This was achieved by introducing an HCMV GFP gene cassette into the TK open reading frame. The resulting virus exhibited no detectable expression of TK activity (Fig. 2c). Furthermore, it resembled the deletion mutant DM21 in its profound attenuation in the peripheral nervous system of mice during acute infection and its inability to reactivate from latency (Fig. 3c). The data demonstrated the necessity for the uninterrupted TK gene for neurovirulence and appeared to eliminate a role for a compensatory gene function. However, although the data appear to argue against compensatory functions, they do not provide any direct evidence for frame-shifting.

It was argued that if frame-shifting explained the neurovirulence of clone C4b, it might be expected that other viruses with mutations in the G-string that induce −2 (or +1) frame-shifts would exhibit similar neurovirulence. However, SC16 GG (−2 frame-shift) showed diminished neurovirulence. Consistent with this are the observations
described in the previous paper on clone C3c, which also exhibits a +1 frame-shift and which appears to lack neurovirulence (Harris et al., 2003).

Another prediction was that if frame-shifting explained the neurovirulence of clone C4b, virus recovered from the nervous system during acute infection and following reactivation from latency would have the phenotype and genotype of the input virus. This was not the case. Expression of TK was observed with viruses recovered from the nervous system of animals infected with clone C4b, intermediate levels with those isolated during the acute phase and almost wild-type levels with viruses recovered from latency. Consistent with these phenotypic observations, virus recovered from latently infected ganglia had acquired an additional mutation in the G-string (a third G residue), ensuring that the TK gene was now translated in frame – although the protein would be expected to have an additional glycine residue.

A single G insertion in the G-string appears to be the most commonly observed TK mutation (Sasadeusz et al., 1997; Harris et al., 2003). If this mutation is capable of reverting clone C4b to wild-type TK functionality, it may not be surprising that it emerges during replication of the virus in the nervous system, where there would be positive selection pressure for functional TK activity. However, it is still unclear why this phenomenon is so marked with clone C4b to wild-type TK functionality, it may not be surprising that it emerges during replication of the virus in the nervous system, where there would be positive selection pressure for functional TK activity. However, it is still unclear why this phenomenon is so marked with clone C4b but at a slower rate. As genetic instability of C4b has been shown to lead to mixed populations of the TK-positive and -negative viruses in vivo, it is likely that the same instability occurs in vitro and accounts for the low level TK activity detected. The lower levels of TK activity detected before passage in mice reflect the lack of selection of TK-positive viruses in tissue culture. In contrast, during replication of the virus in vivo, strong selective pressure would favour the replication of the TK-positive variant of C4b.

In summary, the low level of expression of TK by clone C4b and its considerable neurovirulence result from inherent genetic instability at the TK locus, resulting in significant phenotypic reversion to a virus with wild-type TK activity by the addition of a third G residue (Fig. 5). The phenotypic and genotypic data regarding SC16 GG suggests that this may be due to factors other than the specific GG insertion in the TK gene of clone C4b.

NOTE ADDED IN PROOF

Since the submission of this paper, Griffiths & Coen (2003) (J Virol 77, 2282–2286, 2003) have described the genetic engineering of a double guanine insertion into the G-string region of the TK gene-encoding region of wild-type HSV-1 strain KOS. The majority of plaques isolated from stocks of this virus had a TK-negative phenotype, yet approximately 3% of plaques had high levels of TK activity. These phenotypic revertants were also able to reactivate from latency in mouse ganglia. On further evaluation of these revertants, an additional G insertion was found to be present, which the authors concluded would contribute to the observed pathogenicity.

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REFERENCES


