ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2006, p. 4038–4043 Vol. 50, No. 12
0066-4804/06/$08.00+0 doi:10.1128/AAC.00380-06

Mutations in the E9L Polymerase Gene of Cidofovir-Resistant Vaccinia Virus Strain WR Are Associated with the Drug Resistance Phenotype▽

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Received 28 March 2006/Returned for modification 8 May 2006/Accepted 7 September 2006

Cidofovir (CDV) is an effective drug against viruses of the *Orthopoxviridae* family and is active in vitro against variola virus, the cause of smallpox. However, CDV-resistant poxviruses can be generated by repeated in vitro passage in the presence of suboptimal concentrations of CDV. To determine if mutations in the E9L polymerase gene could confer resistance to this nucleoside analog, this gene was sequenced from CDV-resistant vaccinia virus and found to encode five amino acid changes, centered on an N-terminal region associated with 3′→5′ exonuclease activity. Transfer of this mutant E9L gene into wild-type vaccinia virus by marker rescue sufficed to confer the resistance phenotype. E9L polymerase mutations occurred sequentially during passage in CDV, and an H296Y/S338F double mutant that conferred an intermediate CDV resistance phenotype was identified. In vitro, the marker-rescued CDV-resistant vaccinia virus containing all five mutations grew nearly as well as wild-type vaccinia virus. However, the virulence of this virus for mice was reduced, as 10- to 30-fold more CDV-resistant virus than wild-type virus was required for lethality following intranasal challenge. Cidofovir and hexadecyloxypropyl-cidofovir gave partial protection to mice infected with the virus when used at 50 and 100 mg/kg of body weight given as single treatments 24 h after virus exposure, whereas 2-amino-7-[(1,3-dihydroxy-2-propoxy)methyl]purine (compound S2242) was completely protective at 25, 50, and 100 mg/kg/day when given daily for 5 days. These findings suggest that drug therapy for poxviruses may be complicated by drug resistance but that treatment of the infection with currently known compounds is possible.

A number of poxviruses, including variola virus and monkeypox virus (both orthopoxviruses) and the molluscum contagiosum virus (a molluscipoxvirus), can cause infections of various severities in humans. Of these, variola virus is a particular concern, given the possibility that it could be used as a bioterrorist agent (9). As a result, significant efforts have been made toward reducing the risks associated with a smallpox outbreak, including prophylactic vaccination and the institution of a public health infection control plan. In addition, antiviral chemotherapy against variola virus, the agent of smallpox, has great potential as a means to reduce the morbidity and mortality of smallpox. Of the available agents, cidofovir, (*S*)-1-(3-hydroxy-2-phosphonyl-methoxypropyl)cytosine (CDV), has emerged as the therapeutic of choice (2, 4, 14, 19, 22). CDV is already approved for human use in the treatment of cytomegalovirus infection in AIDS patients, and there is considerable clinical experience with using this drug. In addition, CDV has shown activity against variola, monkeypox, vaccinia, cowpox, and camelpox viruses in vitro (2, 10, 21) and has produced good clinical responses against molluscum contagiosum virus in humans (5).

As with other antimicrobial drugs, there is the potential that antiviral chemotherapy might select for drug resistance, as exemplified by the high incidence of drug resistance in current isolates of human immunodeficiency virus (HIV) type 1 (17) and hepatitis B virus (25). In the case of poxviruses, we previously showed that significant resistance to CDV could develop when vaccinia, monkeypox, camelpox, and cowpox viruses were repeatedly passaged in the presence of suboptimal concentrations of CDV (21, 23). Whereas the wild-type (WT) poxviruses were sensitive to achievable concentrations of CDV, the 50% effective concentration (EC_{50}) values of the resistant viruses were 8- to 27-fold higher. In an in vivo model using CDV treatment for SCID mice, CDV-resistant cowpox (an EC_{50} value 16-fold higher than that of the wild type) killed all of the mice, whereas all treated mice infected with wild-type cowpox survived. Since all of these viruses are related to variola virus (8), further studies were undertaken to identify the molecular basis of the resistance phenotype, beginning with vaccinia virus as the prototypical poxvirus.

The diphosphoryl derivative of CDV is incorporated into DNA by purified vaccinia virus DNA polymerase in vitro (12). While the viral E9L DNA polymerase could be proposed as the site of the CDV resistance mutation, a role for the A20R protein (11) and/or other viral proteins cannot be excluded a priori. Support for the role of E9L in CDV resistance has already been provided by DNA polymerase inhibition assays performed with wild-type and CDV-resistant forms of cowpox

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▽ Published ahead of print on 18 September 2006.

virus (21). Consequently, the E9L gene was cloned from the CDV-resistant vaccinia virus strain Western Reserve (WR) and was found to encode five amino acid mutations, of which two together (H296Y and S338F) confer a phenotype of intermediate CDV resistance. To confirm that these mutations are sufficient to confer the resistance phenotype, the mutant E9L gene was introduced into minimally passaged wild-type virus by marker rescue followed by CDV selection. The EC_{50} of the resulting recombinant virus was ≥10-fold higher than that of the wild-type virus. The wild-type and marker-rescued viruses grew similarly in African green monkey kidney (Vero) cells, but the resistant virus replicated less well in mouse mammary tumor (C127I) cells in culture. These findings make a strong case for the E9L polymerase gene as the site of CDV resistance mutations and provide data showing a reduced but still-appreciable virulence of CDV-resistant vaccinia virus for mice.

MATERIALS AND METHODS

Viruses and cells. CDV-resistant vaccinia virus (strain WR) was derived from vaccinia virus strain WR (obtained from the American Type Culture Collection, Manassas, VA) by 15 consecutive passages in Vero cells under increasing concentrations of CDV (23), as was also done previously with vaccinia virus strain Copenhagen (21). These concentrations of CDV ranged initially from 50 μM at the onset of selection to 1,000 μM at the end. As a control, a CDV-sensitive stock of vaccinia virus was derived by 15 passages in Vero cells in the absence of CDV. A marker-rescued form of the CDV-resistant virus (referred to herein as Vac-CDV-R^{mr}) was prepared by inserting the DNA polymerase from the CDV-resistant virus into a wild-type virus background as described below. African green monkey kidney (Vero) and mouse mammary tumor (C127I) cells were obtained from ATCC. African green monkey kidney (MA-104) cells were purchased from BioWhittaker (Walkersville, MD). The latter cell line was used to propagate high-titer virus for animal experiments.

Antiviral compounds. Three substances shown to possess antiorthopoxvirus activity in animal models were selected for evaluation by use of infected mice. These included cidofovir, hexadecyloxypropyl (HDP)-CDV, and 2-amino-7-[(1,3-dihydroxy-2-propoxy)methyl]purine (compound S2242) (15, 20). Cidofovir was kindly provided by Mick Hitchcock of Gilead Sciences (Foster City, CA). HDP-CDV was synthesized as described previously (3). S2242 was provided through the antiviral testing program of the National Institute of Allergy and Infectious Diseases, NIH. Cidofovir and S2242 were dissolved in sterile saline, whereas HDP-CDV was dissolved in water for animal studies. Each compound was given by the preferred mode of administration; HDP-CDV was given by the oral route (p.o.), whereas the other compounds were administered intraperitoneally (i.p.) for maximum bioavailability. Sterile saline, injected by the i.p. route, served as the placebo control for antiviral studies in mice.

Cloning and sequencing of E9L polymerase regions. To prepare viral DNA, 10 μl of virus stock was added to 90 μl of proteinase K (400 μg/ml in 150 mM NaCl, 20 mM Tris-HCl [pH 7.5], 1% [vol/vol] Triton X-100). The mixture was incubated at 60°C for 30 min to degrade protein and subsequently at 95°C for 15 min to inactivate the proteinase K. For PCR, two primers outside the E9L coding regions were used: forward primer 5′-ATCCGATTATGAGACATTAGCTAA TATTAGTAC-3′ (53449 to 53481) and reverse primer 5′-CTAAACCCGCTT GCGAAAGTACTATA-3′ (56757 to 56732); the numbers in parentheses indicate positions in GenBank sequence AY243312.1. These primers lie outside the coding region and were chosen to hybridize with a wide range of poxvirus sequences. Aliquots of viral DNA were amplified using *Pfu* Ultra Hot Start PCR master mix (Stratagene, San Diego, CA) and the following touch-down thermocycling protocol: 95°C for 2 min; then 20 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 6 min, where the annealing temperature was lowered from 65°C to 55°C at the rate of 1°C every two cycles; then 15 cycles at annealing temperature 55°C; and finally 72°C for 10 min. Following electrophoresis in 1.5% agarose gels, the DNA was isolated and purified using silica gel (QIAquick gel extraction kit; QIAGEN, Valencia, CA) and cloned into pCR2.1 (TopoPCR kit; Invitrogen, San Diego, CA). Plasmids were prepared using an EndoFree plasmid maxi kit (QIAGEN). Sequences were obtained with 11 primers spaced along the 3.3-kb sequence by use of the dye terminator method and an automated sequencer. pE9L-wt is the plasmid that contains the wild-type E9L polymerase sequence, whereas pE9L-R contains the mutations associated with CDV resistance.

Marker rescue insertion of cloned E9L sequences into vaccinia virus. The mutant E9L polymerase gene was inserted into vaccinia virus strain WR by homologous recombination (marker rescue) essentially as described by Earl and Moss (7). Vero cells were plated at 10^5 per well in six-well plates in Dulbecco's minimal essential medium, 2 mM L-glutamine, and 10% fetal bovine serum (D10). The following day, the cells were infected with approximately 10^5 PFU per well of virus at 37°C for 2 h. Meanwhile, 0.8 μg of pE9L-R plasmid DNA was prepared with Lipofectamine 2000 in Opti-MEM I (Invitrogen) according to the manufacturer's instructions and used to transfect the cells. Following an additional 4 days of culturing, the dying cell monolayer was placed in a tube and lysed using three freeze-thaw cycles. Then, aliquots were prepared as serial 10-fold dilutions and plated onto new Vero monolayers in D10 either with or without 200 μM CDV. In this first round of selection, the number of plaques in the CDV-containing wells was about 0.6% of that of wells without CDV. After 3 days of culturing, well-separated individual plaques from the pE9L-R wells were picked using silicone-greased cloning rings and trypsin-EDTA and transferred to wells of fresh Vero cells for expansion by another 4 days of culturing in the presence of 200 μM CDV. Lysates from the wells that showed the most extensive cytopathic effects were aliquoted and stored frozen at −80°C. The newly developed marker-rescued Vac-CDV-R^{mr} virus was prepared with the minimum amount of cell culturing in order to avoid attenuating it for the animal experiments.

Construction of single-mutation E9L polymerase mutants and their insertion into vaccinia virus by marker rescue. Mutations for each of the five nonsynonymous codon changes were individually introduced into the plasmid for wild-type E9L polymerase (pE9L-wt) by use of mutagenesis primers and a QuikChange site-directed mutagenesis kit (Stratagene, Inc., La Jolla, CA). The five single-mutation clones were sequenced on both strands to confirm that only the expected mutations were present. Then, the single-mutation forms of E9L were introduced into vaccinia virus strain WR by marker rescue as described above, except that a 10-fold-lower dose of CDV (20 μM) was used to select for slightly resistant viruses in the Vero cell cultures. Individual plaques were isolated as described above and expanded in Vero cells in the absence of CDV, since they did not replicate upon transfer to new Vero cultures in the presence of 20 μM CDV. As an exception, the virus constructed to contain the S338F mutation was able to grow in the presence of 20 μM CDV, and this isolate was further expanded using a second passage in Vero cells in the presence of 20 μM CDV, following which its E9L region was PCR cloned and resequenced, which identified the acquisition of a second, unplanned H296Y mutation.

Drug susceptibility assays and growth characteristics of marker-rescued virus. The antiviral activity of CDV was determined against the original wild-type virus and recombinant Vac-CDV-R^{mr} virus in Vero and mouse mammary tumor (C127I) cells. These cells were chosen because CDV has shown different degrees of potency against poxviruses grown in these cell lines (20). Plaque reduction assays were performed using our standard assay in 12-well microplates, which required 3 days of virus replication for plaque growth, followed by fixing and staining with 0.2% crystal violet in 10% buffered formalin (20). EC_{50} values were determined by plotting the percentage of plaques versus the drug concentration on semilog paper. It has been previously shown that other CDV-resistant poxviruses (i.e., cowpox and monkeypox viruses) replicate poorly relative to wild-type viruses in mouse but not Vero cell lines (21). Since this may be the case with the marker-rescued Vac-CDV-R^{mr} vaccinia virus strain WR, the replication of this virus and that of the wild-type virus were compared in both C127I and Vero cells. The studies were done by infecting cell monolayers in 12-well microplates with approximately 200 virus plaques per well. After 3 days of incubation, the plates were frozen. Later, the cells and supernates from thawed plates were collected, the cells were pelleted, and the cell pellets sonicated in a small liquid volume for 1 min to release virus. This medium was recombined with the saved supernates, and the titer was determined by plaque assay on fresh monolayers of Vero cells in 12-well microplates. Three independent antiviral and virus titer production experiments were performed, with cell lines tested in parallel.

Mouse experiments. Specific-pathogen-free female BALB/c mice weighing from 13 to 15 g were obtained from Charles River Labs (Wilmington, MA) and studied under a protocol approved by the Institutional Animal Care and Use Committee of Utah State University. Mice were infected intranasally with 0.05 ml of CDV-R^{mr} virus following anesthesia with ketamine (100 mg/kg of body weight by i.p. injection). Virus was given in 3.2-fold (half-$\log_{10}$) dilution increments to groups of BALB/c mice, with the highest dose being $10^{6.5}$ PFU of the CDV-R^{mr} virus. Mice were held for 21 days or euthanized when moribund. From this work, a dose of $10^{6.0}$ PFU per mouse was selected to study the treatment of mice infected with this virus. Treatments were initiated 24 h after virus challenge and administered according to the treatment regimen indicated below (see Table 4). The route and length of each treatment were optimized based upon our previous experience with the compounds in the infection model (19–21, 24).

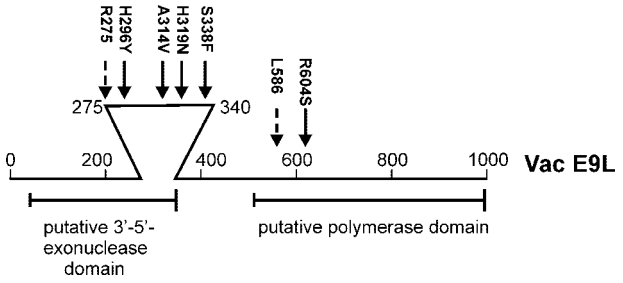

FIG. 1. Location of mutations in CDV-resistant E9L polymerase. Seven mutations that were not present in wild-type vaccinia virus were identified in E9L from CDV-resistant vaccinia virus. Nonsynonymous coding mutations are shown with a solid line, and silent synonymous mutations are shown with a dashed line (at positions R275 and L586). Amino acids associated with these changes are as follows: R275 (CGT→CGC), H296Y (CAT→AAT), A314V (GCC→GTC), H319N (CAC→AAC), S338F (TCT→TTT), L586 (CTC→CTA), and R604S (AGA→AGC).

Statistical interpretations. Increases in animal survivor numbers were statistically analyzed by the two-tailed Fisher exact test. Mean increases in the time to death were analyzed by the two-tailed Mann-Whitney U test. Both tests were performed using the InStat computer program (GraphPad Software, San Diego, CA).

RESULTS

To determine if CDV resistance is associated with mutations in the E9L polymerase gene, the entire coding region of the gene (6) was amplified by PCR and cloned into a plasmid for sequencing. The nucleotide sequence of the E9L gene of vaccinia virus strain WR passaged 15 times in Vero cells in the absence of CDV contained no mutations and was identical to the wild-type sequence for vaccinia virus strain WR (GenBank AY243312.1, 53636 to 56656). However, the E9L gene of the CDV-resistant virus contained seven mutations, as shown in Fig. 1. Two mutations were synonymous, whereas the other five changed the amino acid sequence of the protein (H296Y, A314Y, H319N, S338F, and R604S).

To prove that mutations in the E9L gene were responsible for CDV resistance, the coding sequence of the mutated E9L polymerase from the CDV-resistant virus was transferred into wild-type vaccinia virus strain WR by marker rescue (thus producing Vac-CDV-R^{mr}). We then used media containing 200 μM CDV to select for recombinant viruses exhibiting CDV resistance. Cloned virus was expanded by one passage in African green monkey kidney (MA-104) cells and then analyzed for inhibition by CDV and for replication potential in cells (Table 1). As was shown previously (20), CDV was much more active in C127I cells than in Vero cells. It required 14 times more CDV in C127I cells and 11 times more drug in Vero cells to cause a degree of inhibition of Vac-CDV-R^{mr} viral plaque numbers similar to that for wild-type virus. For comparative purposes, we evaluated HDP-cidofovir and S2242 against these viruses in cell culture (Table 1). Results indicated that the CDV-resistant virus was also resistant to HDP-cidofovir but not to S2242. Since HDP-cidofovir is a prodrug form of cidofovir, these results were not surprising, Similar results with these compounds being used against other cidofovir-resistant poxviruses have been published previously (20, 21, 23). Virus replication performed in the two cell lines demonstrated

TABLE 1. Antiviral activity of compounds against WT and marker-rescued CDV-resistant (CDV-R^{mr}) vaccinia WR viruses

Compound	EC_{50} (μM)[a] of indicated virus in:			
	C127I cells		Vero cells	
	WT virus	CDV-R^{mr} virus	WT virus	CDV-R^{mr} virus
Cidofovir	2.1 ± 0.7	29 ± 6 (14)	92 ± 8	1030 ± 250 (11)
HDP-cidofovir	0.31 ± 0.2		0.24 ± 0.1	4.6 ± 1.1 (19)
S2242	9.5 ± 3.8	10.6 ± 3.1 (1.1)	52 ± 1.0	52 ± 4.9 (1.0)

[a] Fifty percent inhibitory concentration determined by plaque reduction assays (log_{10} PFU/ml). Values represent means ± standard deviations (n = 3). Numbers in parentheses for CDV-R^{mr} virus indicate change (n-fold) in susceptibility to CDV (CDV-R^{mr} virus EC_{50} divided by WT virus EC_{50}).

that Vac-CDV-R^{mr} and the wild-type virus replicated equally well in Vero cells ($10^{6.7}$ PFU/ml versus $10^{6.8}$ PFU/ml, respectively), but the replication of Vac-CDV-R^{mr} was fivefold less than that of the wild-type virus in C127I cells ($10^{6.9}$ PFU/ml versus $10^{7.6}$ PFU/ml, respectively). Thus, the overall efficiency of Vac-CDV-R^{mr} replication was compromised in C127I cells.

Two further experiments with Vero cells were performed in an effort to identify which mutations were most important for the CDV resistance phenotype. First, additional sequences were obtained from the first eight passages under the selection pressure of increasing concentrations of CDV. As shown in Table 2, two mutations (H296Y and S339F) came up by the second passage in CDV. By the sixth passage, all five mutations were present. In a second experiment, individual mutations were introduced into the plasmid for wild-type E9L and transferred by marker rescue into vaccinia virus strain WR. Only very small plaques were identified in Vero cells in the presence of low-dose (20 μM) CDV, and many of these plaques could not be expanded in secondary Vero cell cultures. Only the virus constructed to have the S338F mutation could be expanded in the presence of 20 μM CDV. Consequently, plaques from the other single-mutation marker rescue conditions were expanded in the absence of CDV in order to

TABLE 2. Sequential acquisition of E9L mutations during passage in increasing doses of CDV[a]

Passage no.	E9L mutation				
	H296Y	A314V	H319N	S338F	R604S
0 (wild type)	−	−	−	−	−
1	−	−	−	−	−
2	+	−	−	+	−
3	+	−	−	+	−
4	+	+	−	+	−
5	−	+	−	−	−
6	+	+	+	+	+
7	+	+	+	+	+
8	+	+	+	+	+
15	+	+	+	+	+

[a] To identify the order and rate at which E9L polymerase mutations develop under CDV selection pressure, virus was passaged in Vero cells in ascending concentrations of CDV in a second experiment independent of the one described for Fig. 1. Following E9L PCR amplification and cloning, single clones from each passage were sequenced. As shown, mutations were found after only two passages in CDV. Because only one clone was sequenced at each passage, the single mutation identified at passage 5 may not be representative of the bulk population. Notably, all of the mutations found in the 15-times-passaged virus shown in Fig. 1 were already present from passage 6 onward. +, mutation found; −, mutation not found.

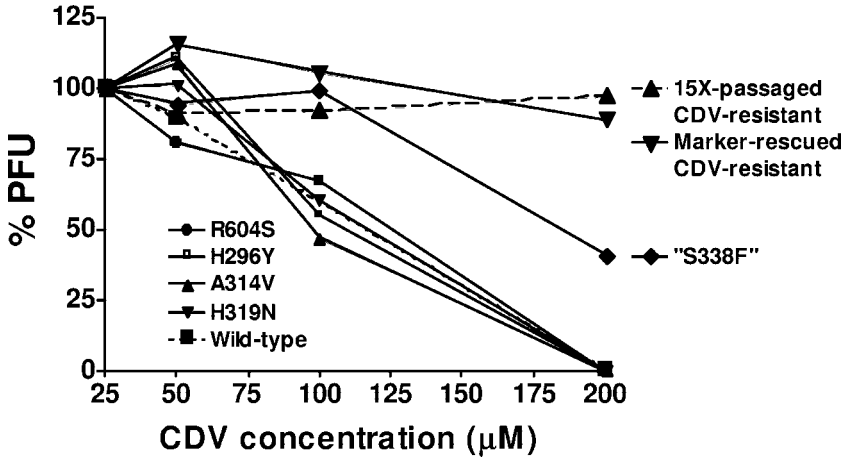

FIG. 2. Intermediate CDV resistance in an H296Y/S338F double E9L mutant. Plasmids containing individual mutations were introduced into vaccinia virus strain WR by marker rescue and selection in Vero cells in low-dose CDV (20 μM). The mutant that was constructed to contain S338F ("S338F") was found by sequencing to have acquired a second H296Y mutation to become H296Y/S338F. This double mutant, which was also identified as an intermediate in the sequential passage study (Table 2), had an intermediate resistance phenotype in Vero cells.

obtain an amount of virus sufficient for further testing. Most of the viruses from these secondary cultures had wild-type sensitivity to CDV in Vero cells (Fig. 2) but, due to limitations of the marker rescue approach used, the absence of resistance in these attempts to rescue single-mutation viruses does not allow us to address the role that the single mutations play in CDV resistance. Interestingly, however, the virus engineered to contain the S338F mutation and expanded by two passages in 20 μM CDV showed a phenotype of intermediate resistance to CDV ($EC_{50} \sim 200$ μM) in Vero cells. Upon cloning and sequencing the E9L polymerase gene from this "S338F" virus, it was found to have acquired a second H296Y mutation. Thus, an H296Y/S338F double mutant was identified in two contexts: first in the second passage in CDV (Table 2), and second in an analysis of single E9L mutations (Fig. 2). These findings, along with the intermediate CDV resistance of the H296Y/S338F virus, suggest that this double mutant represents a transitional step in the evolution of full CDV resistance.

After isolation of the CDV-R^{mr} virus in vitro, two passages of the virus in MA-104 cells were necessary in order to have adequate titers for infection of mice. Minimal virus passaging was done to avoid further attenuation of the virus resulting from extensive virus passage in cell culture (23). The two virus passages were tested for virulence in mice (Table 3). In these experiments, virus passages 1 and 2 were 100% lethal at $10^{5.5}$ and $10^{6.0}$ PFU/mouse, respectively. Compared to the previously published result in which a wild-type virus challenge dose of $10^{4.5}$ PFU/animal killed 100% of the mice (23), this represents a 10- to 30-fold decrease in virulence for the CDV-R^{mr} virus. Thus, the presence of the cidofovir-resistant DNA polymerase gene cloned into wild-type virus is associated with a decrease in viral virulence for mice.

Mice infected intranasally with the CDV-R^{mr} virus were treated with three antiviral agents starting 1 day after virus exposure (Table 4). In the first experiment 20% of placebo-

TABLE 3. Lethality of a marker-rescued cidofovir-resistant vaccinia virus (strain WR) in mice

Virus[a]	Virus challenge (log_{10} PFU/mouse)	Survivors/ total	Mean day of death[b]
CDV-R^{mr} (passage 1)	6.0	0/8	7.0 ± 0.0
	5.5	0/8	8.0 ± 0.5
	5.0	6/8	10.5 ± 2.5
	4.5	8/8	>21
CDV-R^{mr} (passage 2)	6.5	0/8	7.0 ± 0.0
	6.0	0/8	7.0 ± 0.0
	5.5	8/8	>21
	5.0	8/8	>21
	4.5	8/8	>21

[a] Previously we reported that $>10^{4.5}$ PFU of wild-type virus killed 100% of BALB/c mice and that the CDV-R virus passaged in Vero cells was avirulent at $10^{7.0}$ PFU/mouse (23).
[b] Values with standard errors are reported for mice that died prior to day 21 of the infection.

TABLE 4. Treatment of a marker-rescued cidofovir-resistant vaccinia virus (strain WR) in mice with three compounds

Compound (mg/kg/day)	Treatment day(s)[a]	Treatment route	Survivors/ total[b]	Mean day of death[c]
Experiment 1				
Cidofovir (100)	1	i.p.	6/10*	10.5 ± 4.4
Cidofovir (50)	1	i.p.	8/10**	12.0 ± 0.0
Cidofovir (25)	1	i.p.	4/10	9.8 ± 2.5
HDP-CDV (100)	1	p.o.	9/10***	12.0 ± 0.0
HDP-CDV (50)	1	p.o.	8/10**	10.5 ± 2.1
HDP-CDV (25)	1	p.o.	4/10	12.8 ± 1.3
S2242 (100)	1–5	i.p.	10/10***	>21
S2242 (50)	1–5	i.p.	10/10***	>21
S2242 (25)	1–5	i.p.	10/10***	>21
Placebo	1–5	i.p.	4/20	11.0 ± 3.1
Experiment 2				
Cidofovir (100)	1–2	i.p.	10/10***	>21
Cidofovir (30)	1–2	i.p.	1/10	7.7 ± 0.9
Cidofovir (10)	1–2	i.p.	0/10	7.4 ± 0.5
Placebo	1–2	i.p.	0/20	8.3 ± 3.5

[a] Treatment began 24 h after virus exposure for the number of days indicated.
[b] *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
[c] Values with standard errors are reported for mice that died prior to day 21 of the infection.

treated mice survived, and the mean day of death was 11.0 days. Cidofovir doses of 50 and 100 mg/kg protected 60 to 80% of the mice from death. The same doses of HDP-CDV were 80 to 90% protective. The advantage to the use of HDP-CDV was that it could be administered by the oral route. The best response to treatment was afforded by S2242, given daily for 5 days, where 100% protection was seen at doses of 25, 50, and 100 mg/kg/day. A second experiment was conducted in which cidofovir was administered for two consecutive days at doses of 10, 30, and 100 mg/kg/day (Table 4). The same virus challenge dose that was administered in the first experiment resulted in 100% death in the placebo group and a shorter time to death (8.3 days), thus indicating that this infection was more severe than the first. Cidofovir was protective at 100 mg/kg/day but afforded no significant protection at the two lower doses.

DISCUSSION

These studies are consistent with the concept that CDV resistance in vaccinia virus is due to mutations in the E9L polymerase gene. The mutations identified in virus passaged 15 times in the presence of CDV appeared relatively early in culture, since all of the mutations shown in Fig. 1 were also found in an isolate from passage 6 (Table 2). Since CDV only reduces and does not totally eliminate poxvirus replication in vivo (e.g., in the cowpox model in mice [4]), further studies will be needed to determine if CDV-resistant viruses can develop in vivo during drug treatment. As a tool for such studies and as a means of tracking CDV resistance in field isolates in the event of a smallpox epidemic, it may be possible to substitute E9L genotyping in place of traditional culture and sensitivity testing, as is done for tracking drug resistance in HIV, for example (13).

Most of the mutations in E9L occurred in a mutational hotspot region between amino acids 296 to 338. Based on its homology to other B-family DNA polymerases (1), the protein has been proposed to contain an N-terminal $3' \rightarrow 5'$ proofreading exonuclease domain and a C-terminal DNA polymerase domain (26), and most of the mutations were identified within the putative proofreading exonuclease domain. The exonuclease domain has been previously shown by Taddie and Traktman to be the locus for resistance to cytosine arabinoside (26), although their study involved chemical mutagenesis of vaccinia virus rather than analysis of the spontaneous mutations that arise under drug selection pressure. Within this hotspot, most of the mutations that arose in response to CDV selection (H296Y, A314V, and H319N) are located in a region of the protein that, based upon the homologous structure of RB69 DNA polymerase, probably forms an extended β-hairpin loop (18). This loop appears to stabilize DNA within the active site of the proofreading exonuclease, and disrupting this structure might well alter the manner in which the protein interacts with DNAs bearing nucleoside phosphonate linkages. Enzymatic studies of vaccinia virus DNA polymerase have shown that CDVpp can substitute for dCTP in in vitro assays, but after its incorporation into DNA partially inhibits chain extension and strongly inhibits enzymatic proofreading (12). This mechanism of CDV action is compatible with the mapping of resistance mutations to this region.

It should also be realized that additional unrecognized mutations may have contributed to some of the phenotypes described in this report. The marker rescue approach used in this study is less exact than the more time-consuming cosmid-based approach to introducing defined mutations as described by Thompson and Condit (28). Adding to this uncertainty, Taddie and Traktman found that vaccinia virus selected for resistance to aphidicolin through a single amino acid change at A498 in E9L polymerase exhibited a higher rate of spontaneous mutagenesis (27). It would be of interest to determine if the initial H296Y and S338F mutations found in our study (Table 2) reduce the fidelity of this polymerase, setting the stage for the acquisition of additional mutations both within and outside the E9L gene.

Vac-CDV-R^{mr} replicated to significantly higher titers in cultured mouse cells than did the original 15-times-passaged CDV-resistant virus from which the mutant E9L gene was obtained (23). Vac-CDV-R^{mr} was also virulent in mice (but required a high infectious dose), whereas the original CDV-resistant virus was completely attenuated for virulence (23). These data suggest that repeated in vitro passage in Vero cells had an attenuating effect on the growth of the 15-times-passaged isolate in mouse cells and in mice apart from an effect on E9L. The construction of a better-growing CDV-resistant vaccinia virus by marker rescue provides a useful reagent for identifying new drugs that will inhibit such viruses in vivo. Given the possibility of spontaneous evolution of CDV resistance in vivo under drug pressure, it is likely that an ideal regimen for poxvirus therapy will involve drug combinations, analogous to current treatments for HIV infection.

Contrary to the complete attenuation exerted by the CDV-R form of vaccinia virus that was developed and reported previously (23), the CDV-R^{mr} virus was lethal to mice infected by the intranasal route at concentrations of virus that could be easily produced by cell culturing. Compared to the wild-type WR strain, however, the CDV-R^{mr} virus was 10- to 30-fold less potent at causing death in the respiratory infection model. These results support the position that virus carrying a DNA polymerase gene resistant to cidofovir is less fit to replicate in the animals. This has been difficult to demonstrate in previous studies, because attenuation has also been seen in wild-type virus passaged extensively in cell culture (23). In spite of some attenuation present in the CDV-R^{mr} virus, mortality was achieved using a high virus challenge dose. This allowed for the study of treatment of the CDV-R^{mr} virus with antiviral agents. Indeed, we showed that three compounds (cidofovir, HDP-CDV, and S2242) were all efficacious to various degrees in reducing mortality caused by the virus. In the first experiment, where 20% of placebo-treated mice survived, S2242 was the most active but required daily administration for 5 days for its effect, whereas single treatments with cidofovir and HDP-cidofovir had some positive benefits in fighting the infection. In the second experiment, which resulted in a more severe infection, only the highest (100 mg/kg/day) dose of cidofovir given for 2 days offered protection. Previously, wild-type vaccinia virus (strain WR) infection was reported to be treatable with doses of 30 and 100 mg/kg/day (19). Single-dose and multiple-dose treatment regimens with these compounds for optimal activities against wild-type vaccinia virus infections have been reported previously (4, 14–16, 19–24). As was shown in a previous article relating to the cidofovir-resistant vaccinia virus

(strain WR) (23), resistance of the virus to cidofovir is only relative. Single treatments with a sufficiently high dose of drug obviously affords intracellular levels of the active antiviral metabolite that are adequate to confer some protection against infections in mice. The same logic would apply to HDP-CDV, since it is a prodrug of cidofovir. However, it may not be appropriate to directly compare the treatment of wild-type infection in mice to that for infection caused by the CDV-R^{mr} virus, because the latter virus is attenuated in vivo (requiring a higher virus challenge dose and producing a longer mean time to death than wild-type virus infection) and therefore may be more readily treatable.

In summary, a virulent form of a cidofovir-resistant vaccinia virus (strain WR) was developed by a marker rescue method. The DNA polymerase gene from the original resistant virus was characterized, and five mutations were found in two regions of the gene associated with DNA polymerase activity. The marker-rescued virus is attenuated for virulence in mice with respect to the wild-type virus, suggesting that the virus containing the mutated polymerase is less fit in terms of causing severe disease in the animal host. Treatment of the infection was still possible with cidofovir and the cidofovir prodrug HDP-CDV, although the efficacies of these two compounds were reduced relative to what was seen with treatment of wild-type virus infection (reported in the scientific literature). One compound, S2242, was highly effective in treating the CDV-R^{mr} virus infection. Thus, if resistance to cidofovir were ever to develop in poxvirus infections in humans either by natural means or by deliberate human terrorist activity, it may still be feasible to treat such infections with existing antiviral compounds.

ACKNOWLEDGMENTS

The work was supported in part by NIAID grant RO1-AI50489 (R.S.K.); by the Research Center on AIDS and HIV Infection of the VA San Diego Healthcare System (R.S.K.); by contract NO1-AI-15435 from the Virology Branch, NIAID (D.F.S.); by NIH grants RO1-EY11834 and RO1-AI29164 (K.Y.H.); and by Department of the Army grant DAMD17-01-2-007 (K.Y.H.). The U.S. Army Medical Research Acquisition Activity, Fort Detrick, MD, is the awarding acquisition office. The Molecular Biology Core of the UCSD Center for AIDS Research was supported by NIH grant P30 AI036214. K.Y.H. has an equity interest and serves as consultant to Chimerix Inc. The terms of these arrangements have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policy.

DNA sequencing was performed by the Molecular Biology Core of the UCSD Center for AIDS Research. We thank Douglas D. Richman for stimulating discussions and Kathy A. Aldern for assistance with viral cultures.

The content of this article does not necessarily reflect the position of policy of the government, and no official endorsement should be inferred.

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