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Problems Associated with the Use of (*E*)-5-(2-Bromovinyl)-2′-Deoxyuridine for Typing Herpes Simplex Virus

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When 0.5 μg of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVdU) per ml was incorporated directly into cell culture medium inoculated with eight known positive specimens, one herpes simplex virus type 1 (HSV-1) isolate grew in the presence of BVdU and was misidentified. By plaque assay, the titers of 15 HSV-1 strains were reduced by more than 3 $\log_{10}$ by BVdU, and the titers of 16 HSV-2 strains were reduced by less than 2 $\log_{10}$. Titers of HSV-1 acyclovir-resistant strains were reduced by less than 1.5 $\log_{10}$, which was characteristic of HSV-2 strains. Thus, typing of HSV isolates in the presence of BVdU by plaque assay is reliable only if information regarding previous antiviral therapy is obtained.

The greater susceptibility of herpes simplex virus type 1 (HSV-1) to (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVdU) has been exploited by Mayo (9) and others (3, 5, 13) to differentiate HSV-1 from HSV-2. The differential susceptibility of the two HSV types is apparently due in part to differences in viral thymidine kinase (TK) and DNA polymerases (1, 4, 6, 11). Activation of BVdU as well as acyclovir (ACV) is dependent upon phosphorylation by virus-induced TK (4, 6, 7). Therefore, HSV-1 isolates resistant to ACV (ACV^r) might be expected also to be BVdU resistant.

In this paper, we compare the BVdU susceptibilities of clinical isolates of HSV-1 and HSV-2 under different conditions of virus growth. We also tested ACV^r derivatives to determine whether misidentification of ACV^r HSV-1 isolates occurs.

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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 (FBS) and 25 μg of gentamicin sulfate per ml. Primary rabbit kidney cell culture tubes (Flow Laboratories, McLean, Va.) were 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% FBS. Human 143 TK-negative (TK^-) cells obtained from H. Thornton, St. Louis University, St. Louis, Mo., were grown and maintained in modified Eagle medium supplemented with 1% nonessential amino acids (Flow Laboratories) and 10% FBS. These cells were routinely passaged in the presence of 30 μg of 5-bromodeoxyuridine per ml. 5-Bromodeoxyuridine was removed one passage before infection with HSV.

HSV clinical isolates were obtained from the Diagnostic Virology Laboratory of Cardinal Glennon Memorial Hospital for Children, St. Louis, Mo. (SLU), from M. Menegus, University of Rochester Medical Center, Rochester, N.Y. (ROC), from T. Smith, Mayo Clinic, Rochester, Minn. (Mayo), and from Binghamton General Hospital, Binghamton, N.Y. (BGN). Strain BW-R(7047), obtained from C. Dekker (Burroughs Wellcome Co., Research Triangle Park, N.C.), is a clone of an HSV-1 ACV^r strain isolated from an immunodeficient child after several courses of treatment with ACV (10) and has been shown to be TK^- by thymidine plaque autoradiography (12; S. Nusinoff-Lehrman, personal communication). HSV-1 F and HSV-2 G prototype strains were obtained from B. Roizman, University of Chicago, Chicago, Ill.

Restriction endonuclease analysis of NaI gradient-purified viral DNA was performed essentially as previously described (2). All viral DNAs were digested with at least one enzyme (*Hpa*I, *Bgl*II, and *Hin*dIII were from Boehringer Mannheim Corp., Indianapolis, Ind.; *Kpn*I and *Sst*I were from Bethesda Research Laboratories, Gaithersburg, Md.). Restriction enzyme analysis of HSV-1 F and HSV-2 G DNAs was performed simultaneously with each analysis of clinical isolates. Designation of type was made by comparison with the restriction fragment patterns of the prototype strains.

BVdU, obtained from I. Sim, G. D. Searle, High Wycombe, Buckinghamshire, United Kingdom, was suspended in sterile phosphate-buffered saline (pH 7.2) and frozen in 0.5-ml volumes at −70°C for no longer than 6 months. A separate vial was thawed for each assay.

For determination of differential susceptibility of HSV-1 and HSV-2 upon initial isolation, original specimens (stored at −70°C) which yielded eight of the isolates were reinoculated into rabbit kidney or Vero cell culture tubes or both. Medium was removed from the tubes before inoculation of specimens (0.2 ml per tube). Virus was adsorbed for 1 h at 36°C on a roller drum, after which medium with and without BVdU (final concentration, 0.5 μg/ml) was added. Tubes were incubated on a roller drum at 36°C for the first 24 h and thereafter on a stationary rack for a total of 7 days. Tubes were read daily for HSV-specific cytopathic effect. Controls consisted of uninoculated cell culture tubes with and without BVdU.

For plaque assays in the presence and absence of BVdU, 0.5 ml of 10-fold serial dilutions of virus stocks were inoculated in duplicate into confluent Vero cell monolayers (6-cm plates) after removal of the growth medium. Virus was adsorbed for 90 min at 37°C with tilting of the plates at 15-min intervals. One-half of the plates of each dilution were overlaid with medium 199 containing 2% FBS, 25 μg of

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TABLE 1. BVdU susceptibility versus restriction enzyme analysis for typing of HSV

Type	Strain	Source	Titer reduction with BVdU[a] (log_{10} decrease)
HSV-1[b]	F	Face	>4.10
	SLU-110	Eye	>4.77
	SLU-210	Eye	3.05
	SLU-280	Cervix	3.38
	SLU-310	Face	4.37
	SLU-360	Finger	3.79
	SLU-400	Labia	>5.80
	SLU-585	Bronchus	>5.31
	BGN-10	Esophagus	>5.54
	ROC-2200	Genital	4.10
	ROC-2600	CSF[c]	3.72
	Mayo 1197	Throat	3.47
	Mayo 15836	Lung	3.85
	Mayo 15861	Eye	3.12
	Mayo 15879	Vulva	3.08
HSV-2[b]	SLU-190	Vagina	1.18
	SLU-240	Perineum	1.07
	SLU-290	Vulva	1.00
	SLU-340	Hand	1.00
	SLU-450	CSF	1.54
	SLU-520	CSF	1.62
	SLU-540	CSF	1.61
	SLU-565	Labia	1.98
	SLU-635	Liver	1.25
	ROC-2500	Brain	1.73
	Mayo 15862	Skin	1.22
	Mayo 15867	Perinanal	1.31
	Mayo 15872	Cervix	0.73
	Mayo 15877	Vagina	1.88
	Mayo 15882	Penis	1.34
	Mayo 15917	Genital	1.22

[a] Titrations were performed by plaque assay in Vero cells. For HSV-1, the range was 3.05 to >5.80; for HSV-2, the range was 0.73 to 1.98.
[b] Typed by restriction endonuclease analysis.
[c] CSF, Cerebrospinal fluid.

gentamicin sulfate per ml, 0.165% human immune serum globulin (Gammar; Armour Pharmaceutical Co., Kankakee, Ill.) and with 0.5 μg of BVdU per ml. The remaining plates were overlaid with medium without BVdU. Plates were incubated for 48 h at 37°C in 5% CO_2. After removal of the overlay medium, the plates were methanol fixed and Giemsa stained. Plaques were visualized under a dissecting microscope (20 to 40×) and counted.

To generate ACV^r mutants, we passaged six HSV-1 and three HSV-2 clinical isolates sequentially (four to seven times) in Vero cells in the presence of 10 μg of sodium ACV (Burroughs Wellcome Co.) per ml. The final stocks were titrated on Vero cells in the absence of ACV.

For TK assays, human 143 TK$^-$ cells were infected with 10 PFU of the virus stock per cell. At about 20 h postinfection, cells were harvested by scraping, washed in phosphate-buffered saline, and suspended in sterile water. The suspension was sonicated and then centrifuged at 31,000 × *g* for 60 min. The supernatant fluid was assayed for TK in a reaction mixture containing 200 mM sodium phosphate buffer (pH 6.0), 25 mM $MgCl_2$, 15 mM ATP, and 50 μM [^{14}C]-thymidine. The mixture was incubated at 37°C, and samples that were taken at 0, 15, and 30 min were spotted onto DE81 filter paper disks, washed six times in 1 mM ammonium formate and once in 95% ethanol, dried, and counted in a toluene-based scintillation fluid. Activity was calculated as picomoles of [^{14}C]-thymidine converted to [^{14}C]-thymidine monophosphate per microgram of protein in 15 min. Protein was determined by the method of Lowry et al. (8). TK activities are expressed as the percentage of parental TK activity induced by the mutant strains.

To determine whether BVdU could differentiate between HSV-1 and HSV-2 upon initial isolation, eight previously positive original specimens were reinoculated into rabbit kidney or Vero cell culture tubes in the presence (0.5 μg/ml) and absence of BVdU and observed for cytopathic effect. One HSV-1 isolate (SLU-400) grew in the presence of BVdU and thus was misidentified as HSV-2. One isolate (SLU-340) failed to grow in the absence of BVdU, but was identified as HSV-2 on the basis of its growth in BVdU.

A total of 15 HSV-1 and 16 HSV-2 isolates, as determined by restriction endonuclease analysis, were tested for susceptibility to 0.5 μg of BVdU per ml by plaque assay (Table 1). The titers of all HSV-1 strains were reduced at least 3 log_{10} (range, 3.05 to >5.80). Inhibition of HSV-2 strains was also observed, but the titer reduction was less than 2 log_{10} (range, 0.73 to 1.98).

Six HSV-1, three HSV-2 in vitro-generated TK$^-$ ACV^r strains, and one HSV-1 ACV^r strain [BW-R(7047)] derived from an immunocompromised patient after repeated courses of ACV were assayed for BVdU susceptibility. Titer reductions for the HSV-1 ACV^r strains were less than 1.5 log_{10} (Table 2), which was characteristic of HSV-2 strains. The titers of the ACV^r HSV-2 strains were reduced by less than 1 log_{10}. TK activity was reduced to 5.8% or less in all in vitro-generated ACV^r isolates.

A potential problem in employing BVdU for typing of HSV is the ability of high titers of HSV-1 in clinical specimens to overcome BVdU inhibition. When BVdU was incorporated directly into the isolation medium, one HSV-1 isolate was misidentified. Typing results obtained with this technique should be considered preliminary and should be confirmed.

Plaque assays in the presence or absence of 0.5 μg of BVdU per ml determined that HSV-1 clinical isolates were

TABLE 2. BVdU susceptibility of ACV^r TK$^-$ strains

Strain[a]	Actual HSV type by restriction analysis[b]	Titer reduction with BVdU (log_{10} decrease)	Mutant TK activity as % of parental activity[c]
SLU-110 ACV^r	HSV-1	0.3	5.8
SLU-310 ACV^r	HSV-1	0.8	2.9
SLU-360 ACV^r	HSV-1	1.2	<1
SLU-585 ACV^r	HSV-1	0.8	3.6
ROC-2200 ACV^r	HSV-1	0.6	2.3
ROC-2600 ACV^r	HSV-1	1.0	3.2
SLU-450 ACV^r	HSV-2	0.9	4.3
SLU-520 ACV^r	HSV-2	0.7	<1
ROC-2500 ACV^r	HSV-2	0.5	<1
BW-R(7047)	HSV-1	1.4	ND[d]

[a] These isolates, except for BW-R(7047), were rendered ACV^r and thus TK$^-$ by multiple sequential passage (four to seven times) in Vero cells in the presence of 10 μg of ACV per ml.
[b] By BVdU analysis, all strains were typed as HSV-2. The criteria for making this determination are discussed in the text.
[c] All activities were calculated as picomoles of thymidine phosphorylated in 15 min/μg of protein.
[d] ND, Not done.

relatively more susceptible than were HSV-2 isolates. ACV^r TK$^-$ HSV-1 isolates, however, were relatively nonsusceptible to BVdU and would be misidentified. This finding was not surprising since the antiviral activities of both BVdU and ACV are dependent upon phosphorylation by the HSV-induced TK (4, 6, 7). With the recent availability of ACV for treatment of HSV infections, it is possible that ACV^r HSV-1 strains may emerge in the general population. In fact, one of our ACV^r HSV-1 strains, BW-R(7047), was a naturally occurring strain, albeit from an immunodeficient patient. Therefore, if BVdU susceptibility is employed as a typing method, patient information regarding previous antiviral therapy is absolutely essential.

With careful attention to the above details, typing of HSV isolates by plaque assay based on their susceptibilities to BVdU may be a reliable method for use in small laboratories without the capability of performing restriction endonuclease analysis or immunofluorescence tests.

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