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Plaque Autoradiography Assay for the Detection and Quantitation of Thymidine Kinase-Deficient and Thymidine Kinase-Altered Mutants of Herpes Simplex Virus in Clinical Isolates

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A plaque autoradiography assay to detect and quantitate thymidine kinase (TK) mutants of herpes simplex virus type 1 (HSV-1) and HSV-2 in clinical samples is described. This method utilizes the selective incorporation of [^{125}I]iododeoxycytidine, a pyrimidine analog selectively phosphorylated by the HSV TK. Only cells infected with TK-competent virus will efficiently incorporate iododeoxycytidine and are the only cells detected by autoradiography. Furthermore, this assay discriminates between TK$^+$ virus (TK competent) and TK^A virus (TK altered or reduced). This ability to differentiate TK$^+$ from TK^A virus is enhanced when infected cells are labeled with [^{14}C]thymidine in tandem with iododeoxycytidine labeling. Reconstruction experiments with mixtures of TK$^+$ (HSV-1 Patton) virus and TK-deficient (TK$^-$) (B2006) or TK^A (IUDR^r) mutants were performed to determine the limits of detection of this technique. Ten percent TK$^-$ or TK^A virus was the lower limit for the detection of TK mutants in a mixed population, whereas 1 in 1,000 TK$^+$ virus revertants could be detected in a TK$^-$ virus population. In reconstructed populations and 45 clinical samples, a good correlation existed between the increase in 50% inhibitory dose for acyclovir and the percent TK mutant virus present. Similarly, the results of this technique correlated well with the acyclovir phosphorylating activity of extracts from cells infected with isolates or reconstructed mixtures. Plaque autoradiography with [^{125}I]iododeoxycytidine was able to distinguish mixed populations of TK$^+$ and TK$^-$ virus and homogeneous populations of TK^A virus. The tandem use of [^{125}I]iododeoxycytidine and [^{14}C]thymidine readily identified TK^A virus, which appeared as TK$^+$ virus when labeled with [^{14}C]thymidine alone. This technique provides a sensitive screen for antiviral resistance due to alterations in the viral TK and can be used to analyze clinical samples.

Clinical studies with acyclovir (ACV) have stimulated interest in the potential development of antiviral drug resistance and the characterization of herpes simplex virus (HSV) mutants significantly less susceptible to ACV. Although there is no currently established relationship between diminished susceptibility to ACV of an HSV isolate in vitro and lack of response to therapy in vivo for humans and only a preliminary correlation in animals (12), for convenience isolates with significantly reduced susceptibility will be termed "resistant." ACV resistance can be observed in cell culture, and results from the selection of preexisting resistant virus in a mixed population or from the selection of a mutation in the thymidine kinase (TK) or DNA polymerase locus (4, 14, 37, 40). Both the TK and DNA polymerase loci are involved in the mechanism of action of ACV (4, 11, 14, 37). The TK is responsible for the conversion of ACV to ACV monophosphate (21), which is subsequently phosphorylated by cellular enzymes to ACV triphosphate (34, 35). ACV triphosphate then functions as a suicide inactivator of the HSV DNA polymerase (20), causing premature termination of viral DNA synthesis and the accumulation of short fragments of viral DNA (18, 32).

Thus far, only TK mutants have been isolated from clinical samples. However, the term "TK mutant" is misleading; it implies that only thymidine phosphorylation is impaired. In reality, the enzyme is indiscriminant and phosphorylates thymidine (TdR), deoxycytidine, and structural analogs of these and other nucleosides (ACV in particular) (6, 9, 24, 25, 27). A mutation may alter the enzyme's ability to phosphorylate one or more of these compounds. Hence, the enzyme may be able to phosphorylate TdR normally, but not able to phosphorylate one or more of the other substrates. Such viruses have been referred to as "altered substrate specificity" mutants. Viruses that do not appreciably phosphorylate ACV, but can detectably phosphorylate thymidine, are termed "TK altered or reduced" (TK^A) in this text. Resistant variants that do not phosphorylate TdR (TK$^-$) have been shown to have diminished infectivity and virulence and are able to establish latency in mice only with difficulty (13, 16, 42, 44). Laboratory-derived variants that induce an enzyme with an altered substrate specificity may retain their pathogenicity and are capable of establishing latent infections in mice (8, 29). Because ACV therapy in certain clinical settings (1, 7, 38) may select for TK mutants, which occur naturally at a rate of 10^{-4} (36), it would be useful to monitor the emergence of drug-resistant virus during courses of antiviral chemotherapy and to be able to identify and quantitate the various TK mutants in a clinical specimen.

Several methods have been reported to differentiate TK$^+$ virus populations from TK$^-$ virus populations (21, 26, 39, 41, 43). However, none of these techniques can reliably detect and distinguish small numbers of either TK$^-$ or TK^A viruses in an individual clinical isolate. In this paper we describe a method that allows the differentiation and quantitation of TK$^+$, TK$^-$, and TK^A viruses in low-passage clinical isolates.

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This procedure may prove useful in studying the evolution of drug-resistant mutants in patients requiring long-term continuous or intermittent drug therapy and in defining the role that an ACV-resistant TK^A virus assumes in infection.

MATERIALS AND METHODS

Cells. Vero cells (American Type Culture Collection [ATCC], Rockville, Md.) were cultured in Eagle minimal essential medium (MEM) (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 2.5% newborn bovine serum (M. A. Bioproducts, Walkersville, Md.) and 2.5% fetal bovine serum (Hyclone; Sterile Systems, Inc., Logan, Utah). $LMTK^-$ cells, TK-deficient mouse LM cells obtained from R. G. Hughes (Roswell Park Memorial Institute, Buffalo, N.Y.), were cultured in Eagle MEM supplemented with 5% fetal bovine serum and 20 μg of 5-bromodeoxyuridine per ml (31).

Virus. The Patton strain of HSV type 1 (HSV-1) and its DNA polymerase mutant BW^r and the TK^A mutant of strain Patton, $IUDR^r$, were provided by Kendal O. Smith (Department of Microbiology, San Antonio, Tex.) (17, 40). HSV-1 strain SC16 and its mutants, S1 and B3, were obtained from Hugh Field and Graham Darby (Division of Virology, Department of Pathology, University of Cambridge, Cambridge, United Kingdom) (8, 15, 23). Mutants S1 and B3 are resistant to ACV and to ACV and *E*-5(2-bromovinyl)-2′-deoxyuridine, respectively, and encode for TKs with altered substrate specificities (22, 28). HSV-1 strain CL101 and its TK^- mutant B2006 were also obtained from Hugh Field (10). The KOS strain of HSV-1 and its DNA polymerase mutant PAA^r_5 were obtained from Priscilla Schaffer (Dana-Farber Research Center, Boston, Mass.) and have been described previously (2–4, 17). $AraA^r_9$, a polymerase mutant of strain KOS, was obtained from Don Coen (Department of Pharmacology, Harvard Medical School, Boston, Mass.) and has been described previously (3). Clinical samples were obtained from patient isolates submitted to Burroughs Wellcome Co. in the course of clinical trials of ACV. Isolates 2859, 2861, and 8493 were characterized as HSV-1, and isolates 6081, 8269, 8295, 8404, 8405, 8406, 8407, 8408, and 8736 were identified as HSV-2.

Stock cultures of wild-type and mutant strains of HSV-1 and HSV-2 were grown in Vero cells in Eagle MEM supplemented with 5% fetal bovine serum. Titers of virus stocks were determined in Vero cells (5).

[^{14}C]TdR plaque autoradiography. Virus populations were tested for the ability to express TK activity by a modified procedure of Tenser et al. (43). Briefly, confluent $LMTK^-$ cell monolayers were infected with 20 to 200 PFU of virus. After a 1-h adsorption at 37°C in an atmosphere of 5% CO_2, the monolayers were overlaid with 5 ml of Eagle MEM supplemented with 5% newborn bovine serum and 0.5% human immune serum globulin (Gamstan; Cutter Biological, Berkeley, Calif.). When plaques were observed 6 days postinfection, the monolayers were labeled with 0.5 μCi of [^{14}C]TdR (ICN Pharmaceutical Inc.; specific activity, 56 mCi/mmol) for 4 h at 37°C. The label was then removed, and the monolayers were washed twice with phosphate-buffered saline for 15 min at 37°C. After 10% Formalin fixation, staining was performed with 0.8% crystal violet in 95% ethanol. Circumferential rims were removed, and the monolayers were placed in contact with Kodak X-Omat XAR-5 X-ray film for 5 days at room temperature.

^{125}IdC plaque autoradiography. Virus populations were tested for the ability to express TK activity by a modification of the methods of Summers and Summers (41) and Tenser et al. (43). Confluent monolayers of Vero cells in 60-mm petri dishes were infected with approximately 20 to 200 PFU of virus. Adsorption was carried out at 37°C in an atmosphere of 5% CO_2 for 1 h. The plates were then overlaid with 5 ml of Eagle MEM supplemented with 5% newborn bovine serum and 0.5% immune serum globulin (human). After incubation for 4 days at 37°C, plaques were observed. The overlay was removed, and the cell monolayers were labeled with 0.5 μCi of [^{125}I]iododeoxycytidine (^{125}IdC; New England Nuclear Corp., Boston, Mass.; specific activity, 2,200

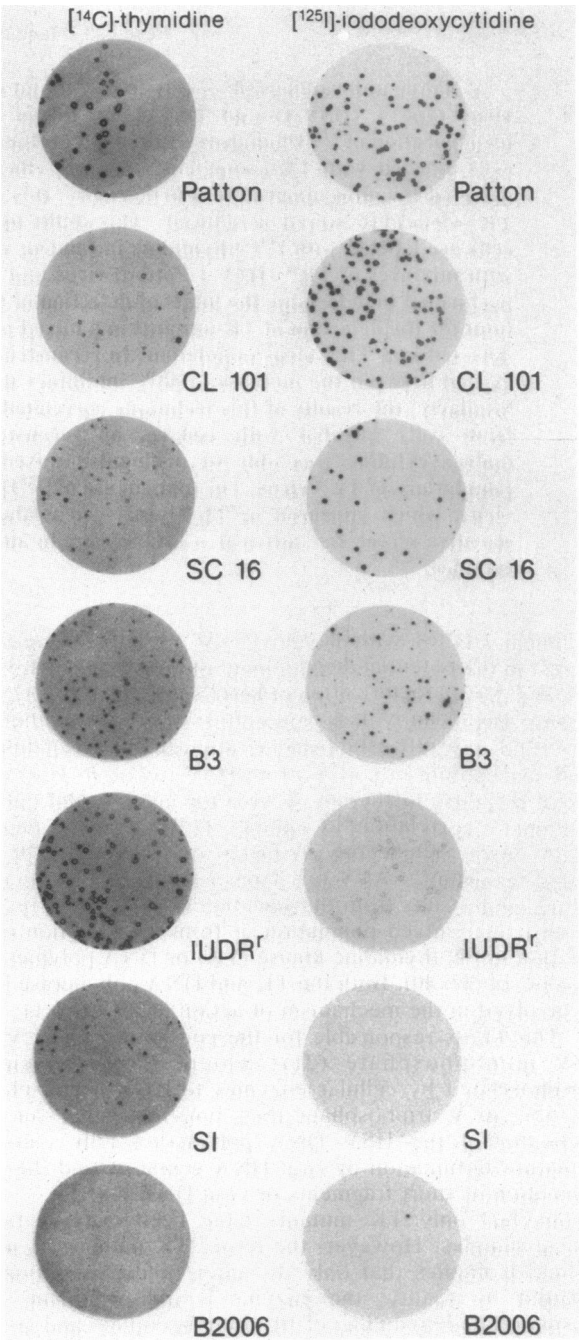

FIG. 1. Comparison of [^{14}C]TdR and ^{125}IdC plaque autoradiography. Procedures performed as described in the text.

Ci/mmol) for 2 h at 37°C. After incubation, the label was removed, and the cell monolayers were washed twice with phosphate-buffered saline for 15 min at 37°C. The monolayers were fixed with 10% Formalin in phosphate-buffered saline for 10 min and then stained with 0.8% crystal violet in 95% ethanol. The circumferential rims were removed from the dishes, and the monolayers were placed in contact with Kodak X-Omat XAR-5 X-ray film for 7 days at room temperature. The films were developed, and plaques were scored by the presence of dark or faintly exposed areas on the film that corresponded to plaques on the stained monolayer. The number of dark and faint plaques was compared with the total number of plaques in the stained monolayer to determine the actual percentage of each virus type in the population.

Antiviral susceptibility assay. Virus populations were tested for their susceptibility to antiviral drugs by using a standard plaque reduction assay (5). After infection, the plates were overlaid with dilutions of drug contained in 5 ml of Eagle MEM supplemented with 5% newborn bovine serum and 0.5% human immune serum globulin. The concentrations of compound required to reduce the plaque numbers by 50% compared with controls lacking drug (ID_{50}s) were obtained by using a computer probability analysis (version 79.3 of procedure PROBIT; Statistical Analysis Systems, Raleigh, N.C.).

Virus inhibition assays. The susceptibility of HSV isolates to inhibition by ACV was also determined with a dye uptake assay (33) that measures quantitative cytopathic effect reduction in microtiter plates with serial twofold dilutions of ACV. Vero cells were challenged with approximately 30 infectious units of the test virus, and the cytopathic effect at 72 h was measured by the uptake of neutral red dye. The ID_{50} was the concentration of ACV producing a 50% reduction in dye uptake compared with cell controls (100%) and virus controls (0%).

TK assay. Extracts of HSV-infected Vero cells were assayed for TK activity by previously described procedures (22).

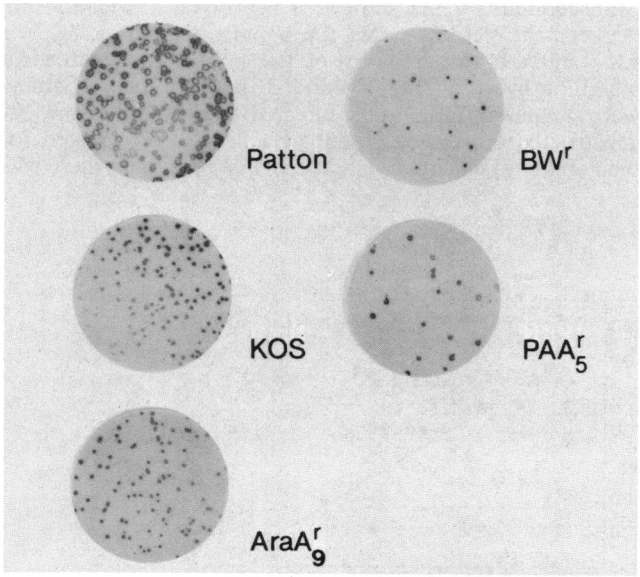

FIG. 2. Autoradiograms of Vero cell monolayers infected with HSV-1 DNA polymerase mutants and their wild-type counterparts. Infected cell monolayers were labeled with ^{125}IdC and processed as described in the text.

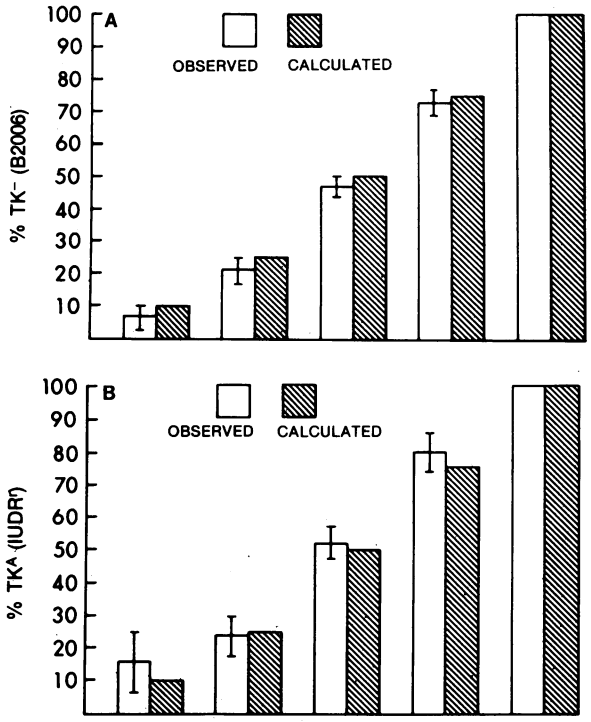

FIG. 3. Detection and quantitation of TK$^-$ (B2006) virus (A) and TK^A (IUDR^r) virus (B) present in a reconstructed mixed population of virus. The total amount of virus was determined by enumerating plaques after staining the monolayer with 0.8% crystal violet in 95% ethanol. The monolayers, which had been labeled with ^{125}IdC before fixation and staining (see the text), were then placed in contact with X-ray film for 7 days. After the film was developed, autoradiographic foci with an intensity similar to Patton controls were enumerated. Since the TK$^-$ virus-infected cells produced no images on the X-ray film, the percent TK$^+$ virus in the mixture was determined. This percent subtracted from 100 represents the observed percent of TK$^-$ virus in the mixed population in panel A. TK^A virus produce autoradiographic foci with an intensity fainter than Patton; therefore, these foci could be counted directly. The total of the individually calculated ratios of TK$^+$ and TK^A virus equaled a total of 100% of the plaques counted on the stained monolayer. The bars represent the standard deviations from the mean values obtained from 10 replicates. The calculated values were determined from the known titer of each virus (Patton and B2006) in the defined mixture.

RESULTS

Comparison of [^{14}C]ACV, ^{125}IdC, and [^{14}C]TdR plaque autoradiography. The ability of [^{14}C]TdR to differentially label cells infected with TK$^+$, TK$^-$, and TK^A virus was tested by using a plaque autoradiography assay (44). Only TK$^-$ viruses could be distinguished with 0.5 μCi of [^{14}C]TdR per ml (Fig. 1).

Several dilutions of [^{14}C]TdR were tested to determine whether lower concentrations of the labeled nucleoside would permit identification of the known TK^A viruses. At all concentrations tested (0.05, 0.1, 0.125, and 0.25 μCi/ml), results with the TK^A viruses were indistinguishable from those obtained with wild-type virus; therefore, differentiation between the TK^A mutants and wild-type virus was not possible with [^{14}C]TdR (data not shown).

Attempts to label plaques with [^{14}C]ACV were unsuccessful because ACV, unlike the TdR, is not incorporated into wild-type viral DNA to any appreciable extent. Therefore, clear identification of infected cells was not possible.

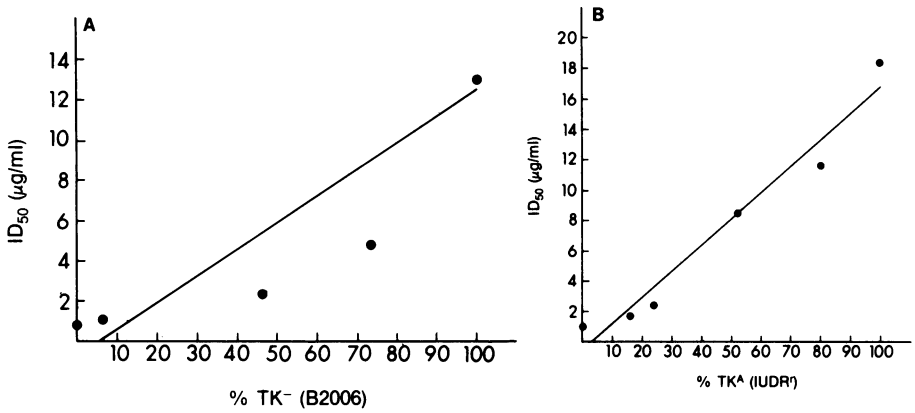

FIG. 4. Dose response of defined mixtures of HSV-1 Patton and B2006 (A) or HSV-1 Patton and IUDR^r (B) to ACV. The composition of the virus mixtures was determined by plaque titration on Vero cells and subsequent labeling with ^{125}IdC. Details are described in the text. ID_{50} values were determined by plaque reduction.

However, autoradiography of these same isolates with both [^{14}C]TdR and ^{125}IdC revealed three patterns of labeling (Fig. 1). Cells infected with ACV-susceptible isolates labeled strongly with ^{125}IdC and [^{14}C]TdR. These included three wild-type strains with multiple in vitro passage histories (Patton, CL101, SC16) and one laboratory-derived isolate selected for its resistance to *E*-5(2-bromovinyl)-2′-deoxyuridine (B3). On the other hand, the TK$^-$ mutant B2006, which lacks any detectable TK-inducing activity and shows no detectable ACV phosphorylation, did not label with either isotope. Two mutants, that express low levels of TK activity and have an impaired ability to phosphorylate ACV labeled strongly with [^{14}C]TdR, but only faintly with ^{125}IdC (IUDR^r and S1).

DNA polymerase mutants PAA$^r_5$, BW^r, and AraA$^r_9$ were tested to determine whether a mutant coding for an altered polymerase would give false-negative results due to an altered ability to incorporate IdC monophosphate into DNA. Figure 2 indicates that no differences were observed in the ability of these polymerase mutants to incorporate ^{125}IdC compared with the wild-type strains, KOS and Patton.

Reconstructed populations. To determine the reliability and lower limits of detection of the assay, reconstruction experiments with mixtures of TK$^+$ (HSV-1 Patton) and either TK$^-$ (B2006) or TK^A (IUDR^r) mutants were performed. The observed percentage of TK$^-$ virus (B2006) (Fig. 3A) and the observed percentage of TK^A virus (IUDR^r) (Fig. 3B) in the mixed population as determined by ^{125}IdC plaque autoradiography was compared with the calculated percentage of TK$^-$ or TK^A virus actually contained in the virus pool. The calculated percentage is based on the actual amount of each virus in the mixture used to infect the cells. Standard deviation lines on the observed bars show that the autoradiogram percentages fall within the calculated values. The results in Fig. 3 suggest that the reproducible lower limit for the quantitative detection of TK$^-$ or TK^A virus by this assay method appears to be 10% of the virus population. This value is reflective of the assay-to-assay variation of the autoradiography technique. However, the ability of the assay to detect TK$^+$ revertants in a pure population of TK$^-$ virus is much more sensitive, approximately 10^{-3}. This increased sensitivity is illustrated in Fig. 1, where one TK$^+$ revertant is visible in the field of plaques produced by B2006. From this B2006 TK$^-$ stock, 3 TK$^+$ plaques were identified in the 2,157 plaques counted on 10 replicate plates.

Susceptibility tests showed a nearly linear relationship between the percentage of the population that is TK$^-$ or TK^A and the susceptibility of the population to in vitro inhibition by ACV (Fig. 4A and B, respectively). Extracts were prepared from cells infected with these same reconstructed populations, and the extracts were tested for their ability to phosphorylate ACV (Fig. 5). Again, there was

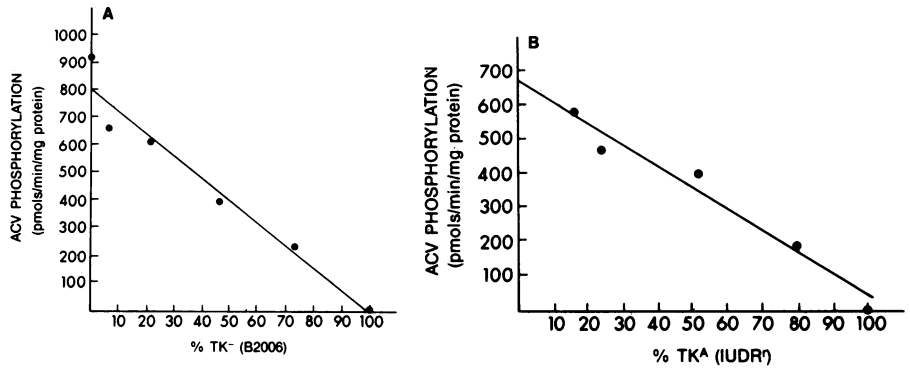

FIG. 5. ACV phosphorylation activities of extracts of cells infected with defined mixtures of HSV-1 Patton and B2006 (A) or HSV-1 Patton and IUDR^r (B). Details are described in the text.

a good correlation between the amount of TK^- (Fig. 5A) or TK^A (Fig. 5B) virus present in the sample and the ability of the extract to phosphorylate ACV.

^{125}IdC and [^{14}C]TdR autoradiography with clinical isolates. In a limited number of isolates, a precise correlation existed between susceptibility to ACV and the ability to incorporate [^{14}C]TdR and ^{125}IdC as determined by plaque autoradiography. However, this direct correlation between ACV susceptibility and uptake of label was not always found in clinical isolates. Three major patterns are presented in Table 1. Group 1 viruses (Patton and clinical isolate numbers 2860, 8493, 8736, and 6081) are characteristic of TK^+ viruses. The extracts of virus-infected cells show a significant amount of ACV phosphorylating ability, the isolates demonstrate in vitro susceptibility to ACV, and the autoradiograms of each isolate show incorporation of [^{14}C]TdR and ^{125}IdC. Group 2 viruses (B2006 and clinical isolate numbers 8295 and 2859) represent the other extreme (TK^-). The extracts do not phosphorylate ACV to an appreciable extent, the viruses are resistant to ACV in vitro, and autoradiograms show no incorporation of either isotope. Group 3 viruses ($IUDR^r$ and clinical isolate numbers 8405, 8406, 8407, and 8408) are resistant to ACV, the infected cell extracts of each exhibit negligible ACV phosphorylation, and autoradiograms of each virus indicate that ^{125}IdC is phosphorylated poorly whereas [^{14}C]TdR phosphorylation appears to be comparable to wild-type levels. A fourth group is also represented in Table 1. Isolates 8269, 8404, and 2861 represent a spectrum of mixed populations of TK^- or TK^A with TK^+ virus. For example, 8269 has both TK^- and TK^A virus, demonstrating that they can coexist in the same population. Subsequent cloning of this population confirmed this observation.

TABLE 1. Comparison of isolate ID_{50} values, [^{14}C]TdR and ^{125}IdC plaque autoradiography results, and ACV phosphorylation data

Isolate no.	ACV ID_{50} (μg/ml)[a]	Plaque autoradiography		ACV phosphorylation (pmol/min per mg of protein)
		^{125}IdC, %TK^+[b]	[^{14}C]TdR, %TK^+	
Patton	1.05	100	100	920
2806	2.6	100	100	1,700
8493	0.61	100	100	1,700
8736	1.3	100	100	1,500
6081	1.3	100	100	900
B2006	>40.0	0	0	<10
8295	12.2	0	0	<20
2859	36.0	0	0	<10
$IUDR^r$	32.2	0[c]	100	<10
8405	11.3	0[c]	100	<20
8406	12.4	0[c]	100	<20
8407	12.2	0[c]	100	<30
8408	8.4	0[c]	100	<20
8269	10.0	31	82	1,400
8404	>40.0	27	35	120
2861	1.2	83	100	2,000

[a] ID_{50} values were determined by dye uptake.
[b] The percent of plaques on the stained monolayer that had corresponding autoradiographic foci with an intensity similar to wild-type Patton.
[c] TK^A.

DISCUSSION

The technique described in this study will aid in the detection of ACV-resistant viruses that may occur naturally as part of the virus pool. Plaque autoradiography utilizing a standard plaque titration assay and capitalizing upon the selective incorporation of ^{125}IdC permitted the detection and quantitation of TK^+, TK^-, and TK^A herpesvirus in clinical samples. This technique enabled the reproducible determination of proportions of each virus variant to a lower limit of 10%. It also permitted the detection of 1 in 1,000 TK^+ virus revertants in the TK^- virus population examined. The method relies on the incorporation of ^{125}IdC, which is phosphorylated by the HSV TK and readily incorporated into viral DNA (41). A previous method that utilized the selective incorporation of ^{125}IdC into the DNA of TK^+ virus-infected cells was described by Summers and Summers (41). However, their technique did not reveal distinct plaques and therefore permitted a qualitative and not a quantitative analysis of the virus population. Tenser et al. (43) modified the method of Summers and Summers and developed a technique that employed [^{14}C]TdR plaque autoradiography to distinguish plaques formed by TK^+ virus from plaques formed by TK^- virus. Their procedure eliminated the drawbacks of the biochemical TK assay; however, their technique did not identify TK^A virus.

During the course of these studies, we found three major categories of virus populations in the samples examined: (i) isolates susceptible to ACV and able to phosphorylate both ^{125}IdC and [^{14}C]TdR, (ii) isolates resistant to ACV and not able to phosphorylate ^{125}IdC or [^{14}C]TdR, and (iii) isolates resistant to ACV and not able to phosphorylate ^{125}IdC, but able to phosphorylate [^{14}C]TdR. These categories are indicative of TK^+, TK^-, and TK^A virus phenotypes, respectively. Interestingly, none of the viruses that phosphorylated ^{125}IdC failed to phosphorylate [^{14}C]TdR.

A comparison of the autoradiograms utilizing ^{125}IdC or [^{14}C]TdR readily identifies the TK^A virus. These procedures used in tandem will allow the detection of TK^A mutants selected during therapy that acquire resistance associated with alterations in substrate specificity or a reduction in the amount of TK.

Viruses resistant to ACV occasionally have emerged during drug therapy, primarily in immunocompromised patients (1, 7, 38). Thus far, these variants have been identified as either TK^- or as TK mutants that express low levels of TK. A virus that may code for a TK with altered substrate specificity has been recovered from a clinical isolate containing a mixed population of viral phenotypes (unpublished data, Table 1). This virus was obtained from a patient during chronic suppressive oral ACV therapy. Despite recovery of this TK^A virus, the patient healed his lesion normally and continued on suppressive oral drug therapy without further recurrence. After discontinuation of ACV therapy, viruses isolated during recurrences were susceptible to ACV in vitro and contained no TK^A virus by plaque autoradiography. Similarly, patients who have shed TK^- virus during ACV therapy have generally responded well to continued drug treatment. Viruses from posttherapy recurrences in these patients have also been susceptible to ACV in vitro. Nevertheless, it is important to be able to detect the development of such resistant viruses during the course of therapy, so that when resistant variants do arise, therapy can be modified accordingly. The plaque autoradiography technique described here can be applied directly to clinical samples for the identification and quantitation of TK^+, TK^-, and TK^A virus. Moreover, this method should be useful in studies designed to follow the development of TK mutants in vitro and in vivo. Such studies could provide insight into the role

that the host and the immune status of the host play in the development of clinical resistance.

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