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Antiviral Research 37 (1998) 17–28

Antiviral Research

Penciclovir and pathogenesis phenotypes of drug-resistant Herpes simplex virus mutants

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Received 30 June 1997; accepted 18 September 1997

Abstract

We compared the penciclovir susceptibilities and pathogenesis phenotypes of mutants of Herpes simplex virus type 1 that are resistant to acyclovir and/or foscarnet. The mutants, which were derived from laboratory strain KOS, included six DNA polymerase mutants, a thymidine kinase negative mutant, a thymidine kinase partial mutant, and a double mutant. Two of four polymerase mutants not previously examined for penciclovir susceptibility exhibited modest resistance to this drug. A thymidine kinase negative mutant exhibited ~20-fold resistance while a thymidine kinase partial mutant was penciclovir-sensitive. Following intracerebral inoculation of 7-week old CD1 mice, the mutants ranged from exhibiting near wild-type neurovirulence (thymidine kinase partial) to modest attenuation (e.g. thymidine kinase negative) to more severe attenuation. Following corneal inoculation, three polymerase mutants exhibited modest deficits (relative to those of thymidine kinase negative mutants) in their abilities to replicate acutely in the ganglion and reactivate from latency. For mutant AraA^{r}13, the deficit in ganglionic replication was shown to be due to its polymerase mutation by analysis of recombinant viruses derived by marker rescue. These results may have implications for issues of penciclovir action and resistance, for drug resistance in the clinic, and for the interactions of herpes viruses with the peripheral and central nervous systems. © 1998 Elsevier Science B.V.

Keywords: Thymidine kinase; DNA polymerase; Neurovirulence; Latency

1. Introduction

Herpes simplex virus (HSV) is an important human pathogen. Fortunately, HSV infections can be treated with antiviral drugs, most of which target the virus-encoded DNA polymerase (Pol) that is essential for virus replication (reviewed in Coen, 1992). Some drugs such as foscarnet (phos-

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PII S0166-3542(97)00054-5

phonoformic acid, PFA) act by direct inhibition of Pol. Certain other drugs such as acyclovir (ACV) and penciclovir (PCV, the active form of famciclovir) inhibit Pol following conversion to triphosphates by the virus encoded thymidine kinase (TK) and cellular enzymes.

Drug-resistant HSV mutants can be isolated in the laboratory and they have arisen in the clinic, where they pose an increasingly serious problem in patients with AIDS and other immunocompromised individuals (reviewed in Safrin, 1996). PFA-resistant (PFA^r) HSV mutants contain *pol* mutations, while ACV^r and PCV^r mutants contain *pol* or *tk* lesions or both (reviewed in Coen, 1996b). The *pol* mutants express Pol that is less susceptible to inhibition by drug. Because TK is not essential for replication in cell culture, *tk* mutants can be TK-negative (TK^-; completely devoid of TK activity); TK-partial (low levels of TK activity); or TK-altered (enzyme that fails to phosphorylate drug, but can phosphorylate thymidine). Most drug-resistant clinical isolates of HSV have thus far been TK-deficient; whether such isolates are truly TK^- or TK-partial has been controversial as it can be difficult to distinguish these phenotypes (reviewed in Coen, 1994). There have been fewer *pol* mutants recovered from patients; however, with the growing use of drugs such as PFA to treat ACV-resistant infections, the number of these mutants is increasing (Safrin et al., 1994a,b). One important issue is whether drug resistant mutants are susceptible or resistant to newer antiviral drugs such as PCV. A second issue is how drug resistant mutants that arise in patients evade drug therapy, yet retain sufficient Pol and/or TK function to cause disease. Studies of drug-resistant mutants in animal models may have predictive value should such mutants arise in the clinic and may provide insights into virus biology.

HSV pathogenesis has been most thoroughly studied in mouse models. One assay of pathogenesis in mice entails infection of the central nervous system (CNS) via intracerebral (i.c.) inoculation, which leads to encephalitis and death (neurovirulence). In a second assay of pathogenesis, HSV is inoculated at a peripheral site such as the cornea. HSV replicates productively at this site, gains access to nerve terminals and is transported axonally to sensory ganglia of the peripheral nervous system (PNS), such as the trigeminal ganglion, which is a secondary site of replication. Subsequently, HSV establishes and maintains a latent infection, during which infectious virus is not detectable (Stevens, 1989). In mouse models of latency, reactivation is usually assessed by explanting ganglia in culture, which triggers the production of infectious virus. Although the second assay appears to mimic the natural course of HSV infection of humans more than the first, it is not known which is more predictive of pathogenicity of drug resistant mutants in patients.

When drug-resistant HSV mutants have been examined with either of these assays, nearly all have exhibited some attenuation. Truly TK^- mutants (e.g. containing a *tk* deletion mutation) are impaired for neurovirulence (Martuza et al., 1991) and they are exceedingly impaired for acute replication in the PNS and for reactivation from latency (Coen et al., 1989a; Efstathiou et al., 1989; Jacobson et al., 1993). However, *tk* deletion mutants have not been examined quantitatively for their impairment for neurovirulence. TK-partial and TK-altered mutants are less impaired for neurovirulence and replicate in the PNS and reactivate to varying extents depending on the amount of TK activity (reviewed in Coen, 1994). Drug-resistant *pol* mutants have exhibited some attenuation of neurovirulence and have been qualitatively competent for reactivation from latency, (Klein and Friedman-Kien, 1975; Field and Darby, 1980; Klein et al., 1981; Darby et al., 1984; Larder and Darby, 1984; Field and Coen, 1986; Jacobson et al., 1995). However, most have not been assessed for their ability to replicate in the PNS.

In this study, we sought to determine the degrees of cross-resistance to PCV of certain mutants characterized previously for resistance to other anti-HSV drugs, particularly PFA-resistant *pol* mutants, a TK^- deletion mutant, and a TK-partial mutant, and to measure the degrees of pathogenicity of these mutants in mice, particularly in terms of neurovirulence and replication in the PNS. Our results point to a rather broad spectrum of PCV and pathogenesis phenotypes. Possible implications of our results for issues of

Table 1
Properties of viruses in this study

Virus	Mutation[a]	Drug phenotypes (fold-resistance[b])			References
		ACV	PCV	PFA	
KOS (wild type)	None	S	S	S	Smith, 1964
pol mutants					
PFA^{r}2	A605V, δ-region C	R (4)[c]	S[c]	R (10)[c]	Derse et al., 1982; Gibbs et al., 1988
PFA^{r}1	R700G, region II	R (2)[c]	S[c]	R (6)[c]	Derse et al., 1982; Gibbs et al., 1988
PFA^{r}5	S724N, region II	R (8)[c]	R (4)[c]	R (6)[c]	Derse et al., 1982; Gibbs et al., 1988
AraA^{r}13	V813M, region III	R (5)[d]	R (3)[d]	R (4)[d]	Chiou et al., 1995; Coen et al., 1985, 1982; Gibbs et al., 1988
F891C	F891C, region I	R (10)[c]	R (2)[c]	R (10)[c]	Marcy et al., 1990
AraA^{r}9	N961K, region V	R (4)[d]	HS[d]	S[d]	Chiou et al., 1995; Coen et al., 1985, 1982; Gibbs et al., 1988
tk mutants					
*dl*sptk	deletion of nts 497–857	R (50)[c,d]	R (20)[c]	S[d]	Coen et al., 1989c; Martuza et al., 1991
KG111	amber at codon 44	R (8)[c]	S[c]	S[c]	Irmiere et al., 1989
Double mutant					
PKG7	*pol* R842S, *tk* amber 44	R (150)[c]	R (4)[c]	R (4)[c]	Coen et al., 1986; Jacobson et al., 1995
Recombinant viruses					
A13Aph$^+$K/b	None	S	ND[e]	ND	Chiou, 1988; Chiou et al., 1986
A13Aph$^+$X/c	None	S	ND	ND	Chiou, 1988; Chiou et al., 1986

[a] This column provides the relevant codon changes and conserved motifs altered for *pol* mutants and the relevant nucleotide or codon changes for *tk* mutants, where nucleotide (nt) 1 is the mRNA start site.
[b] Mutants are characterized as resistant (R), sensitive (S), or hypersensitive (HS) using our previously published criteria (Chiou et al., 1985; Coen et al., 1985). Fold-resistance values (in parenthesis) represent the dose required to reduce plaque formation by 50% (ED_{50}) of the mutant divided by the ED_{50} for the corresponding wild-type virus.
[c] Phenotype (including fold-resistance where applicable) determined in this study. In some cases, the fold-resistance values obtained differ somewhat (usually no more than 2-fold) from published values.
[d] Phenotype determined in a previously published study, which is included among the citations in the right-most column.
[e] ND, not done.

drug action and resistance, for antiviral drug resistance in the clinic, and for the interactions of herpesviruses with the peripheral and central nervous systems are discussed.

2. Materials and methods

2.1. Cells and viruses

African green monkey kidney cells (Vero) were propagated as described (Weller et al., 1983). Viruses used in this study include wild type HSV type 1 strain KOS, drug resistant mutants, and recombinant viruses. These viruses are listed in Table 1 and were propagated and titered as described (Coen et al., 1985) except that high-titer stocks were prepared by pelleting infected cells by low-speed centrifugation and resuspending them in 1/10th the volume of the supernatant prior to freezing, thawing, and sonication.

2.2. Assays of drug susceptibilities

Plaque reduction assays to assess the resistance, sensitivity, or hypersensitivity of mutants to ACV,

PCV, and PFA were performed using Vero cells and analyzed as described previously, using criteria (including statistical analysis) for terming a virus resistant (Chiou et al., 1985; Coen et al., 1985; Chiou et al., 1995). Briefly, a mutant that reproducibly exhibits an ED_{50} (the concentration that inhibits plaque formation by 50%) two-fold or more greater than the corresponding wild type and that reproducibly exhibits a plating efficiency two-fold or more greater than the corresponding wild type at at least one drug concentration is considered resistant to the drug. Fold-resistance was calculated by dividing the ED_{50} of a mutant by the ED_{50} of the corresponding wild type virus.

2.3. Assays of neurovirulence

Seven-week old CD1 mice (Charles River Laboratories, Wilmington, MA) were anesthetized with pentobarbital. For each virus dilution tested, at least six mice were each inoculated i.c. in the right hemisphere with 30 μl of the particular inoculum using a tuberculin syringe and a 30-G needle. Titers of inocula were verified by simultaneous plaque assays. The numbers of deaths and surviving mice were recorded daily for 2 weeks. To determine the dose of each isolate that resulted in 50% lethality (LD_{50}), ten-fold serial dilutions of virus (in medium) were assayed and the data analyzed by the method of Reed and Muench (1938).

2.4. Assays of acute and latent infections

Assays of acute replication and reactivatable latency following corneal inoculation of 7-week old CD1 mice were performed as described, assaying acute replication in eyes and trigeminal ganglia at 3 days post-inoculation and latency in ganglia at 30 days post-inoculation (Leib et al., 1989, 1991; Jacobson et al., 1993).

3. Results

3.1. Drug-resistance phenotypes of mutants

In this study, we sought to compile drug susceptibility and pathogenesis data for a number of drug-resistant mutants derived from a single strain of HSV. In particular, we wished to determine whether certain ACV- and/or PFA-resistant mutants were susceptible or resistant to PCV. To that end, we examined six *pol* mutants, two *tk* mutants, and a *pol-tk* double mutant derived from strain KOS, whose mutations and phenotypes are listed in Table 1. These mutants have previously been shown to be either ACV- and/or PFA-resistant. For each *pol* mutant, a single codon change has been identified and shown to confer ACV- and/or PFA-resistance phenotypes (Gibbs et al., 1988; Marcy et al., 1990). For each *tk* mutant, the mutation has been shown to confer ACV-resistance (Coen et al., 1989c; Irmiere et al., 1989). Of the *tk* mutants, one, *dl*sptk, is a TK^- deletion mutant (Coen et al., 1989c), while the other, KG111, contains a nonsense mutation between the first and second methionine codons, yet synthesizes low levels of active TK via initiation of translation at the second methionine codon (Coen et al., 1989b; Irmiere et al., 1989). The double mutant, PKG7, contains the *pol* mutation of mutant PAA^r5 and the *tk* mutation of KG111 (Coen et al., 1986; Jacobson et al., 1995).

All of the mutants have previously been reported to be ACV^r. We report here the degree of resistance to ACV of five of these (PFA^r2, PFA^r1, PFA^r5, KG111, and PKG7) at 37°C, along with a revised value for *pol* mutant F891C (previously published values for the other mutants are also presented) in Table 1. Wild type strain KOS exhibited an ED_{50} for ACV of 3 μM, similar to previous results (Coen et al., 1989a,c). The fold-resistances to ACV varied from slight (2-fold) for *pol* mutant PFA^r1 to very high (150-fold) for the double mutant, PKG7. As anticipated from previous results with a double mutant (Darby et al., 1984), the fold ACV-resistance of PKG7 was roughly the product of the fold-resistances of its PAA^r5 and KG111 parents (Table 1 and Coen et al., 1985). All but one of the *pol* mutants and the double mutant exhibited resistance to PFA with ED_{50}s > 550 μM (100 μg/ml); in these experiments KOS exhibited an ED_{50} of 190 μM, similar to previous results (Coen et al., 1985). Fold-resistances to PFA varied from 4-fold to 10-fold. The *tk* mutants, as expected, remained susceptible to PFA.

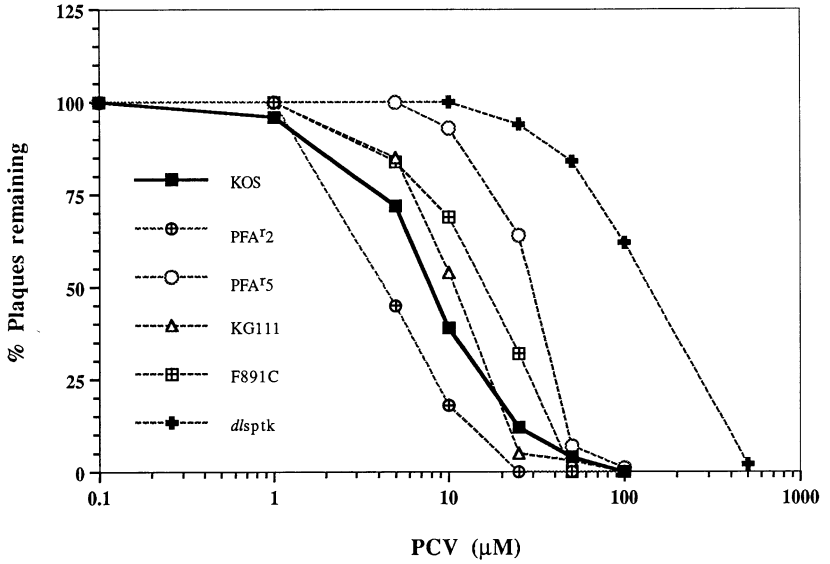

Fig. 1. Susceptibilities of viruses to penciclovir. Plaque reduction assays were performed on the indicated viruses as described (Coen et al., 1985). The data are the average of two separate assays each performed in duplicate.

To our knowledge, only two of the mutants (both *pol* mutants) had been previously examined for susceptibility to PCV. Of these, AraA^{r}13 was modestly resistant and AraA^{r}9 was hypersensitive (Chiou et al., 1995). In the present study, wild type strain KOS exhibited an ED_{50} of ~ 10 μM in Vero cells [higher than that observed in human cells (Boyd et al., 1993)], similar to previous results (Chiou et al., 1995). Of the remaining *pol* mutants, we found that two were sensitive to PCV and two were modestly resistant (2- to 4-fold) (Fig. 1 and Table 1). The TK$^-$ mutant was ~ 20-fold resistant to PCV; interestingly, KG111 was sensitive to PCV. The double mutant, like its *pol* mutant parent PAA^{r}5 (Chiou et al., 1995), was ~ 4-fold resistant to PCV.

3.2. Neurovirulence of drug-resistant mutants in CD1 mice

We wished to examine the neurovirulence of these drug-resistant mutants to determine their degree of attenuation, if any. As our studies of pathogenesis following peripheral inoculation entailed corneal inoculation of 7-week old CD-1 mice (Coen et al., 1989b,c; Jacobson et al., 1993, 1995; also see below), we used the same mice to measure the dose that resulted in 50% lethality (LD_{50}) for several of our drug-resistant mutants (Table 2). The *pol* mutant PFA^{r}5 was only slightly attenuated with an LD_{50} just five-fold higher than that of KOS. Interestingly, the TK$^-$ mutant, *dl*sptk, exhibited an LD_{50} only ~ 20-fold higher than that of KOS. We also examined five other *pol* mutants and *dl*sptk following i.c. inoculation at a single dose (1×10^6 PFU/mouse) that was ~ 50-fold higher than the LD_{50} for strain KOS. Five out of six animals died following inoculation with mutant PFA^{r}1, indicating that it was not severely attenuated (Table 3). The other *pol* mu-

Table 2
LD_{50} determinations

Virus	LD_{50}
KOS	2×10^4
PFA^{r}5	1×10^5
KG111	3×10^4
*dl*sptk	4×10^5
PKG7	2×10^7

Table 3
Neurovirulence of *pol* and *tk* mutants at 10^6 PFU/mouse

Virus	Number of deaths/Number inoculated
KOS	5/6
PFA^{r}2	1/6
PFA^{r}1	5/6
AraA^{r}13	0/6
F891C	2/6
AraA^{r}9	3/6
*dl*sptk	5/6

tants exhibited lower virulence ranging from no deaths (AraA^{r}13) to three deaths (AraA^{r}9) out of six animals inoculated (Table 3). Interestingly, most of these *pol* mutants were evidently more attenuated for neurovirulence than *dl*sptk, the TK-negative deletion mutant, which killed five out of six animals at this dose.

The TK-partial mutant, KG111, exhibited near wild type neurovirulence with a <2-fold higher LD_{50} than that of KOS (Table 2). In contrast, the double mutant, PKG7, which is severely impaired for ganglionic replication and reactivation from latency (Jacobson et al., 1995), was also severely attenuated for neurovirulence with an LD_{50} ~ 1000-fold higher than that of KOS and KG111 and ~ 30-fold higher than that of its *pol* mutant parent, PAA^{r}5 (E. Pelosi, F. Rozenberg, D.M. Coen, unpublished results). This is consistent with *pol* and *tk* mutations acting synergistically to attenuate neurovirulence, in accordance with previous results (Field and Darby, 1980; Darby et al., 1984; Jacobson et al., 1995).

3.3. Behavior of pol mutants following corneal inoculation

Very few *pol* mutants have been examined for their replication and reactivation following corneal inoculation and there is little information regarding the replication of such mutants in the PNS following inoculation by any route. One mutant that we previously examined, PAA^{r}5, exhibited little attenuation following corneal inoculation (Jacobson et al., 1995) despite being 30-fold attenuated for neurovirulence in CD1 mice (E. Pelosi, F. Rozenberg, D.M. Coen, unpublished results). We, therefore, compared three other *pol* mutants that exhibited varying degrees of neurovirulence (PFA^{r}5, PFA^{r}2, and AraA^{r}13) with their parental strain, KOS, following corneal inoculation of CD1 mice. The three KOS-derived *pol* mutants replicated in the eye, exhibiting titers at 3 days post-infection that were slightly lower than those of KOS, although this difference may have been due to assay variability (Table 4). However, the titers of mutant viruses in trigeminal ganglia at three days post-infection were clearly reduced compared to KOS with mutants PFA^{r}2 and AraA^{r}13 exhibiting the least ganglionic replication (~ 1000-fold lower titers than KOS; Table 4). Indeed, infectious virus was recovered from only one of four ganglia acutely infected with AraA^{r}13. Interestingly, mutant PFA^{r}5, which was the least defective for ganglionic replication, contains the same mutation as mutant TP2.5 (Larder et al., 1987). TP2.5 was indistinguishable from wild-type for zosteriform spread, which depends upon efficient primary replication, passage through the PNS, and further replication in the skin to form secondary lesions (Larder and Darby, 1985). All three KOS-derived *pol* mutants reactivated from latency, although PFA^{r}2 and AraA^{r}13 reactivated with somewhat lower effi-

Table 4
Ganglionic replication and reactivation following corneal inoculation

Virus	Acute replication[a]		Reactivation[b]
	Eyes	Ganglia	
KOS	3.5 ± 1.2	4.7 ± 0.3	8/8
PFA^{r}5	2.4 ± 0.6	3.6 ± 0.3	12/12
PFA^{r}2	3.0 ± 0.5	1.9 ± 1.0	9/12
AraA^{r}13	3.3 ± 0.7	1.1 ± 0.7	5/10
A13Aph$^+$K/b	3.4 ± 0.7	4.0 ± 0.9	10/10
A13Aph$^+$X/c	3.9 ± 0.1	3.1 ± 1.3	9/9

[a] Log mean titers $\pm$ log standard deviations of virus in eyeswabs and ganglionic homogenates measured at 3 days post-inoculation.
[b] Number of ganglia reactivating/number assayed.

ciencies. Thus, these three mutants appear more attenuated following corneal inoculation than does PAA^{r}5 (Jacobson et al., 1995). Interestingly, PFA^{r}5 was less attenuated for neurovirulence (5-fold, Table 2) than was mutant PAA^{r}5 (30-fold; E. Pelosi, F. Rozenberg, and D.M. Coen, unpublished results) despite being more attenuated following corneal inoculation (compare Table 2 with Jacobson et al., 1995).

3.4. The attenuation of AraA^{r}13 following corneal inoculation is due to the pol mutation

Because the behaviors of these mutants following corneal inoculation contrasted with that of PAA^{r}5 (Jacobson et al., 1995), we asked whether the attenuation of the most impaired of them, AraA^{r}13, was due to a *pol* mutation. We utilized recombinant viruses, A13Aph + K/b and A13Aph + X/c (Table 1) in which the *pol* mutation of AraA^{r}13 was rescued by wild-type sequences that overlap within the *pol* gene (Chiou et al., 1985, 1986). The only sequence difference within this overlap between AraA^{r}13 and KOS is at codon 813 (Gibbs et al., 1988). The recombinant viruses behaved very much like KOS following corneal inoculation (Table 4). Thus, the inefficient replication of AraA^{r}13 in the PNS, is due to its *pol* mutation at codon 813 (Gibbs et al., 1988).

4. Discussion

This study brings together data regarding the susceptibilities to antiviral drugs and the pathogenic behaviors of a number of drug resistant mutants. Below, we discuss possible implications of our results for antiviral drug mechanisms, for the relative importance of different viral functions in the PNS vs. the CNS, and for the potential pathogenicity of drug-resistant mutants in the clinic.

4.1. Potential implications for mechanism of action of PCV

The TK-partial mutant, KG111, expresses ~10% the amount of TK polypeptide expressed by wild type virus at 34° due to an amber mutation at codon 44 and subsequent initiation at a downstream initiation codon (Irmiere et al., 1989). The resulting truncated polypeptide appears to be fully active for thymidine kinase activity; for example, there is ~10% the thymidine anabolism in KG111-infected cells as there is in KOS-infected cells (Coen et al., 1989b). Based on several studies with this and related mutants (Coen and Schaffer, 1980; Coen et al., 1980; Summers et al., 1983; Haarr et al., 1985; Coen et al., 1989b; Irmiere et al., 1989), we estimate that it expresses ~5% the amount of active TK polypeptide as wild type virus at 37°. Despite that, the mutant is fully sensitive to PCV. This argues that only ~5% of wild-type TK levels are sufficient for full PCV-sensitivity. This result is similar to that previously obtained with certain other nucleoside analogs (Coen et al., 1989b) and indicates that HSV expresses far more TK than is necessary to convert PCV to phosphorylated species that inhibit viral replication. However, we note that KG111 was not selected for resistance to PCV. Other TK-partial mutants that express ~5% TK activity due, for example, to point mutations, need not be sensitive to PCV as such mutants might be more affected for PCV phosphorylation than for thymidine phosphorylation.

It is interesting that *dl*sptk exhibited only ~20-fold resistance to PCV. This and other TK-negative mutants exhibit higher fold-resistances to other antiviral nucleosides such as ACV in Vero and BHK cells (Table 1 and Coen et al., 1989b; Larder and Darby, 1984). Similarly, another *tk* deletion mutant, DM2.1 (Efstathiou et al., 1989), exhibited ~50-fold resistance to PCV but ~300-fold resistance to ACV in human foreskin fibroblasts (Boyd et al., 1993). If, as we have previously argued (Chiou et al., 1995; Coen, 1996a,b), the maximum degree of resistance to a drug conferred by a mutation is a measure of how important the gene product is for drug selectivity, then the lower degree of resistance to PCV exhibited by *tk* null mutants suggests lower selectivity of activation of PCV by HSV TK. This would be consistent with the higher amounts of PCV triphosphate than

ACV triphosphate found in uninfected human cells and the greater phosphorylation of PCV than ACV by mitochondrial deoxyguanosine kinase (Lowe et al., 1995). Whether this is true in Vero cells is not known. A thorough comparison of drug phosphorylation and drug susceptibilities in various cells infected with wild type and *tk* null mutants would help test these ideas.

In this study, we identified *pol* mutants PFA^{r}5 and F891C as 3- to 4-fold resistant to PCV. Thus, there are now ten *pol* mutants that have exhibited PCV-resistance, all modestly (Boyd et al., 1993; Chiou et al., 1995; Ertl et al., 1995). If one accepts the argument above regarding maximum degree of resistance and selectivity, than these added data bolster the contention that inhibition of HSV Pol by PCV-TP is not very selective (Hannah et al., 1989; Chiou et al., 1995). It still remains possible, however, that a *pol* mutation that confers high resistance to PCV could be identified; perhaps this would require selection for resistance to PCV. We also observed that the double mutant, PKG7, is 3- to 4-fold resistant to PCV consistent with the behavior of its parents, PAA^{r}5, which is $\sim$3-fold resistant (Chiou et al., 1995) and KG111, which is sensitive. We note that if PKG7 had been analyzed solely by a TK assay it might be incorrectly assumed that its resistance to PCV was due to a *tk* mutation rather than to a *pol* mutation. This emphasizes the value of genetic as well as biochemical analyses of drug-resistant mutants.

4.2. Pathogenicity of tk mutants

In neurovirulence studies in 3-week or older mice, mutants described as TK$^-$ (based on enzyme or drug resistance assays) have been highly attenuated with LD_{50}s 10^4 to $>10^5$ higher than those of their corresponding wild-type strains and absolute LD_{50} values reaching $>10^6$ (Field and Wildy, 1978; Field and Darby, 1980; Darby et al., 1981; Tenser, 1983; Chrisp et al., 1989; Suzutani et al., 1995). In contrast, *dl*sptk, which is TK$^-$ due to a large deletion mutation, was only $\sim$20-fold attenuated with an absolute LD_{50} value of 4×10^5 (Table 2). It is exceedingly unlikely that this was due to reversion or to the presence of contaminating wild type virus as the mutation is a large deletion and no contaminating virus has been detected in other mouse experiments (e.g. reactivation from latency) or by plaque autoradiography (E. Pelosi, J.G. Jacobson, M.F. Kramer, S.-H. Chen, D.M. Coen., unpublished results). The earlier mutants may have exhibited greater fold-attenuation relative to their wild-type parents because the particular wild-type virus may be more neurovirulent in the particular mice used (e.g. strain SC16 in 3-week old Balb/c mice). However, it is difficult to ascribe the higher absolute LD_{50} values of some of these *tk* mutants to such strain differences. An alternative explanation is the presence of additional mutations in these *tk* mutants.

Certain TK-partial mutants have been tested for neurovirulence in mice and have shown some attenuation (Field and Darby, 1980; Darby et al., 1984). We have shown here that KG111 is essentially fully neurovirulent following i.c. inoculation (Table 2), much as it was not detectably impaired for PNS replication or reactivation following corneal inoculation (Coen et al., 1989b; Jacobson et al., 1995). Although it is possible that it would exhibit greater attenuation relative to wild type in a more susceptible host and/or if the *tk* mutation were placed in a more virulent viral background, KG111 appears to be the least attenuated ACV^r mutant tested to date following either corneal or i.c. inoculation of mice.

4.3. Pathogenicity of pol mutants

We found that most *pol* mutants exhibited meaningful attenuation of neurovirulence following i.c. inoculation of 7-week old CD1 mice (Tables 2 and 3) and that the degrees of attenuation varied substantially. These results are consistent with previous studies in four week Balb/c mice (Darby et al., 1984; Field and Coen, 1986; Field and Darby, 1980). About 14 known or putative *pol* mutants have previously been assessed for pathogenicity following inoculation in the skin of mice, although not directly for ganglionic replication (Darby et al., 1984; Field and Coen, 1986; Field and Darby, 1980; Klein and Friedman-Kien, 1975; Klein et al., 1981; Larder and Darby, 1985; Larder et al., 1986). Of these, only one,

RSC-26, exhibited any substantial defect, evidently due to the *pol* mutation (Darby et al., 1984; Larder et al., 1986). [Interestingly, by this route, TK^- mutants are substantially impaired (Efstathiou et al., 1989)]. Following corneal inoculation, the three *pol* mutants examined here were substantially defective for ganglionic replication (Table 4). For mutant $AraA^r13$, this defect is clearly due to the *pol* mutation (Table 2). In other studies, we have found that while *pol* mutant PAA^r5 exhibited little impairment in ganglionic replication (Jacobson et al., 1995) another *pol* mutant, 615.8, did, similar to the mutants studied here (E. Pelosi, D.M. Coen, unpublished results). Our data do not allow us to distinguish whether the defects in ganglionic replication manifested by $AraA^r13$ are a consequence of somewhat less efficient primary replication in the cornea or whether they are due to a specific requirement for certain aspects of Pol function in the PNS or both. Interestingly, *pol* sequences might be responsible for the different abilities of certain HSV strains to replicate in the eye and trigeminal ganglia following corneal inoculation (Day et al., 1988; Lausch et al., 1990).

It is interesting to compare the neurovirulence phenotypes of *pol* and *tk* mutants with their abilities to replicate acutely in ganglia. There was a good correlation between ganglionic replication in the PNS with neurovirulence in the CNS for the three *pol* mutants that were tested for both phenotypes. However, *pol* mutants, $AraA^r13$ and PFA^r2 were more attenuated for neurovirulence than the TK^- mutant, *dl*sptk (Table 3), yet were more competent for ganglionic replication and reactivation following corneal inoculation in ganglia (compare Table 4 and Coen et al., 1989c). This suggests that there are different requirements for TK and Pol in the PNS versus the CNS.

4.4. Potential implications for clinical drug-resistance

The emergence of HSV mutants that are resistant to antiviral drugs is an increasing problem, particularly in patients with AIDS (reviewed in Safrin, 1996). Although the mutants we have studied are laboratory isolates, studies of such mutants can presage results of clinical studies. Indeed, several mutations found in clinical isolates have been identified earlier in laboratory-derived mutants (Collins et al., 1989; Nugier et al., 1991; Sasadeusz et al., 1997). Correlation of the drug susceptibility and pathogenesis phenotypes of laboratory mutants with the behavior of similar mutants in patients should be valuable for understanding clinical drug-resistance. We think it is quite possible that some of the mutations studied here—especially those that retain substantial pathogenicity—will arise in patient isolates and that the data reported here may be useful in understanding the clinical course of any such patients.

All but one of the mutants we studied exhibited $\geq$ 4-fold resistance to ACV with ED_{50}s > 2 μg/ml (> 10 μM). All but one of the *pol* mutants we studied exhibited $>$ 4-fold resistance to PFA with ED_{50}s > 100 μg/ml. These degrees of resistance are predictive of poor clinical response to these drugs (Safrin et al., 1994a). It is not yet known what degree of resistance to PCV is clinically relevant. However, given that only $\sim$ 4-fold resistance appears to be clinically relevant for ACV and PFA (Safrin et al., 1994a), it seems quite possible that the resistance to PCV of several *pol* mutants we studied could be clinically relevant, too.

Nevertheless, a mutant that causes clinical drug resistance must both evade drug therapy and retain pathogenicity. The mutants that we have studied vary widely in their behaviors in the two assays of pathogenicity in mice that we used. If these assays in mice are predictive for pathogenicity in humans, then one might expect that those mutants that were most attenuated (e.g. $AraA^r13$) would be less likely to be associated with severe drug-resistant disease. On the other hand, those that were least attenuated might be expected to be most problematic. In this regard it is interesting that several PFA^r mutants from patients who exhibited a poor clinical response to PFA have exhibited relatively low ED_{50}s for ACV (Safrin et al., 1994b) similar to the KOS-derived mutant, PFA^r1 (Table 1), which was not severely attenuated (Table 3). It will be interesting to see whether the *pol* mutations of PFA^r clinical isolates resemble those of laboratory-isolated PFA^r mutants.

Of particular interest for clinical resistance to ACV is mutant KG111. This virus exhibits wild-type replication and reactivation from latency following corneal inoculation (Coen et al., 1989b; Jacobson et al., 1995) and wild-type neurovirulence (Table 2). It exhibits TK activity as low as or lower than several clinical ACV^r isolates that have been reported (Erlich et al., 1989; Nugier et al., 1991; Kost et al., 1993). It will be interesting to see if a similar mutant is found associated with ACV-resistant disease. Although most clinical ACV^r mutants have been cross-resistant to penciclovir, should a mutant like KG111 arise in a patient, it may be possible to treat it with penciclovir or its prodrug, famciclovir.

Acknowledgements

We thank Y.-C. Cheng for providing mutants PFA^{r}1, PFA^{r}2, and PFA^{r}5 and for initial collaboration in their characterization; J. Jacobson and T. Astor for help with the mouse experiments, K. Tyler for instruction in i.c. inoculation, and NIH grants AI24010, NS35138, AI26077, and AI19838 for support. E.P. gratefully acknowledges support from the University of Verona and a Bristol-Myers Fellowship in Pharmacology. Although none of the work described herein was funded by any company, D.C. acknowledges gifts and research grants to his laboratory from Glaxo Wellcome and SmithKline Beecham and that he has been a paid consultant for SmithKline Beecham.

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