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Case Report

Acyclovir-Resistant Varicella-Zoster Virus: Phenotypic and Genetic Characterization

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A man with acquired immunodeficiency syndrome (AIDS) developed zoster of the right arm which was resistant clinically to acyclovir. Varicella-zoster virus (VZV) was cultured from a skin biopsy performed at the beginning of acyclovir therapy (isolate 1) and after its failure (isolate 2). The emergence of acyclovir resistance during treatment was investigated by developing a simple and rapid drug sensitivity assay based on the plaque reduction reference method. This late-antigen synthesis reduction assay involved serial dilutions of cell-associated virus. The 50% inhibitory concentration (IC50) of acyclovir was 16 ± 7.5 μM for the susceptible reference strain OKA, in agreement with published data. The acyclovir IC50 increased from 6.5 μM for isolate 1 to 100 μM for isolate 2. In comparison with the sequence of isolate 1, isolate 2 had a single mutation consisting of a C to T change at position 907 of the thymidine kinase gene, which changed a glutamine codon into a stop codon at position 303 of the thymidine kinase protein. These results show the emergence of acyclovir resistance through a single previously undescribed mutation in the thymidine kinase gene, and confirm the heterogeneity of mutations inducing acyclovir resistance. ***J. Med. Virol. 55: 250–254, 1998.***

KEY WORDS: varicella-zoster virus; acyclovir; resistance; susceptibility assay; thymidine kinase

INTRODUCTION

The genome of varicella-zoster virus (VZV), a member of the herpesvirus family, encodes a 35-kDA protein called thymidine kinase (TK), which has a similar amino acid sequence and molecular weight to the kinase encoded by herpes simplex virus (HSV) types 1 and 2. Acyclovir is a guanosine analogue of choice for the treatment of primary and recurrent VZV infection in immunocompromised patients [Balfour et al. 1983]. VZV TK selectively phosphorylates acyclovir to its monophosphate derivative, which is further phosphorylated by cellular kinases to its triphosphate form (the active metabolite). Acyclovir-resistant strains of VZV have been isolated from patients with acquired immunodeficiency syndrome (AIDS) after long-term acyclovir therapy for chronic or recurrent VZV infection [Pahwa et al., 1988; Jacobson et al., 1990; Snoeck et al., 1994] and from patients who become unresponsive to acyclovir [Sawyer et al., 1988; Linnemann et al., 1990; Talarico et al., 1993]. The molecular basis of VZV resistance to acyclovir has been studied primarily with laboratory mutants [Sawyer et al., 1988; Roberts et al., 1991]. Some resistant clinical isolates have also been examined [Talarico et al., 1993]. Most acyclovir-resistant VZV isolates do not express a functional TK, or have a TK with altered activity. Molecular studies showed that resistance was conferred by single mutations located throughout the TK gene [Talarico et al., 1993; Boivin et al., 1994].

The acyclovir sensitivity of two VZV strains isolated before and after acyclovir treatment of an AIDS patient with clinical resistance to the drug was examined. A simple and rapid drug sensitivity assay was developed based on the plaque reduction reference method. The TK gene of the two isolates was sequenced to detect the emergence of mutations during treatment.

CASE REPORT

The patient was a 24-year-old man known to have been infected by human immunodeficiency virus (HIV)

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since 1988. In November 1990, he developed zoster of the right arm and received oral acyclovir (unknown dose). In June 1992, he developed a new episode of vesiculobullous zoster on the right arm. The CD4+ cell count was 9/mm^3. VZV was cultured from a skin biopsy performed on June 12, 1992 (isolate 1). The patient was treated subsequently with intravenous acyclovir, 10 mg/kg thrice daily (1.5 g/day) from June 12. Sinus lymphoma was diagnosed at the same time and he received solumedrol (80 mg/day) from June 13 to July 3. He also received two courses of polychemotherapy (doxorubicin, bleomycin, vindesin, and cyclophosphamide) on June 22 and July 14. Because the zoster on the right arm was not cured after 3 consecutive weeks of intravenous acyclovir followed by 1 week of oral acyclovir (4 g daily), this treatment was stopped on July 7. Again VZV was cultured from a skin biopsy of a crusted hyperkeratic lesion of the right arm on July 16 (isolate 2). Cytomegalovirus sinusitis was diagnosed on July 21, and he received intravenous foscarnet (200 mg/kg/day). The zoster healed and had not relapsed when the patient died in December 1992.

MATERIALS AND METHODS

The drug susceptibility of the VZV isolates and the reference strain OKA was determined by using the late-antigen synthesis reduction assay in 24-well plates containing confluent human fibroblasts at 48 hr of culture. The inoculum was obtained from 25 cm^2 infected cell layers with approximately 50 recent cytopathic effects. After trypsinization, infected cells were resuspended in 4 ml of minimum essential medium (MEM) without fetal calf serum. Serial 10-fold dilutions (10^{-1}–10^{-4}) of infected cell suspension were inoculated. Five antiviral concentrations were tested. Antiviral drugs were diluted in MEM containing 10% fetal calf serum (Gibco BRL, Paisley, Scotland). Antiviral drug dilutions could be stored at +4°C for no more than a few hours. The culture medium from 6 wells in line was removed by aspiration and 200 μl of 10^{-4} viral dilution was added per well. The same operation was carried out with the 10^{-3}–10^{-1} dilutions. Plates were incubated for 1 hr at 37°C with 5% CO_2. The isolates (in duplicate) and the OKA strain were tested in parallel. After incubation, viral dilutions were removed and 1 ml of antiviral drug dilution (acyclovir or foscarnet) was added per well. The titer of the inoculum was determined in two columns free of antiviral drug. Final drug concentrations were 100, 20, 5, and 1 μM acyclovir and 200, 100, 50, and 20 μg/ml foscarnet. After 48 hr of incubation at 37°C with 5% CO_2, the culture medium was removed by aspiration and the cell layer was fixed with acetone. After 2 washes in phosphate buffer solution (PBS), cells were treated for antigen detection with 200 μl of VZV-specific mouse antibody (clone 1U1, Argène Biosoft, Varilhes, France) at 1/150 dilution for 45 min. This antigen, probably a late antigen, was characterized by staining VZV-infected epithelial cells in an immunofluorescence procedure with this monoclonal antibody. The cytoplasm and cytoplasmic membrane of VZV-infected cells were stained with this antibody and with a fluorescein-conjugated monoclonal antibody (Martine Harzic, personal communication). After 2 washes in PBS, the antigen-antibody complex was detected with a peroxidase-conjugated anti-mouse antibody in the presence of diaminobenzidine (Sigma, St Louis, MO). Foci were counted under an optical microscope. To differentiate a focus of late-antigen synthesis from staining due to the inoculum, a conglomerate of at least four infected cells was needed to define a focus (Fig. 1). No foci were clearly identified after 24 hr of incubation, whereas confluent foci preventing accurate reading were observed after 72 hr. Fifty percent inhibitory concentrations (IC50) were calculated by means of linear regression.

The polymerase chain reaction (PCR)-DNA products of the TK gene were sequenced according to Lacey et al. [1991]. The main 1,181-bp product was excised from the gel, eluted with the Jetsorb kit (Genomed, Bad-Oeynhausen, Germany), and cleaved with *Eco*RI and *Xho*I (Boehringer Mannheim, France). After purification through a Microspin column (Pharmacia, Gaithersburg, MD), the restriction fragments were ligated into plasmid Blue Script KS+ (Stratagene, La Jolla, CA) and cloned into XL1-blue (*Escherichia coli,* Stratagene). Sequencing was performed as previously described [Mabillat et al., 1990] with the single-strand binding protein modified T7 sequencing kit (Pharmacia). Sequencing was done on sense and nonsense strands of one clone and confirmed on a PCR product to eliminate errors due to Taq DNA polymerase.

RESULTS

The IC50 values for the OKA strain and clinical VZV isolates are shown in Table I. The mean IC50 of acyclovir was a little higher than the mean value described for the cell-associated virus OKA in the plaque reduction assay [Sawyer et al., 1988; Pawha et al., 1988], while that of foscarnet was similar. Isolate I was sensitive, with acyclovir IC50 values lower than those of OKA, as previously described for wild strains [Crumpacker et al., 1979; Biron and Elion, 1980]. In contrast, the isolate 2 IC50 values were clearly increased (10.6–27-fold). Both isolates were fully sensitive to foscarnet, with IC50 values in the range of OKA. The increase in the acyclovir IC50 and the absence of any change in foscarnet susceptibility were highly suggestive of a TK defect [Talarico et al., 1993; Boivin et al., 1994]. This prompted us to sequence the TK gene for mutations similar to those previously identified (Table II).

A comparison of the TK gene sequences of these two isolates and those of the Dumas strain described by Davison and Scott [1986] showed that both isolates bore the S-288 to L substitution found in all VZV strains except for one isolate [Boivin et al., 1994] and the Dumas strain. In comparison with the sequence of isolate 1, isolate 2 had a single mutation consisting of a

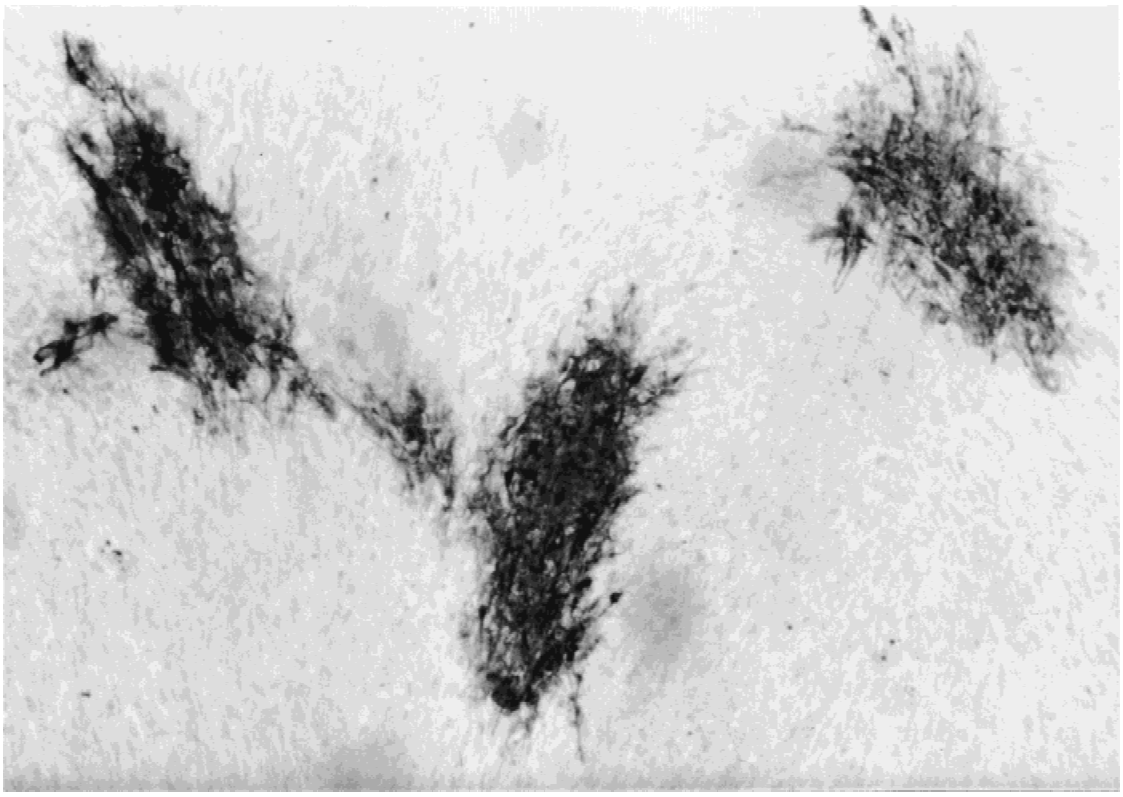

Fig. 1. Different aspects of late-antigen synthesis foci under the optical microscope. ×40.

TABLE I. IC50 Values in the Late-Antigen Assay and Sequence Changes Found in the TK Gene of the Acyclovir-Resistant Isolate (2) Relative to the Acyclovir-Sensitive Isolate (1)

	IC50		Type of change in TK gene[a]	
VZV strain	Acyclovir (μM)	Foscarnet (μg/ml)	Nucleotide	Amino acid
OKA	16 ± 7.5[b]	20.9 ± 10.8[b]		
Isolate 1	3.7–9.4[c]	20–24[c]	No change	No change
Isolate 2	100 –> 100[c]	27–28[c]	C-907 → T	Q-303 → stop

[a]Compared with the published sequence of VZV [Davison and Scott, 1986] and excluding the widespread S-288 → L change (see text).
[b]Mean values ± SD.
[c]Results of 2 experiments.

C to T change at position 907 of the TK gene, which changed the glutamine into a stop codon at position 303 of the protein. This change predicted the synthesis of a truncated enzyme, like other reported mutations (Table II).

DISCUSSION

This clinical case of acyclovir resistance is unusual because it occurred after only 3 weeks of intravenous acyclovir therapy at the appropriate dose. This may be explained partly by the patient's severe immunodeficiency (CD4 cell count 9/mm^3), simultaneous polychemotherapy, and corticosteroids for lymphoma. Acyclovir-resistant clones of VZV might have been selected during previous oral treatment (unknown dose) and remained present. No resistant viruses were detected in isolate 1, probably because they were rare, but they represented the majority of viruses in isolate 2, after acyclovir therapy.

The results of the late-antigen synthesis reduction assay were in keeping with the clinical course. The assay appeared to be reliable for the detection of VZV resistance to acyclovir. A case of foscarnet-resistant multidermal zoster correlating with foscarnet resistance in this assay has been reported [Fillet et al., 1995]. The assay can be used with other anti-VZV drugs, and might prove useful for testing new agents. IC50 values were obtained after 48 hr of culture, with fair reproducibility. As cytopathic effects are measured

TABLE II. Known Mutations in the TK Gene Associated With Acyclovir Resistance*

TK gene change			
Nucleotide	Peptide	Frequency	Reference
/	/	1	Boivin et al. [1994]
del ATTT47-50	A37 - stop	1	Talarico et al. [1993]
G71 - A	G24 - E	1	Boivin et al. [1994]
del A72	138 - stop	1	Boivin et al. [1994]
add A72	R54 - stop	1	Boivin et al. [1994]
A74 - G	K25 - R	1	Talarico et al. [1993]
del A76	I38 - stop	1	Talarico et al. [1993]
A176 - G	E59 - G	1	Talarico et al. [1993]
G385 - A	D 129 - N	1	Talarico et al. [1993]
G389 - A	R130 - Q	1	Sawyer et al. [1988]
T412 - C and C725 - T	C138 - R and S242 - F	1	Talarico et al. [1993]
A427 - G	R143 - G	1	Talarico et al. [1993]
G428 - A	R143 - K	1	Talarico et al. [1993]
T461 - C	L154 - P	1	Sawyer et al. [1988]
del C493	V171 - stop	1	Boivin et al. [1994]
add C493	V194 - stop	1	Boivin et al. [1994]
G675 - A	W225 - stop	2	Sawyer et al. [1988]
del A677, C678	C231 - stop	1	Boivin et al. [1994]
del A681, C682	C231 - stop	1	Talarico et al. [1993]
del C682, T683	C231 - stop	1	Boivin et al. [1994]
add T889, C890	L298 - stop	1	Boivin et al. [1994]
C907 - T	Q303 - stop	1	Our results
G922 - C[a] and T412 - C	E308 - Q and C138 - R	1	Boivin et al. [1994]
del 873 bp	del R14 - Q303	1	Snoeck et al. [1993]

*del = deletion; / = no mutation.
[a]No change in TK activity if alone.

by reading dark-brown foci, the assay is objective. However, it requires strain isolation, which is hindered by the fragility of VZV.

The results of the genotypic characterization are in keeping with previous reports that a single mutation is sufficient to produce resistance to acyclovir and that changes can appear almost anywhere on the genome (Table II). The mutation observed now, which changed the glutamine into a stop codon at position 303 of the TK protein, has not been reported previously, but lies in a region whether other mutations have been described [Boivin et al., 1994]. In particular, a mutation creating a stop codon has been described at position 298 (Table II).

These findings show the emergence of acyclovir resistance through a single mutation in the TK gene, and confirm the heterogeneity of mutations conferring resistance, a phenomenon that rules out the detection of resistance by a point mutation PCR assay or by sequencing a short part of the TK gene.

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