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Molecular analysis of clinical isolates of acyclovir resistant herpes simplex virus

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Abstract

We characterised the antiviral phenotype and genotype of 41 herpes simplex virus (HSV) strains from patients clinically resistant to acyclovir (ACV). Our results confirm recognised mutational sites as being major determinants of thymidine kinase (*tk*)-associated ACV resistance, in particular insertions and/or deletions at homopolymer stretches of Gs and Cs (59% of all isolates). Previously described amino acid substitutions in functional sites of the *tk* were also identified (7% of all isolates). In addition, we identified several stop codons in novel locations on the amino acid sequence (7% of all isolates) and amino acid substitutions (15% of all isolates) likely to be directly responsible for conferring resistance to ACV. When there were no mutations detected in the *tk* gene (12% of all isolates), mutations in the DNA polymerase gene likely to be important in the generation of resistant virus were identified.

Keywords: Herpes simplex virus; Acyclovir; Resistance mutation; Polymorphism; Thymidine kinase; DNA polymerase

1. Introduction

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are responsible for a variety of clinical manifestations, including mouth and genital ulcers (Rustigian et al., 1966; Nahmias et al., 1967), eye infections (Brown, 1971), meningoencephalitis (Murray et al., 1966) and encephalitis (Rawls et al., 1966). The symptoms associated with both virus types are usually self-limiting in healthy individuals, but can be extensive and prolonged in immunocompromised individuals, including patients with HIV infection and recipients of solid organ or bone marrow transplants (Whitley et al., 1984; Englund et al., 1990; Hill et al., 1991; Morfin et al., 2000). In these cases, prolonged antiviral therapy may be required for management of the infection, resulting in the generation of drug resistant virus (Erlich et al., 1989; Birch et al., 1990; Chatis and Crumpacker, 1992; Chibo et al., 2002) in approximately 6–7% of immunocompromised patients (Christophers et al., 1998; Chen et al., 2000).

Acyclovir (ACV), a guanine nucleoside analogue, has been widely used for the management of herpes virus infections (Whitley and Gnann, 1992; Elion, 1993). Its preferential phosphorylation by the HSV-encoded thymidine kinase (*tk*) makes it a truly selective antiviral drug (Balfour, 1983). Three types of ACV resistance involving the viral *tk* have been reported: '*tk*-negative' mutants that produce no functional protein, '*tk*-partial' mutants that produce levels of *tk* too low for efficient phosphorylation and '*tk*-altered' strains that produce an enzyme that phosphorylates ACV less efficiently than the natural substrate thymidine. A fourth type of resistance generated only rarely in clinical isolates involves mutations in the HSV DNA polymerase (*pol*) (Balfour, 1983; Larder et al., 1987; Sacks et al., 1989; Coen, 1991; Gaudreau et al., 1998).

ACV resistant (ACVr) mutants have been studied genotypically and phenotypically using laboratory-derived strains and, more recently, clinical isolates. These studies have identified various resistance hot spots in the *tk* gene, notably homopolymer nucleotide stretches of guanines (Gs) and cytosines (Cs) in which insertions or deletions result in frameshift mutations and, as a consequence, truncated *tk* (Collins and Darby, 1991; Sasadeusz et al., 1997). Other *tk* mutations include random nucleotide substitutions in those regions of the gene that are critical for enzyme function (Schmit and Boivin, 1999).

In the study reported here we characterised the antiviral phenotype and genotype of a panel of 41 ACVr HSV clinical isolates. We also studied ACV sensitive (ACVs) isolates as

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a means of distinguishing heterogeneity in the *tk* and DNA *pol* from mutations associated with drug resistance and as a result identified several novel mutations that are unlikely to contribute to ACV resistance. We confirm recognised mutational sites to be major determinants of *tk*-associated ACV resistance and show that several novel mutations in both the *tk* and DNA *pol* genes appear to directly contribute to ACV resistance in HSV strains.

2. Materials and methods

2.1. Patients and specimens

Clinical material was sent to the laboratory from patients chronically infected with HSV, in particular bone marrow transplant recipients and patients infected with HIV, who were refractory to therapy with ACV. Clinical information for each patient and the phenotypic susceptibility of their HSV isolates are shown in Table 1 (HSV-1 strains) and Table 2 (HSV-2 strains). A total of 55 clinical isolates were studied, comprising 7 ACVs HSV-1, 18 ACVr HSV-1, 7 ACVs HSV-2 and 23 ACVr HSV-2 strains. Two ACVs isolates (isolate 1 for HSV-1 and isolate 26 for HSV-2) were chosen to provide reference consensus sequences for wild-type *tk* and *pol* genes.

The 14 ACVs strains were isolated from immunocompetent patients experiencing acute, untreated infection with HSV-1 or HSV-2. Of the 18 patients infected with ACVr HSV-1, 12 were males and 4 were females (Table 1) (information on the gender of 2 patients was not provided). Where clinical information was available, four of these patients were males infected with HIV, four males and three females were recipients of bone marrow transplants and one male patient had leukaemia. The age range of patients infected with ACVr HSV-1 was 8–78 years. Of the 23 patients with ACVr HSV-2 infection, 15 were males and 1 was female (Table 2); information on the gender of 7 individuals was not provided. Thirteen males and one female were infected with HIV. The age range of patients infected with

Table 1
Age, gender and clinical details of patients from whom HSV-1 strains were isolated

Isolate number	Patient details			IC_{90} (μg/ml)[a]	Resistance mutation (nt position in *tk* gene)	Mechanism of resistance (size of truncated protein in amino acids)
	Gender	Age (years)	Source of virus (associated illness)			
1	F	25	Genital	1.0		
2	F	18	Vulva	0.1		
3	F	36	Oral	1.0		
4	M	27	Penis	1.0		
5	F	28	NA[b]	1.0		
6	M	47	NA	1.0		
7	F	14	Genital	1.0		
8	M	12	NA	100	G ins (nt 435)	F[c] (227)
9	F	16	Lip (BMTx[d])	200	G ins (nt 435)	F (224)
10	M	56	NA (BMTx)	200	G del (nt 435)	F (181)
11	F	13	Mouth (BMTx)	100	G del (nt 435)	F (181)
12	M	30	NA (HIV+)	100	C ins (nt 553)	F (227)
13	M	60	Nasal (HIV+)	100	C ins (nt 553)	F (227)
14	M	17	BAL[e] (BMTx)	100	C del (nt 899)	F (elongated)[f]
15	F	41	Labial	100	R51W	
16	M	78	Cornea	100	R176Q	
17	M	58	Mouth (CLL)[g]	100	G del (nt 880)	F (elongated)
18	M	45	Saliva (BMTx)	100	Y53stop	SC[h]
19	NA	NA	NA	100	Q341stop	SC
20	M	65	Eye	100	D162A	
21	M	29	Ear (HIV+)	100	R220C	
22	F	9	NA (BMTx)	200	A189V; L227F	
23	NA	NA	NA	200	R220C	
24	M	8	Mouth (BMTx)	100	None	
25	M	40	Buttock (HIV+)	200	None	

Isolates 1–7 are ACVs; isolates 8–25 are ACVr.

[a] IC_{90}: concentration of ACV reducing HSV-specific cytopathic effects by at least 90%.

[b] NA: not available.

[c] F: frameshift mutation.

[d] BMTx: bone marrow transplant.

[e] BAL: bronchio-alveolar lavage.

[f] Elongated: stop codon generated downstream of wild-type stop codon.

[g] CLL: Chronic lymphocytic leukaemia.

[h] SC: stop codon.

Table 2
Age, gender and clinical details of patients from whom HSV-2 strains were isolated

Isolate number	Patient details			IC_{90} (μg/ml)[a]	Resistance mutations (nt position in *tk* gene)	Mechanism of resistance (size of truncated protein in amino acids)
	Gender	Age (years)	Source of virus (associated illness)			
26	F	17	Vaginal	1.0		
27	F	40	NA[b]	1.0		
28	F	37	Genital	1.0		
29	F	23	Vaginal	1.0		
30	F	36	Genital	1.0		
31	F	42	Buttock	1.0		
32	F	NA	Buttock	1.0		
33	M	50	Anal	200	G ins (nt 439)	F[c] (228)
34	M	42	Back (HIV+)	100	G ins (nt 439)	F (228)
35	NA	NA	Anal	200	G ins (nt 439)	F (228)
36	M	33	Genital (HIV+)	200	G ins (nt 439)	F (228)
37	M	38	NA (HIV+)	200	G ins (nt 439)	F (228)
38	M	26	Penile (HIV+)	200	G del (nt 439)	F (181)
39	M	30	Perianal (HIV+)	200	C ins (nt 556)	F (228)
40	M	36	NA (HIV+)	100	C ins (nt 556)	F (228)
41	M	27	Perianal (HIV+)	100	C ins (nt 556)	F (228)
42	M	39	NA (HIV+)	200	C dels (nt 556)	F (262)
43	NA	NA	NA	100	G ins (nt 439)	F (228)
44	NA	NA	NA	200	C ins (nt 556)	F (228)
45	M	32	Perianal (HIV+)	100	R177W	
46	F	23	Vulva (HIV+)	200	G del (nt 222)	F (85)
47	NA	NA	Genital	200	C del (nt 812)	F (347)
48	M	26	Anal	100	C ins (nt 591)	F (228)
49	M	29	Perianal (HIV+)	200	T ins (nt 628)	F (228)
50	NA	NA	NA	200	Y239Stop	SC[d]
51	M	29	NA (HIV+)	200	P85S; N100H; V192M	
52	NA	NA	NA	100	R34C	
53	M	47	Rectal (HIV+)	100	None	
54	M	30	NA (HIV+)	200	None	
55	NA	NA	NA	100	None	

Isolates 26–32 are ACVs; isolates 33–55 are ACVr.
[a] IC_{90}: Concentration of ACV reducing HSV-specific cytopathic effects by at least 90%.
[b] NA: not available.
[c] F: frameshift mutation.
[d] SC: stop codon.

ACVr HSV-2 was 23–50 years. Of the remaining 9 patients shedding ACVr HSV, clinical information supporting evidence of HIV infection or other immunosuppressive disease was not supplied.

2.2. Virus isolation and susceptibility testing

HSV strains were isolated from clinical samples as previously described (Birch et al., 1990) and the serotype determined either by neutralisation with HSV type-specific antiserum or using a polymerase chain reaction (PCR) assay capable of distinguishing HSV-1 and HSV-2 (Druce et al., 2002). Drug susceptibility testing and determination of ACV and foscarnet inhibitory concentration was as previously described (Birch et al., 1992). Briefly, ACV and foscarnet were diluted in minimal essential medium containing 2% foetal calf serum at concentrations of 200, 100, 25, 10, 1 and 0.1 μg/ml. Each drug dilution was added to confluent monolayers of human fibroblasts in a 96-well microtiter plate which was then incubated for 2 h at 37 °C. Virus diluted to contain 100–300 tissue culture infected doses was then added to each drug dilution and the plate incubated for 72 h at 37 °C. HSV strains replicating in the presence of ≥10 μg/ml ACV were considered to be ACVr; viruses replicating in the presence of ≥200 μg/ml of foscarnet were considered to be foscarnet resistant.

2.3. PCR and nucleotide sequence determination

Because the original phenotypically resistant isolates were likely to contain mixtures of wild-type and resistant viruses, prior to sequencing a pure population of each virus was selected by a single round of plaque purification (Chibo et al., 2002) and retested to ensure it was phenotypically resistant. An identical purification approach was applied to the ACVs strains used as controls. HSV DNA was obtained by the addition of 40 μl of culture supernate from plaque-purified virus to 2 μl of 1% NP-40, followed by incubation at 70 °C for 45 s. The sequences of primers used in the PCR and sequencing reactions for characterisation of the HSV *tk* and

Table 3
Primers used and their nucleotide position relative to the human HSV-2 genome (accession number NC_001798)

Region targeted	Primer name	nt position[a]	Primer sequence
HSV-1 and -2 *tk* gene (full length)	HSVTKF[b]	48049–48029[a]	5′-TGG CGT KRA ACT CCC GCA CCT-3′
	TK-AR	47569–47587[a]	5′-GAY ATG AGG AGC CAR AAC G-3′
	TKINT-FOR	47629–47611[a]	5′-CGC CCA GAT AAC AAT GRG C-3′
	TKINT-REV	47278–47297[a]	5′-MYC GTA RAC RCG GCG AAA TSG-3′
	TK-BF	47269–47250[a]	5′-CAA YAC GGT GCG GTA YCT GC-3′
	HSVTKR[b]	46811–46832[a]	5′-TCT GTY YTT TTA TTG CCG TCA T-3′
HSV POL (amino terminus)	HSV2-BEGPF[b]	63239–63257	5′-GTA TAG CAG GAC AAC GAC C-3′
	HSV1-BEGPF[b,c]	62769–62790	5′-CAT TCC CCT CTT TAG GGG TTC G-3′
	HSV-BEGPA2R	63822–63803	5′-CAG RAG CGT GAT GAC GGT CC-3′
	HSVBEGP-BF	63709–63727	5′-CAC GTG TAC GAC ATC CTG G-3′
	HSVBEGP-CR	64301–64284	5′-CAG TTA AAC TCG ACG TCG-3′
	HSVBEGP-DF	64288–64305	5′-GTC GAG TTT AAC TGY ACG-3′
	HSV-BEGPR[b]	64846–64827	5′-CGT ACA TGT CGA TGT TCA CC-3′
HSV POL (middle)	HSVPOLF[b]	64801–64822	5′-CGC AGC AAG ATM AAG GTG AAC G-3′
	POL-A2R	65446–65428	5′-GGT ACA GGC TGG CAA AGT C-3′
	POL-BF	65157–65176	5′-CCA GAA GGG CTT YAT YCT GC-3′
	POL-CR	65648–65627	5′-GAG CGG ATC TGC TTT CGC ATG G-3′
	POL-DF	65530–65548	5′-GAC TAC CTG GAG ATC GAG G-3′
	POL-ER	66072–66052	5′-GGT GAA CGT YTT TTC GCA CTC-3′
	POL-FF	66003–66021	5′-GAT GGC GAG CCA CAT CTC G-3′
	HSVPOLR[b]	66376–66355	5′-GGA CAA AGT CCT GGA TGT CCC TG-3′
HSV POL (carboxy terminus)	HSVENDP-F[b]	66205–66225	5′-CTG CTG TTT TAC GAC GAT ACC-3′
	HSVENDIN-F	66684–66702	5′-CAA GCT GCT GGT GTC CGA G-3′
	HSVENDIN-R	66771–66753	5′-GTG SGA GAA GTA ATA GTC C-3′
	HSVENDP-R[b]	67214–67195	5′-CAT GCT GTA CGT CAT CTT CC-3′

[a] On the human HSV-2 complete genome, the *tk* gene is in the complement.
[b] Corresponds to PCR and sequencing primers.
[c] Specific primer with nucleotide position relative to HSV-1 genome (accession number X14112).

DNA *pol* genes are shown in Table 3. PCR reactions were carried out using a QIAGEN Taq polymerase kit (QIAGEN, Hilden, Germany). The cycling program consisted of an initial denaturation of 4 min at 94 °C, followed by 40 cycles of 30 s at 94 °C and 30 s at 60 °C. An extension time dependant on the size of the PCR product was carried out at 72 °C, with a final extension of 5 min at 72 °C. PCR products were purified using a QIAquick PCR purification kit (QIAGEN). Purified products were sequenced in the forward and reverse directions using a cycle sequencing reaction (ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin-Elmer, Applied Biosystems Division, Foster City, CA). The reaction products were analysed using an ABI Prism 377 automatic DNA sequencer. Derived nucleotide sequences were analysed and the amino acid sequence determined using the SeqEd version 1.0.3 program (Applied Biosystems).

3. Results

3.1. Virus isolates and drug susceptibility

Tables 1 and 2 show clinical information and drug susceptibility data for the patients and viruses studied. Although complete clinical information was not always available, many of the drug resistant isolates were from patients likely to be immunocompromised, in particular bone marrow transplant recipients or individuals infected with HIV. All isolates were susceptible to foscarnet (results not shown). Chromatograms obtained for plaque-purified viruses did not reveal mixtures of bases, suggesting that sequencing was performed on homogeneous populations.

3.2. HSV tk polymorphisms

Polymorphisms identified in phenotypically ACVs and ACVr isolates for each HSV type are shown in Table 4 and represented in Fig. 1. There were 12 amino acid substitutions in HSV-1 strains and 7 amino acid substitutions in HSV-2 strains that were different to the reference wild-type isolates (1 and 26, respectively) and occurred in either ACVs strains only or in both ACVr and ACVs strains. These substitutions were therefore unlikely to be directly associated with resistance. Fourteen additional polymorphisms were detected only in ACVr isolates (HSV-1 ($n = 8$) and HSV-2 ($n = 6$)). However, these mutations were unlikely to be associated with resistance because the same isolates already contained either insertions, deletions or substitutions in sites critical for enzyme function, or had been previously shown by others to occur in ACVs viruses (Table 4; Morfin et al., 2000).

Table 4
Polymorphisms found in the *tk* genes of HSV-1 and HSV-2 ACVs and ACVr isolates

HSV-1 *tk* polymorphism	Phenotype (number of isolates)	HSV-2 *tk* polymorphism	Phenotype (number of isolates)
G6C	ACVs (2); ACVr (11)	R26H	ACVs (1)
R41H	ACVs (1); ACVr (6)	E39G	ACVs (1); ACVr (3)
L42P	ACVs (2); ACVr (1)	D78N	ACVs (2); ACVr (15)
R89Q	ACVs (3); ACVr (9)	A119T	ACVs (1)
V192A	ACVs (5); ACVr (12)	F140L	ACVs (5); ACVr (18)
G240E	ACVs (1); ACVr (3)	A215T	ACVs (1)
C251G	ACVs (3); ACVr (10)	R220K	ACVs (1)
L267V	ACVs (4); ACVr (10)		
T268P	ACVs (4); ACVr (10)		
E286D	ACVs (5); ACVr (9)		
G302S	ACVs (1)		
H376N	ACVs (5); ACVr (10)		
S23N[a,b]	ACVr (2)	A19V[b]	ACVr (1)
E36K[a,b]	ACVr (2)	A27T[b]	ACVr (1)
A140V[b]	ACVr (1)	S29A[b]	ACVr (1)
F161L[b]	ACVr (2)	T159I[b,c]	ACVr (1)
R281Q[a,b]	ACVr (2)	N245S[b,c]	ACVr (1)
T265A[a,b]	ACVr (1)	R284S[b,c]	ACVr (1)
S276G[b,c]	ACVr (1)		
V348I[b,c]	ACVr (1)		

Isolates 1 (Table 1) and 26 (Table 2) were used as reference sequences.
[a] Not seen in the ACVs isolates studied here, but previously reported as HSV-1 *tk* polymorphisms (Morfin et al., 2000).
[b] Occurring in the presence of mutations known to confer ACVr.
[c] Revealed after insertion or deletion in sequence was manually optimised.

3.3. HSV-1 tk mutations conferring ACVr

To enable intrastrain comparison of *tk* sequences, the viruses studied were compared to a wild-type HSV-1 strain (isolate 1) or a wild-type HSV-2 strain (isolate 26). Comparison of the HSV *tk* sequence of these two reference isolates with that of wild-type viruses registered with GenBank revealed close nucleotide similarity irrespective of serotype (results not shown).

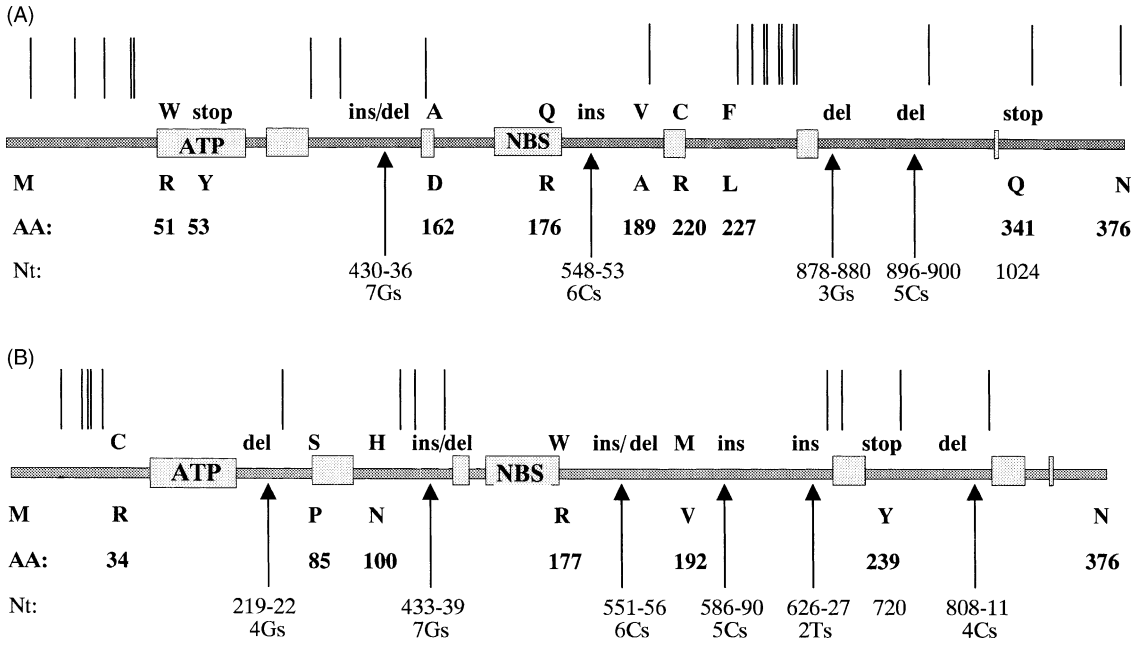

Fig. 1. Mutations identified in the *tk* gene of HSV-1 (a) and HSV-2 (b) isolates resistant to ACV (not to scale). ATP-binding site (ATP), nucleoside-binding site (NBS) and other regions of the *tk* gene conserved among herpesviridae are indicated by the grey boxes. The codon (AA) and nucleotide (Nt) numbers of the wild-type HSV sequence are noted below each gene. Mutational homopolymer runs and the Nts involved are indicated by vertical arrows. Amino acid changes and the relative positions of stop codons (stop), insertions (ins), deletions (del) and either an insertion or a deletion (ins/del) are indicated above each gene. Bars above each gene indicate the locations of polymorphisms.

Of the 18 HSV-1 ACVr isolates studied, 9 carried previously described mutations that accounted for their resistance (isolates 8–16 in Table 1; Gaudreau et al., 1998; Schmit and Boivin, 1999; Morfin et al., 2000). Isolates 8–14 contained insertions and/or deletions at G and C rich homopolymer sites (Fig. 1). Isolates 15 and 16 had amino acid substitutions in the conserved 'active site' of the enzyme, namely the ATP-binding site and the nucleoside-binding site, respectively (Fig. 1). Seven HSV-1 isolates contained mutations not previously described (isolates 17–23 in Table 1). A G deletion from a triple G stretch at nucleotide position 880 was observed in isolate 17 and was likely to produce a non-functional protein as a result of a stop codon generated downstream of the wild-type stop codon. Isolates 18 and 19 contained nucleotide substitutions that produced stop codons; isolates 20–23 contained amino acid substitutions not previously documented as conferring resistance. Two isolates (24 and 25) had no obvious mutation or substitution in their *tk* gene, suggesting that changes in the DNA *pol* gene were the likely basis for the resistance.

3.4. HSV-2 tk mutations potentially conferring ACVr

Of the 23 HSV-2 ACVr isolates characterised, 13 carried previously described *tk* mutations that accounted for their resistance (isolates 33–45 in Table 2; Gilbert et al., 2002) (Fig. 1). Isolates 33–44 contained insertions and/or deletions at G and C rich homopolymer sites. Isolate 45 contained an R177W substitution located in the nucleoside-binding site of the enzyme (Gaudreau et al., 1998). Seven HSV-2 strains contained mutations that have not been previously described (isolates 46–52). Isolates 46–49 contained insertions and/or deletions of single G, C or T nucleotides causing frameshift mutations. Isolate 50 contained an amino acid substitution (Y239Stop) which produced a stop codon in a novel location; isolates 51 and 52 carried amino acid substitutions whose mechanistic role in resistance requires further study. Isolates 53, 54 and 55 contained no detectable *tk* mutations.

Table 6
Mutations identified in DNA *pol* of HSV-1 and HSV-2 ACVr isolates

Patient	HSV-1 DNA *pol* resistance mutations (region)	Patient	HSV-2 DNA *pol* resistance mutations (region)
24	E771Q[a]	53	E250Q[a]
25	K532T (δ-region C)	54	E678G[b]
25	L583V (Exo III)[b]	55	R628C (δ-region C)
		55	D912N[b]

[a] Only mutation seen in DNA *pol* of ACVr, foscarnet sensitive virus.
[b] Associated with resistance in previously reported studies (Kuhn and Knopf, 1996; Hwang et al., 1998; Sarisky et al., 2000; Duffy et al., 2002; Gilbert et al., 2002).

3.5. HSV DNA pol polymorphisms

Polymorphisms in the *pol* gene identified in phenotypically ACVs isolates are shown in Table 5. Analysis of the coding region of the DNA *pol* gene of these ACVs strains and others obtained from Genbank (accession numbers AB070848, AB070847, HE1CG, HS1POL, AB02389, HS2POL, HSV2HG52) revealed 12 amino acid substitutions in HSV-1 strains and 9 amino acid substitutions in HSV-2 strains. After these polymorphisms were excluded from the analysis, amino acid substitutions likely to confer resistance to ACV in the DNA *pol* gene were identified. No polymorphisms were detected in ACVr viruses that did not also occur in ACVs strains (Table 5).

3.6. HSV DNA pol mutations potentially conferring resistance to ACV

The entire open reading frame of the DNA *pol* gene was sequenced in HSV-1 ACVr ($n = 2$) and HSV-2 ACVr ($n = 3$) viruses that did not contain mutations in the *tk* gene (Table 6). Each of these viruses were susceptible to foscarnet. HSV-1 isolate 24 contained a single E771Q substitution; amino acid substitutions K532T and L583V were found in isolate 25. The latter mutation occurred in the Exo III motif, a site conserved among many DNA *pols* and which

Table 5
Summary of polymorphisms in the DNA *pol* of ACVs and ACVr HSV-1 and HSV-2 isolates with no mutations in the *tk* gene

HSV-1 DNA *pol*	Phenotype (number of isolates)	HSV-2 DNA *pol*	Phenotype (number of isolates)
G33S	ACVs (3)	A9T	ACVs (3)
F171S	ACVs (1); ACVr (1)	S15P	ACVs (1)
R330A	ACVs (2)	P60L	ACVs (1)
N522S	ACVs (1)	E139K	ACVs (1); ACVr (1)
A566T	ACVs (2)	D716N (C-II)[a]	ACVs (1)
T639I	ACVs (1)	P801T	ACVs (2)
E684D	ACVs (1)	A904G	ACVs (2)
M906V	ACVs (3)	A905E	ACVs (2)
K908R	ACVs (1); ACVr (1)	G906A	ACVs (2)
H1124P	ACVs (4); ACVr (1)		
A1203T	ACVs (3); ACVr (1)		
A1208T	ACVs (3)		

[a] Mutation occurring in conserved region II.

has been shown to be integral to enzyme function (Hwang et al., 1998). Of the HSV-2 strains, E250Q, E678G and R628C/D912N were present in isolates 53, 54 and 55, respectively (Table 6).

4. Discussion

This study reports on the phenotype and genotype of 41 ACVr HSV clinical isolates. Phenotypically sensitive isolates were also studied to assess heterogeneity within the *tk* and *pol* genes and help determine the frequency of polymorphisms not likely to be related to ACV resistance, although there remains a possibility that these may contribute to the generation of resistance in the presence of other mutations.

Polymorphisms in the *tk* gene of HSV-1 strains have been previously described (Kudo et al., 1998; Morfin et al., 2000). Our study confirmed several of these and also revealed novel substitutions unlikely to be directly associated with ACVr, namely R41H, S276G, G302S and V348I, each of which occurred either in both ACVs and ACVr strains or in the presence of a mutation known to cause resistance.

Heterogeneity in the *tk* gene of HSV-2 isolates has not been thoroughly described apart from F140L (Lee et al., 1999). We identified 12 amino acid substitutions in HSV-2 ACVr strains unlikely to be associated with resistance (Table 4). One of them, E39G, was found in three ACVr isolates and one ACVs isolate. Two of the ACVr isolates harbouring this mutation also contained a G insertion at the G homopolymer sequence hot spot; the other ACVr isolate had no *tk* mutation, but did contain an E250Q mutation in DNA *pol*. The E39G change has been previously reported as causing resistance to ACV (Sasadeusz et al., 1997; Bestman-Smith et al., 2001), although in both cases another mutation was present which could have accounted for the resistance. The observation and its presence in an ACVs isolate suggests its role may be indirect. Of note, none of the polymorphisms we detected in HSV-1 and HSV-2 strains occurred in *tk* 'active sites' (the ATP-binding site between amino acids 51 and 63, the nucleoside-binding site at codons 168–176 and 169–177 of HSV-1 and HSV-2, respectively, and the highly conserved cysteine residue at amino acid 336 (Graham et al., 1986)).

ACVr HSV strains used several mechanisms to confer resistance. The most common was a frameshift mutation caused either by nucleotide insertions or deletions at homopolymer (G or C) repeat sites. These mutations result in the formation of a premature stop codon downstream from the shift and a truncated protein with no *tk* activity (Sasadeusz et al., 1997). They appear to retain replicative capacity using mechanisms that may include inaccurate replication by the DNA *pol* of the extended homopolymeric sequence (leading to the emergence of a subpopulation of virus that could produce sufficient *tk* activity to allow reactivation (Sasadeusz et al., 1997)), or occasional translational frameshifting in the homopolymer stretch resulting in low level production of *tk* (Hwang et al., 1994).

Previously unreported insertions or deletions leading to truncated *tk* were also found in homopolymer repeats of Gs, Cs and Ts in other regions of the gene, including a C deletion detected in isolate 14 which has been previously documented in a laboratory-derived mutant but not in a clinical isolate (Gaudreau et al., 1998). Overall, 59% of the HSV strains analysed utilised the homopolymer insertion/deletion mechanism to generate clinical resistance to ACV.

Nucleotide substitutions in the *tk* that produced either stop codons and a truncated *tk* or a new codon at the site of the substitution occurred less commonly than the homopolymer insertion/deletion mechanism. Two HSV-1 isolates (isolates 18 and 19) and one HSV-2 isolate (isolate 50) contained nucleotide substitutions that produced previously undescribed stop codons. Two HSV-1 and one HSV-2 isolates contained previously reported amino acid substitutions in the ATP-binding site (isolate 15) and the nucleoside-binding site (isolates 16 and 45) (Gaudreau et al., 1998). Analysis of the crystal structure of the HSV *tk* shows that specific amino acids in these functional sites are essential to maintain structural integrity and their mutation renders the virus resistant to ACV (Kussmann-Gerber et al., 1998).

Several substitutions whose role needs to be investigated by mutagenesis were detected in the *tk* gene. Substitutions in conserved regions likely to have caused ACVr in HSV-1 isolates were D162A and R220C. In contrast, the A189V and L227F mutations identified in isolate 22 were located in non-conserved regions (Balasubramaniam et al., 1990). In ACVr HSV-2 isolates, of the detected substitutions R34C, P85S, N100H and V192M, all but the P85S substitution were located in non-conserved regions (Balasubramaniam et al., 1990). The presence of these mutations outside conserved regions does not necessarily rule out their role (either singly or together) in ACVr, and needs to be confirmed in further experiments.

Heterogeneity within the HSV DNA *pol* has not previously been assessed extensively. Amino acid sequence alignment of the ACVs viruses studied here as well as others obtained from Genbank revealed a number of polymorphisms for each serotype. No HSV-1 strains contained polymorphisms in conserved regions. The same observation was made for all but one substitution in the HSV-2 strains, the exception being wild-type HSV-2 isolate 26 which contained a D716N substitution in conserved region II yet remained sensitive to ACV. Amino acid sequence alignments of various herpesviruses shows that the position is not conserved among this group, and includes N716 in HSV-1 DNA *pol* and S716 in cytomegalovirus enzyme.

We identified amino acid substitutions in both conserved and non-conserved regions of DNA *pol* in 5 ACVr isolates (12%) with wild-type *tk* sequence. In HSV-1 isolates these involved an E771Q mutation occurring immediately adjacent to conserved region VI in isolate 24 and both K532T and L583V located at the beginning of the conserved δ-region

C and in the Exo III motif within δ-region C, respectively, in isolate 25. Viruses carrying mutations within the Exo III motif have been previously associated with foscarnet resistance and ACV sensitivity (Hwang et al., 1998). However, the L583V mutation in isolate 25 was associated with ACVr and foscarnet sensitivity. This suggests that individual mutations within this site may have different influences on drug resistant phenotype. Further studies including mutagenesis will help to test this hypothesis.

Three HSV-2 ACVr strains contained mutations in the DNA *pol*. An E250Q mutation was detected in a non-conserved region encoding the amino-terminal end of the protein in isolate 53. No mutation conferring drug resistance has been previously associated with this portion of the DNA *pol* gene and mutagenesis experiments will be required to establish its role in ACVr. An E678G substitution was also identified in a non-conserved region between δ-region C and region II in isolate 54. The same mutation has been previously identified in a resistant HSV-2 strain (Sarisky et al., 2000; Duffy et al., 2002). However, that virus also had the substitutions K943R in conserved region VII and D1021G in a non-conserved region. It is therefore unclear whether one or more of these mutations are responsible for the ACVr phenotype. Isolate 55 harboured two amino acid substitutions, R628C located one amino acid from the end of δ-region C and D912N located in the non-conserved region between I and VII. A D912V/A substitution has been previously noted in a clinical isolate resistant to ACV, foscarnet and adefovir (Gilbert et al., 2002), suggesting an important role for this site in the generation of ACV resistance. In this study, we did not investigate the DNA *pol* sequence of isolates that already harboured a *tk* mutation, but focussed on the DNA *pol* of those isolates we could not resolve by sequencing the *tk* alone. In a previous study we sequenced the DNA *pol* and *tk* genes of a patient who was resistant to both ACV and foscarnet (Chibo et al., 2002). We identified a large deletion in the *tk* gene that could account for the ACV resistance and a mutation in conserved region II of the DNA *pol* gene which could account for the foscarnet resistance.

Our results demonstrate the diversity of the mechanisms involved in HSV resistance to ACV. Most mutations conferring resistance to ACV in HSV-1 and HSV-2 clinical isolates occurred in the *tk* gene, usually in homopolymer stretches of Gs and Cs (59% of all ACVr isolates). Nucleotide substitutions producing stop codons in novel locations (7%) or novel amino acid substitutions (15%) were also identified. Previously described mutations in *tk* active sites were also found (7%). Less commonly, resistance mutations occurred in conserved and non-conserved regions of the DNA *pol* gene (12% of all ACVr isolates). These studies highlight the heterogeneity existing within HSV strains through the identification of a number of previously undescribed polymorphisms that do not confer resistance, and also identify novel mutations likely to result in phenotypically ACVr HSV.

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