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Improved DNA Hybridization Method for Detection of Acyclovir-Resistant Herpes Simplex Virus

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A simplified DNA hybridization method was developed to detect acyclovir-resistant isolates of herpes simplex virus. Herpes simplex virus-infected cell cultures in microtiter plates were treated with concentrations of acyclovir ranging from 8 to 0.015 μg/ml. At 48 h postinfection, infected cells were lysed by a one-step procedure and lysates were absorbed to membranes. Without further treatment, membranes were hybridized by using a herpes simplex virus-specific radioiodinated probe. The membranes were then washed and counted in a gamma counter. The elapsed time for assay performance was 4 h. Parallel plaque reduction assays were performed for comparison. The mean 50% inhibitory dose of in vivo- and in vitro-derived acyclovir-resistant, thymidine kinase-negative isolates was greater than 2 μg/ml by DNA hybridization. The 50% inhibitory dose of acyclovir-susceptible, thymidine kinase-positive isolates ranged from 0.01 to 1.1 μg/ml. This assay is simple and objective and should facilitate antiviral susceptibility testing in diagnostic laboratories.

Acyclovir (ACV), a purine nucleoside, is used for treatment and suppression of infections due to herpes simplex virus (HSV; 4, 9, 15, 23, 24). The drug is phosphorylated by viral thymidine kinase (TK) to acyclovir monophosphate and then phosphorylated by cellular enzymes to the triphosphate form (7, 16). The triphosphate form inhibits the viral DNA polymerase which ultimately results in termination of HSV DNA synthesis (6, 12). Clinical resistance is encountered infrequently and is generally associated with mutations in the TK gene (11). However, with recent Food and Drug Administration approval of oral ACV, widespread use of the drug is likely to develop and may create a selective environment favoring the emergence of resistance. Greater use of ACV may stimulate increased interest in screening HSV isolates for ACV resistance.

Gadler et al. (8) successfully employed a conventional DNA hybridization procedure which measured the effects of antiviral compounds on HSV replication. The procedure these investigators described used ^{32}P, required many tedious manipulations, and is not practical for routine use. The present study describes the application of a recently developed, simplified DNA hybridization procedure to detect acyclovir-resistant (ACV^r) HSV isolates and compares the sensitivity of this new procedure with that of the present reference method of plaque reduction. This hybridization assay is applicable by diagnostic laboratories and allows convenient testing of multiple antiviral agents.

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MATERIALS AND METHODS

Cells. Vero cells (American Type Culture Collection, Rockville, Md.) were grown and maintained in medium 199 (KC Biologicals, Lenexa, Kans.) containing 10% heat-inactivated fetal bovine serum and 25 μg of gentamicin sulfate per ml. CV-1 cells (obtained from W. Blue, Ohio University, Athens) were grown and maintained in modified Eagle medium with 0.4% sodium bicarbonate, 100 U of potassium penicillin G per ml, 25 μg of gentamicin sulfate per ml, and 10% heat-inactivated fetal bovine serum.

Virus isolates. HSV patient isolates were obtained from the Diagnostic Virology Laboratory of Cardinal Glennon Children's Hospital and from St. Louis University School of Medicine, St. Louis, Mo. (SLU); from M. Menegus, University of Rochester, Rochester, N.Y. (ROC); from C. Gleaves, Fred Hutchinson Cancer Research Center, Seattle, Wash. (HCR); and from S. Nusinoff Lehrman, Burroughs Wellcome Co., Research Triangle Park, N.C. (BW). Strains BW 9722 and BW 9723 are HSV type 1 (HSV-1) isolates from a child with severe combined immunodeficiency (18). Strain BW 9722 was recovered after multiple courses of ACV; strain BW 9723 responded to ACV therapy. Strains BW 9787, BW 9911, and BW 10069 were isolated from a patient with recurrent HSV-2 genital disease (M. N. Ellis, P. M. Keller, S. W. Strauss, S. N. Lehrman, and D. W. Barry, Abstr. Ninth Int. Herpes Virus Workshop, 1984, p. 225). HSV-1 F was obtained from W. Blue.

In vitro-derived ACV^r mutants were obtained from M. Arens, St. Louis University, St. Louis, Mo., and were generated by sequential passage of patient isolates four to seven times in Vero cells in the presence of 10 μg of sodium acyclovir per ml (19). The final stocks were titrated on Vero cells without ACV.

Virus type was determined by restriction endonuclease analysis of gradient-purified viral DNA (1) or by immunofluorescence assay with monoclonal antibodies (20).

TK activities of isolates and patient histories from Burroughs Wellcome and the Fred Hutchinson Cancer Research Center were unknown to the investigators until the completion of this study.

TK assays. TK activities of the in vitro-derived ACV^r mutants were previously determined by quantitation of the conversion of [^{14}C]thymidine to [^{14}C]TMP (19). The TK activities of the isolates from the Fred Hutchinson Cancer Research Center were determined by plaque autoradiography using [^{125}I]iododeoxycytidine as the substrate (11, 21).

ACV. Sodium acyclovir (Burroughs Wellcome) was diluted in sterile deionized water to a concentration of 1,000 μg/ml and was stored frozen at −70°C. Frozen stocks were used for up to 3 months after preparation.

Plaque reduction assay (PRA). Confluent Vero cell monolayers on 6-cm plates were infected with 80 to 300 PFU of virus after removal of the growth medium. The virus was adsorbed for 60 to 90 min at 37°C with tilting of the plates at 15-min intervals. After the inoculum was removed, duplicate plates were overlaid with twofold serial dilutions of ACV (usually 8 to 0.015 μg/ml) in medium 199 containing 2% fetal bovine serum, 25 μg of gentamicin sulfate per ml, and 1.0% human immune serum globulin (Gammar; Armour Pharmaceuticals, Kankakee, Ill.). Control cultures containing virus without ACV were also included. Infected cultures were incubated for 48 to 64 h at 37°C in 5% CO_2. After the overlay medium was removed, the monolayers were methanol fixed and Giemsa stained. Plaques were examined and counted under a dissecting microscope (×20 to ×40).

DNA probe. Purified HSV-1 F DNA was digested with the restriction endonuclease *Pst*I and cloned into bacteriophage M13mp19 (14). Hybridization analysis of randomly selected clones against 28 HSV-1 and 32 HSV-2 isolates resulted in the identification of two clones (800 and 1,200 base pairs of HSV DNA) which hybridized with equal efficiency to all isolates of both HSV types tested (unpublished observation). DNAs from these two HSV clones were chemically labeled with ^{125}I by the method of Commerford (5) to an average specific activity of 3.5×10^7 cpm/μg. Iodinated probe DNA was used for up to 2 months from the date of preparation.

DNA hybridization. Confluent monolayers of CV-1 cells in 96-well microtiter plates were inoculated with sufficient virus in 0.05 ml of medium to produce 50 to 100% cytopathic effect in untreated wells after 48 h at 37°C. In practice, a 10- or 100-fold dilution of an infected cell culture tube producing 100% CPE was used. After adsorption of virus for 60 to 90 min, the inocula were removed and duplicate wells were overlaid with medium containing serial dilutions of ACV, as described above. Infected wells without ACV and uninfected wells were also included as controls. Plates were incubated for 48 h at 37°C in 5% CO_2. Medium was aspirated from all wells, and cells were lysed by the addition of 15 μl of lysing reagent (Diagnostic Hybrids Inc.; Fig. 1). Lysates were either tested directly or frozen in the microtiter plates at −70°C. Lysates were absorbed onto GeneScreen Plus membranes (5 by 20 mm; Du Pont NEN, Boston, Mass.) placed vertically in each microtiter well. Without further treatment, membranes were placed in a reaction solution (Diagnostic Hybrids Inc.) containing 50% formamide, 10% dextran sulfate, 100 mM Tris hydrochloride (pH 7.5), 160 μg of sonicated salmon sperm DNA per ml, 0.5 M NaCl, 0.05 M sodium citrate, and 40 ng of probe per ml (approximately 1.4×10^6 cpm/ml). Typically, 10 membranes were incubated in 1.0 ml of hybridization reaction solution. Hybridization reactions were incubated at 60°C for 2 h. Membranes were washed for 45 min per wash at 73°C in successive washes of 2% sodium dodecyl sulfate–0.3 M NaCl–0.03 M sodium citrate (wash 1) and 0.03 M NaCl–0.003 M sodium citrate (wash 2). Membranes were blotted dry and counted in a gamma counter for 1 min each (Compac 120; Picker Corporation, Northford, Conn.). A minimum of 1,000 cpm per untreated virus control well and a maximum of 300 cpm per uninfected cell control well were considered acceptable test performance.

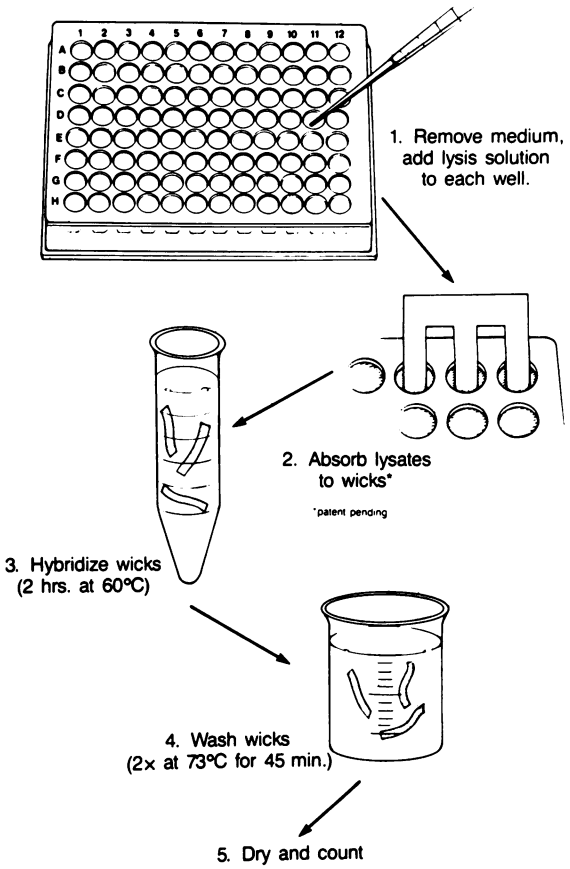

FIG. 1. Improved DNA hybridization method for detection of ACV^r HSV.

Calculation of ID_{50}. The concentration of ACV that caused a 50% reduction of plaque numbers or of counts per minute (ID_{50}) compared with untreated virus controls was calculated by using the linear regression probability program PROBIT (Statistical Analysis Systems, Cary, N.C.).

RESULTS

To determine the effect of inoculum size on ID_{50} by DNA hybridization, 10-, 100-, and 1,000-fold dilutions of strain SLU 360 were inoculated into wells containing twofold dilutions of ACV. These dilutions corresponded to approximately 30,000, 3,000, and 300 PFU per well, respectively. The ID_{50}s were 0.15 μg/ml at an inoculum of 30,000 PFU per well, 0.14 μg/ml at an inoculum of 3,000 PFU per well, and 0.08 μg/ml at an inoculum of 300 PFU per well.

Five coded HSV isolates obtained from Burroughs Wellcome Co. were then tested for their susceptibility to ACV by both the PRA and the DNA hybridization assay

TABLE 1. ID_{50}s of parent and in vitro derived ACV^r HSV isolates

Isolate	TK activity	ID_{50} (μg/ml)	
		Plaque reduction	DNA hybridization
BW 9722	Negative[a]	>2	>2
BW 9723	Positive[a]	0.46	0.10
BW 9787	Negative[a]	3.5	2.7
BW 9911	Altered[a]	2.0	1.2
BW 10069	Altered[a]	2.7	0.45
SLU 110 parent[b]		0.09	0.05
SLU 110 ACV^r	5.8[c]	>4	>4
SLU 310 parent[b]		0.07	0.04
SLU 310 ACV^r	2.9[c]	>8	>8
SLU 360 parent[b]		0.07	0.03
SLU 360 ACV^r	<1[c]	5.5	5.5
SLU 450 parent[d]		0.06	0.01
SLU 450 ACV^r	4.3[c]	>8	>8
SLU 520 parent[d]		0.06	0.06
SLU 520 ACV^r	<1[c]	>8	>8
ROC 2500 parent[d]		0.06	0.12
ROC 2500 ACV^r	<1[c]	>8	>8

[a] S. N. Lehrman, personal communication.
[b] HSV-1 strains.
[c] TK activity was calculated as picomoles of thymidine phosphorylated in 15 min per μg of protein and is expressed as percentage of parental activity.
[d] HSV-2 strains.

(Table 1). Strains BW 9722 and BW 9787, with deficient TK activity, had ID_{50}s of >2 μg/ml by both methods. Strains BW 9911 and BW 10069, which expressed a TK with altered substrate specificity, had an ID_{50} of >2 by PRA. By DNA hybridization, ID_{50}s for these strains were 1.2 and 0.45 μg/ml, respectively. Strain BW 9723, with normal TK activity, had ID_{50}s of <1 μg/ml by both methods.

To further compare the capability of each assay to detect ACV^r isolates, six patient isolates (three HSV-1 and three HSV-2) that were initially acyclovir susceptible (ACV^s) were passaged four to seven times in the presence of 10 μg of ACV per ml to select for resistance. TK activity was absent or reduced for all ACV^r mutants. By both assays, the ID_{50}s for the in vitro-generated ACV^r strains were approximately 100 times greater than that of the parental strains.

There was close correlation between assays (Table 1). The Pearson coefficient of correlation for ID_{50}s by PRA and hybridization was 0.86. The correlation coefficient was 0.64 when ID_{50}s by PRA were compared with the ID_{90} by DNA hybridization (data not shown).

To determine if ACV resistance emerged subsequent to ACV therapy, isolates obtained from immunocompromised patients during or after completion of antiviral therapy were assayed by DNA hybridization. Nine HSV isolates were obtained from six bone marrow transplant patients who shed virus during or shortly after completion of intravenous ACV therapy (Table 2). Isolates shed at the beginning of ACV therapy had ID_{50}s by DNA hybridization of <0.1 μg/ml, while isolates shed later in therapy had ID_{50}s of >2 μg/ml. For example, isolates obtained from patient 2 (strain HCR 3091) and patient 5 (strain HCR 15820), who shed virus 1 and 2 days after initiation of ACV therapy, respectively, had ID_{50}s by DNA hybridization of 0.02 and 0.05 μg/ml, respectively. These isolates were TK$^+$ by plaque autoradiography. (All of approximately 800 plaques examined for each strain incorporated [^{125}I]iododeoxycytidine.) Isolates HCR 5878 (patient 3), HCR 101 (patient 4), and HCR 16484 and HCR 16562 from patient 6, obtained after prolonged ACV therapy, had ID_{50}s by hybridization ranging from 2.1 to >8 μg/ml. A minimum of 780 plaques of each strain were examined by plaque autoradiography (range, 705 to 3,200); no plaques incorporated [^{125}I]iododeoxycytidine. Patient 5, whose initial isolate, HCR 15820, was ACV susceptible and TK$^+$, shed TK$^-$, ACV-resistant virus (strain HCR 15912; ID_{50}, 3.9 μg/ml by DNA hybridization) 5 days after initiation of therapy. Of approximately 1,300 plaques examined, none incorporated [^{125}I]iododeoxycytidine. Moreover, virus recovered 2 days after termination of therapy (strain HCR 15953) remained TK$^-$ and ACV resistant (ID_{50}, 5.2 μg/ml by DNA hybridization). No plaques of over 6,000 examined by plaque autoradiography were TK$^+$. In contrast, virus recovered from patient 1 (HCR 3074) 5 days after completion of therapy had ID_{50}s of 1.3 μg/ml by PRA and 1.1 μg/ml by hybridization. Of 552 plaques examined, all incorporated [^{125}I]iododeoxycytidine.

DISCUSSION

The DNA hybridization assay described herein detected both in vitro- and in vivo-derived ACV^r mutants deficient in or lacking TK activity. In this study, an ID_{50} of ≥2 μg/ml by DNA hybridization correlated with diminished TK activity. To date, alterations in the level or affinity of viral TK have been reported only in isolates from patients failing to respond to ACV therapy (11). With the exception of strains BW 9911 and BW 10069, all isolates in this study with ID_{50}s of <2 μg/ml by DNA hybridization expressed normal TK activity. Strains BW 9911 and BW 10069 expressed a TK with reduced affinity for ACV, although the patient from whom these strains were isolated had responded normally to ACV treatment (Ellis et al., Abstr. Ninth Int. Herpes Virus Workshop). Long-term ACV therapy of severely immunocompromised patients did appear to select for the emergence of ACV^r strains (Table 2).

Good correlation was obtained between DNA hybridization and the PRA, the reference method for antiviral susceptibility testing. Unlike the PRA, the use of a DNA probe requires neither titration of the virus prior to testing nor tedious enumeration of plaques. Moreover, the microtiter plate format conserves medium and antiviral agent.

Varying the inoculum size did not affect ID_{50}s when inocula of 300, 3,000, and 30,000 PFU per well, which corresponded to 1000-, 100-, and 10-fold dilutions of virus stock, respectively, were used. We have found that the mean titer of clinical isolates of HSV in primary rabbit kidney cell cultures displaying 100% cytopathic effect was approximately 6×10^6 PFU per ml (data not shown). A 10- or 100-fold dilution of a clinical isolate for which titers have not been determined would yield an inoculum of 3,000 to 30,000 PFU per well, which is in the range of inocula producing reproducible ID_{50}s. Therefore, it should be possible to use such clinical isolates of HSV to perform the hybridization assay. That dilution producing 50 to 100% cytopathic effect in the untreated control well at 48 h postinoculation should then be used to calculate the ID_{50}.

This DNA hybridization system offers several advantages over a previously described hybridization assay for antiviral susceptibility testing (8). Traditional hybridization procedures use ^{32}P-labeled probes of short half-life and require filtration of infected cells onto nitrocellulose filters, high-temperature baking of filters to denature DNA, prehybridization of filters, and overnight hybridization with probes, followed by extensive washes. Moreover, radioactivity must

TABLE 2. ID_{50}s of HSV isolates from ACV-treated bone marrow transplant patients

Patient no.	Isolate[a]	Source of isolate	Duration of therapy[b] (days) prior to taking of viral culture	ID_{50} (μg/ml) by:		TK[c] activity
				PRA	DNA hybridization	
1	HCR 3074	Throat	18; stopped 5 days prior to viral shedding	1.3	1.1	Positive
2	HCR 3091	Throat	1	0.05	0.05	Positive
3	HCR 5878	Skin	34	5.9	2.1	Negative
4	HCR 101	Lung	30	>8	>8	Negative
5	HCR 15820	Throat	2	0.03	0.05	Positive
	HCR 15912	Sputum	5	3.3	3.9	Negative
	HCR 15953	Sputum	Stopped 2 days prior to viral shedding	>4	5.2	Negative
6	HCR 16484	Mouth	18	ND[d]	3.5	ND[d]
	HCR 16562	Mouth	23	>8	3.5	Negative

[a] All isolates were HSV-1 as determined by immunofluorescence.
[b] Patients were on intravenous ACV therapy for either treatment or suppression of HSV infections.
[c] TK activity was measured by plaque autoradiography with [^{125}I]iododeoxycytidine. All plaques (100%) of TK^+ strains and no plaques (0%) of TK^- strains incorporated [^{125}I]iododeoxycytidine.
[d] ND, Not done.

be monitored by autoradiography or by scintillation counting, procedures that are not available to most clinical laboratories.

The procedure we describe obviates the need for the prehybridization steps detailed above. Infected cells are lysed and the DNA is denatured simultaneously upon addition of the lysing reagent. Lysate is absorbed onto membranes by wicking, and the membranes are hybridized without further treatment. The hybridization reaction requires 2 h followed by two 45-minute washes. Radioactivity is monitored in a gamma counter, equipment which is available in many clinical laboratories. The ^{125}I-labeled DNA probe is a familiar reagent in clinical laboratories which routinely perform radioimmunoassays with ^{125}I.

Other previously described methods for antiviral susceptibility testing also have drawbacks for routine use. The neutral-red-dye uptake assay (13) requires test virus for which titers have been determined and shows significant variation in ID_{50}s with increasing quantities of virus. Enzyme immunoassays require multiple reagents and manipulations (3, 17). Plaque autoradiography detects virus with decreased or altered TK activity but not DNA polymerase mutants (11). Although DNA polymerase mutants have not yet been detected in clinical isolates, they may arise when ACV is used extensively. DNA hybridization, however, should detect both TK and polymerase mutants, although we did not specifically examine ACV^r polymerase mutants.

With the advent of an effective oral antiviral agent, ACV, and the potential for its widespread use, it is essential that viral susceptibility testing be performed to monitor the emergence of resistance (10). Reports of ACV resistance, particularly in severely immunocompromised patients, have already appeared despite the restricted use of ACV to date (2, 22).

This DNA hybridization system is simple and objective and correlates well with the currently available reference method, the PRA. The stability of the probe (2 months) further facilitates routine susceptibility testing. An additional advantage is that this system can be expanded to encompass testing of antiviral agents for other viruses when appropriate probes are available.

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