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A colorimetric assay for the measurement of the sensitivity of herpes simplex viruses to antiviral agents

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A quantitative colorimetric method for measuring the inhibition of viral cytopathic effects has been adapted to the assay of antiviral compounds. Drug-treated, virus-infected cultures in microtiter plates were stained with the vital dye neutral red and the amount of dye incorporated was determined in a multichannel spectrophotometer. The technique required smaller volumes of reagents, was more easily automated than the standard plaque reduction assay and had good reproducibility. Standard conditions of 30 infectious units of challenge virus and 72-h incubation were judged to be optimal. Median inhibitory concentrations (ID_{50}) for a number of compounds were approximately tenfold higher in the dye-uptake assay compared with the plaque reduction assay, possibly related to the higher multiplicity of infection required to give the desired level of cytopathic effect in the microtiter method.

colorimetric assay; drug sensitivity; herpes simplex virus; antiviral agents; neutral red dye; acyclovir

Introduction

The increasing availability of effective antiviral agents for the treatment of some viral infections underlines the need for rapid methods for determining the sensitivity of viruses to these agents. There are, however, no well-accepted standardized assays for determining virus sensitivity and the results from existing assays have not been correlated with the clinical response of treated patients. Although the plaque reduction assay is used extensively, the results obtained can be influenced by factors such as the cell type used in the assay [3,5]. This variable is especially important when the drug under test is dependent on the action of cellular enzymes to convert it into a final active form, as is the case for acyclovir which is converted from the monophosphate to the triphosphate by cellular enzymes [6]. Plaque reduction assays have the disadvantages

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of requiring large amounts of cells and of being relatively time-consuming to perform. We have therefore adapted a previously described dye-uptake assay [4] to a microtiter assay for the measurement of the sensitivity of clinical herpes simplex virus isolates to inhibition by antiviral agents. This assay was convenient, required small volumes of reagents, was able to be automated to a great extent and was reproducibly able to detect changes in in vitro sensitivities of clinical herpes simplex virus isolates.

Materials and methods

Virus strains

A reference laboratory strain of type 1 herpes simplex virus (HSV-1) (strain KOS, kindly supplied by P. Furman) and a number of low-passage clinical HSV isolates, cloned by three cycles of plaque picking where indicated, were grown in Vero cells (continuous line of African Green monkey kidney cells). When cultures showed 3 to 4 plus cytopathic effect (CPE) they were frozen and thawed three times and aliquots were stored at −70°C. Before use, all virus samples were briefly sonicated in an ultrasonic cleaning bath (Sonicor Instrument Corp., Copiague, NY, U.S.A.).

Plaque inhibition assay

Vero cell monolayers in 60-mm culture dishes were inoculated with 100 PFU of the virus under test and then overlayed with 5 ml of Eagle's MEM medium containing 0.3% immune serum globulin USP, Lot No. NF0014.2 (Cutter Laboratories, Emeryville, CA, U.S.A.) and 2-fold dilutions of drug. After incubation for 72 h in an atmosphere of 5% CO_2 in air, the cultures were stained with a solution of 1% crystal violet in a mixture of formalin, acetic acid, methanol and water (2:1:16:6). The concentration of drug-inhibiting plaque formation by 50% was determined from a probit analysis of the number of plaques in duplicate plates at each drug dilution compared with the number of plaques obtained in duplicate plates without any drug. The ID_{50} (μg/ml) of the drug for that virus was the concentration of drug-reducing plaque numbers by 50%.

Dye-uptake assay

The infectivity titer of virus pools was determined by inoculation of 50 μl volumes of serial 10-fold dilutions of the virus into wells of a 96-well, flat-bottom culture plate (Costar No. 3596, Cambridge, MA, U.S.A.), eight wells/dilution, containing 200 μl of a suspension of Vero cells (1×10^5 cells/ml of Eagle's minimal essential medium containing 5% newborn calf serum, 0.075% sodium bicarbonate, 75 U/ml penicillin, 75 μg/ml streptomycin, 2 mM L-glutamine and buffered with HEPES to pH 6.5). Control wells containing no virus (cell control) or no cells (blank control) were included in each plate in 8 replicates. To prevent excessive evaporation from the peripheral wells, the plates were sealed with sterile tape (Dynatech, Alexandria, VA,

U.S.A.) and incubated for 72 h at 37°C in a humidified atmosphere of 5% CO_2 in air.

After removal of the sealing tape, 50 µl of neutral red dye (Sigma Chemical Co., St. Louis, MO, U.S.A.; 0.15% in saline, pH 5.5) was dispensed into each well and the cultures were incubated for a further 30–45 min at 37°C. Unincorporated dye was removed by rinsing with PBS pH 6.5 using a Mini-Mash II apparatus (MA Bioproducts, Walkersville, MD, U.S.A.) to dispense and aspirate the rinse buffer which was collected into side-arm flasks for subsequent autoclaving. The dye incorporated by viable cells was then eluted into the well using 100 µl of phosphate ethanol buffer (pH 4.2) and the optical density (OD) of the solution was determined at 540 nm using a multichannel spectrophotometer designed for use with 96-well plates (Titertek Multiskan, Flow Laboratories, McLean, VA, U.S.A.). The mean OD of the cell-control wells was assigned a value of 100%, the control blank wells a value of 0% and the dilution of virus producing a 50% OD reading, i.e. 50% inhibition of cell growth, was determined from a linear regression analysis of the data using a computer program kindly designed by R. Harvey. The titer of each virus pool was expressed as a DU_{50} value, i.e. the reciprocal of the dilution of virus producing a 50% reduction in neutral red dye-uptake by the cells.

For virus inhibition assays 2-fold dilutions of drug were prepared in 50-µl volumes of culture medium in the wells of a 96-well microtiter plate using automatic dispensing and diluting apparatus (Dynatech, Alexandria, VA, U.S.A.). The usual dilution series for acyclovir gave final concentrations of 0.0178 to 5 µg/ml and 8 replicates were used for each dilution. Two hundred microliters of a suspension of Vero cells (1×10^5 cells/ml) were then dispensed into each well using similar dispensing apparatus. Fifty-microliter volumes of MEM containing approximately 30 DU_{50} of the test virus were added to the respective wells using sterile, disposable 50-µl dropping pipettes (Dynatech, Alexandria, VA, U.S.A.). Control wells containing no drug and no virus (cell control), no virus but the highest drug concentration (drug toxicity control) or no drug (virus control) were included in each plate in 8 replicates. The challenge virus was back-titrated in a separate microtiter plate to determine the exact dose used in the assay. After incubation for 72 h, neutral red dye was added and the incorporation of the dye into the monolayers was determined as described above. Linear regression analysis of the data was used to determine the concentration of drug producing a 50% reduction in viral CPE compared with cell controls (0%) and virus controls (100%). This concentration was the ID_{50} value i.e. 50% inhibitory dose.

Where specified, drug inhibition assays were performed using monolayers of Vero cells which had been incubated for 24 h in serial dilutions of drug before the addition of the challenge virus.

Quantitation of the levels of TK^+ virus in HSV pools

The presence of TK^+ virions in pools of HSV containing both TK^+ and TK^- variants was determined by plaque titration on TK^- mutant human cells (line 143 obtained from Dr. C. Croce, Wistar Institute, Philadelphia, PA, U.S.A.) in the presence and absence of aminopterin as described by Campione-Piccardo et al. (1979).

Results

Comparison of plaque reduction and dye-uptake assays

The infectivity titers of 10 virus pools (laboratory strain KOS and 9 early passage clinical isolates) were determined in plaque assays and in the quantitative CPE assay. The results (Table 1) indicate that PFU titers were an average of 16-fold (range 2–42) higher than the titers obtained in a dye-uptake assay. The sensitivities of these viruses to inhibition by acyclovir were assayed in parallel by both plaque reduction and dye-uptake assays. ID_{50} values from dye-uptake assays were an average of 9 times higher than those obtained in plaque reduction assays (Table 2). The sensitivities of a TK^+ and a TK^- HSV-1 clinical isolate to inhibition by Ara-A, IUDR and PAA, in addition to ACV, were also determined in parallel dye-uptake and plaque-reduction assays. The results obtained from dye-uptake assays were approximately 10-fold higher than those obtained in plaque reduction assays for all the compounds (Table 3).

Influence of challenge dose and incubation period

The sensitivity of HSV-1 strain KOS to inhibition by acyclovir was determined in parallel assays in which the cultures were infected with a range of virus challenge doses and stained with neutral red dye at 24, 48, 72 and 96 h. Although virus challenge doses

TABLE 1

Comparison of infectivity determinations in plaque and dye-uptake assays

Virus strain[a]	Infectivity titer (log_{10}/200 µl)		Ratio PFU/DU_{50}
	DU_{50}[b]	PFU[c]	
1498 (KOS)	5.94	6.93	10
2022	5.38	6.48	12.6
2023	4.56	5.35	6.2
2025	5.04	6.66	41.7
2035	4.81	6.17	22.9
2038	5.11	6.57	28.8
2039	4.60	4.95	2.2
2041	4.60	5.70	12.6
2067	5.96	6.28	2.1
2068	4.86	6.20	21.9
		Mean	16.1

[a] With the exception of 1498 all virus strains are early passage clinical isolates.

[b] Dye-uptake assay performed in microtiter plates with Vero cells as described in 'Materials and Methods'.

[c] Plaque-forming assay performed on Vero cell monolayer as described in 'Materials and Methods'.

TABLE 2

Comparison of virus-inhibition assays

Virus strain[a]	ACV ID_{50} (μg/ml)		Ratio dye/plaque
	Plaque[b]	Dye uptake[c]	
1498 (KOS)	0.015	0.26	17.3
2022	0.41	11.7	28.5
2023	0.028	0.17	6.1
2025	0.16	0.83	5.2
2035	0.05	0.36	7.2
2038	0.06	0.37	6.2
2039	0.03	0.11	3.7
2041	0.03	0.09	3.0
2067	0.07	0.35	5.0
2068	1.76	14.8	8.4
		Mean	9.1

[a] With the exception of 1498 all virus strains are early passage clinical isolates.

[b] Plaque reduction assay performed on Vero cell monolayers as described in 'Materials and Methods'.

[c] Dye-uptake performed in microtiter plates with Vero cells as described in 'Materials and Methods'.

were based on the infectivity titer of the virus pool obtained from a 72-h incubation assay, back titrations were done to determine the actual challenge dose used at 24, 48, 72 and 96 h. The virus challenge doses were not significantly altered after 24 h incubation. At 24 h a valid result was obtained only with a theoretical challenge dose of 3 000 DU_{50} (Table 4). Insufficient CPE was measured with the lower virus doses. By 48 h CPE was present in all plates except those receiving 3 DU_{50} of virus; the ID_{50}

TABLE 3

Determination of inhibitory activity of different compounds in two assay systems

Compound	ID_{50} (μg/ml) for virus strain[a]					
	KOS		WDB^S		WDB^R	
	Plaque	Dye	Plaque	Dye	Plaque	Dye
ACV	0.015	0.22	0.05	0.48	0.9	16
Ara-A	5.7	22	1.1	7	0.86	7
IUDR	0.51	3.6	0.38	1.5	2.28	>20
PAA	8.5	17	2.7	24	1.5	16

[a] KOS is an ACV-sensitive (TK^+) laboratory HSV-1 strain, WDB^S and WDB^R are sensitive (TK^+) and ACV-resistant (TK^-) early passage clinical HSV-1 isolates, respectively.

TABLE 4

Effect of challenge dose and incubation period on ID_{50} determinations

Incubation time (h)	Challenge dose (DU_{50})[a]	ACV ID_{50} (μg/ml)
24	3	NR[b]
	30	NR
	300	NR
	3000	0.9
48	3	NR
	30	0.1
	300	0.4
	3000	2.9
72	3	0.2
	30	0.6
	300	1.7
	3000	5.8
96	3	0.6
	30	1.1
	300	5.8
	3000	8.9

[a] Based on prior titration using 72-h incubation period.
[b] NR = No result.

values increased from 0.1 to 2.9 μg/ml with increasing amounts of virus. At 72 and 96 h all doses of virus produced significant levels of CPE. At each dose of virus, the ID_{50} increased with the incubation period, in parallel with the increasing CPE produced, and at each time point the ID_{50} increased with the challenge dose. The results obtained with a greater range of challenge doses in assays incubated for 72 h are shown in Fig. 1. Within the range of 3–300 DU_{50} of virus an approximately 4-fold increase in ID_{50} was observed; this variation was acceptable and within the range of experimental variation found in many biological asays. Results from experiments with challenge doses <3 or >300 DU_{50} showed a greater level of variation and with the higher doses a sensitive virus such as KOS would have an ID_{50} usually found with ACV-resistant, TK^- strains. For routine use a 72-h incubation period and challenge dose of 30 DU_{50} were subsequently employed, with limits of 3 and 300 DU_{50} being applied to allow for experiment-to-experiment variation in the challenge dose.

Reproducibility

For control purposes, HSV-1 strain KOS was routinely tested in all assays perform-

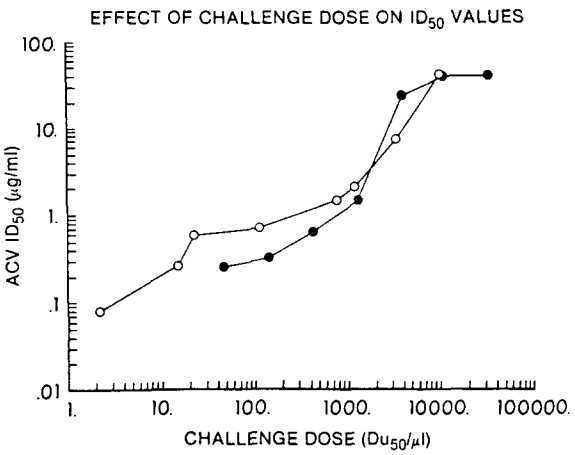

Fig. 1. Influence of virus challenge dose on ACV-inhibition assays. ACV-treated Vero cells in microtiter plates were infected with dilutions of HSV-1 strain KOS. CPE after 72 h of incubation was quantitated by the uptake of neutral red dye and the ACV ID_{50} determined for each challenge dose of virus. ●———● = Experiment 1; ○———○ = Experiment 2.

ed in this laboratory for screening the sensitivity of clinical isolates. The results of 70 sequential assays for this virus with ACV are shown in Table 5. These data indicate a mean ACV ID_{50} value of 0.26 μg/ml and a SD of 0.19 μg/ml. Any assay in which the ID_{50} of strain KOS was outside the range of the mean ± 2 SD, i.e. 2.5-fold greater than the mean was repeated. This has happened in <1% of assays over a 1-yr period. For comparison of paired clinical isolates, a difference of 5-fold was arbitrarily selected as being significant.

The plate-to-plate variation of the assay was measured in 10 parallel assays performed on the same day for strain KOS; these results (Table 5) showed a variation of only 50% from plate-to-plate.

TABLE 5

Reproducibility of dye-uptake assay for determination of virus sensitivity

	ACV ID_{50} (μg/ml)				
	n	Mean	SD	Median	Range
Experiment-to-experiment variation[a]	70	0.26	0.19	0.24	0.03–0.82
Plate-to-plate variation[b]	10	0.52	0.13	0.47	0.39–0.74

[a] Results of 70 consecutive assays.

[b] Results of 10 assays performed on the same day.

HSV-1 strain KOS was assayed for its sensitivity to acyclovir inhibition in microtiter plates in which monolayers of Vero cells were allowed to form during 24 h incubation in serial dilutions of ACV in culture medium and in plates in which Vero cells in suspension were added to the dilutions of drug a short time before the challenge virus was added. The ID_{50} values obtained were similar with both techniques (results not shown). Assays were routinely performed using the latter technique.

Effect of mixed virus populations on ID_{50} determinations

Mixed populations of acyclovir-sensitive and -resistant HSV have been found in some clinical specimens (M.N. Ellis, unpubl. obs.). To determine what effect such mixtures may have on the measurement of ID_{50} values, mixtures of ACV-resistant (TK^-, ID_{50} = 18.9 μg/ml by dye uptake) and ACV-sensitive (TK^+, ID_{50} = 0.5 μg/ml by dye-uptake) viruses were tested in both dye-uptake and plaque reduction assays. The dose–response curves with both techniques were parallel, with the dye-uptake ID_{50} being slightly more sensitive to the influence of the TK^- virus population (Fig. 2).

In such mixed populations it is possible that the ID_{50} of the mixed population is a mean of the ID_{50} values of the individual component fractions, weighted according to the relative frequency of the components. Therefore, theoretical ID_{50} values were calculated for each population and compared with the observed ID_{50} (Fig. 3). Within the limits of experimental variation the observed values paralleled the calculated values.

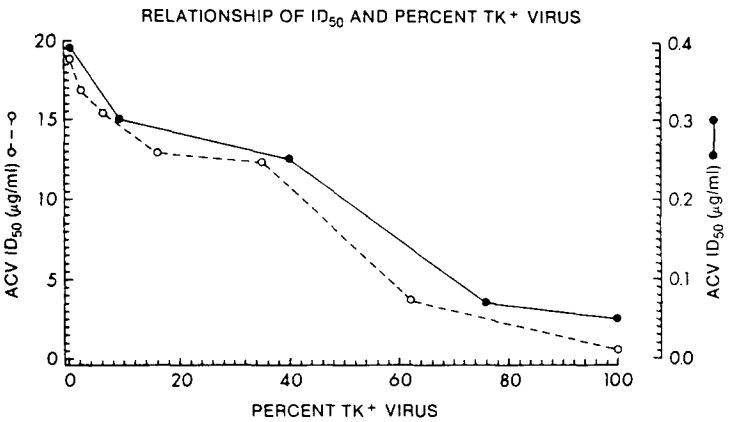

Fig. 2. ACV-dose responses for defined mixtures of TK^+ and TK^- strains of HSV-1. Mixtures of TK^+ and TK^- variants of a clinical isolate were tested for their sensitivity to inhibition by ACV in a quantitative CPE-reduction assay. The composition of the virus mixtures was determined by plaque titration on TK^- 143 cells in the presence or absence of aminopterin. o---o = dye uptake; ●——● = plaque reduction.

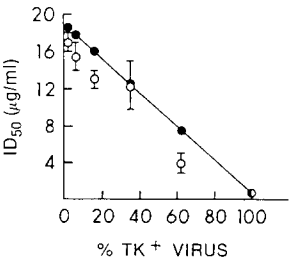

Fig. 3. Correlation between theoretical and observed ID_{50} values for defined mixtures of TK^+ and TK^- viruses. The ID_{50} values for defined mixtures of TK^+ and TK^- HSV were obtained in a quantitative CPE-reduction assay. The theoretical ID_{50} values were calculated from the known ID_{50} values of the component viruses in the proportion of the viruses in the defined mixture. ● = calculated ID_{50}; ○ = observed; ID_{50}.

Discussion

We are currently entering an era where effective antiviral chemotherapy is available for some diseases. The effectiveness of such therapy may, however, be compromised by the presence of drug-resistant virus populations. To help define whether treatment failures are the result of such specific drug resistance or of other factors, it is important that the specific sensitivities of viral isolates against antiviral agents be determined. Ideally, the methods for measuring virus sensitivity would involve rapid techniques which would be correlated with clinical observations. The present situation, however, is far from this ideal in that methods for determining virus sensitivity are not usually rapid, involving perhaps 2 to 3 days or longer, and are not standardized, with variation in assay results depending on the method of assay and cell strain used. In addition, there are currently no accepted definitions of what is a 'resistant' strain of virus.

In our laboratory, we have a specific need to compare the sensitivities of large numbers of clinical isolates of herpes simplex virus isolated from patients treated with acyclovir or placebo in clinical studies. These laboratory studies are essentially retrospective in nature but because of the number of specimens to be examined, we require an assay that is able to be automated, requires few cell culture reagents, is reproducible and can specifically detect changes in virus sensitivity. The dye-uptake assay satisfies all of these requirements.

Procedures in which the degree of CPE was determined by visual observation of unstained or crystal-violet stained monolayers in microtiter culture plates have been described for the assay of ACV [9] and for the assay of interferon [8,11]. The uptake of neutral red dye, a vital stain which is taken up by living cells but not by virus-killed cells, was initially described for the assay of interferon [4]. Measurement of the eluted dye can be easily quantitated using multichannel spectrophotometers designed for ELISA-type assays. The use of automatic diluters and dispensers further increases the convenience of the assay permitting 40 plates or more to be set up each day. The addition of cells in suspension to the diluted drug in the microtiter plates saves an

additional handling step compared with using preformed monolayers in the plates and does not significantly alter the sensitivity of the assay.

Standard conditions adopted for the assay were a 72-h incubation period and a virus challenge dose of 30 DU_{50}. Our results with different challenge doses indicate that problems in interpretation of data may occur if untitered clinical isolates are used in rapid assays to detect potential drug-resistant isolates. With our assay one could use a shorter incubation period with a higher challenge dose but this did not offer any advantage in the present setting since only 3 drug assays/wk, with 15–20 viruses tested/assay, could still be performed in a normal 5-day working week.

Reproducibility of the assay was good and as a control the laboratory strain KOS was included in all assays; the assay in total was repeated if the ID_{50} for KOS was > 2 SD from the mean observed in a series of 70 titrations. With clinical specimens we have adopted an arbitrary 5-fold limit of acceptable variation between paired isolates. We have observed some reproducible, low-level variations in ID_{50} between different sequential isolates from a patient but the use of a 5-fold limit has worked well in practice in discriminating between significant and insignificant variations in ID_{50}. In addition we employ a cutoff value of 2 μg/ml as a useful threshold separating sensitive and in vitro-resistant viruses because we have found 95% of clinical isolates to be inhibited by 2 μg/ml or less [7].

ID_{50} values determined in the dye-uptake assay were approximately 10-fold higher than those obtained in a comparable plaque reduction assay for the drugs acyclovir, IUDR, Ara-A and PAA. The difference in ID_{50} as determined in dye-uptake compared with plaque-reduction assays did not appear to be a function of the use of preformed monolayers in the plaque-reduction assay as similar results were obtained when preformed monolayers were used in the dye-uptake assay. The higher dye-uptake ID_{50} values may rather reflect the relatively greater challenge dose of virus used in that assay, with each well receiving 30 DU_{50} of virus equivalent to approximately 500 PFU (30×16, Table 1). A major difference between the two assays is that in the dye-uptake assay the liquid overlay does not inhibit the extracellular spread of virus which is restricted in a plaque-reduction assay by either agarose or immune serum globulin. Potentially a small fraction of drug-resistant virus could replicate and be transmitted extracellularly in the dye-uptake assay to give a disproportionate amount of viral CPE by the end of the incubation period.

We do not, however, view this as a disadvantage since it is a good indication of the presence of drug-resistant virus in the test virus population. If such virus is present in the initial patient lesion, then it would have an equal opportunity to be expressed in the presence of drug as in our in vitro system. However, even the dye-uptake assay would not be able to detect levels of TK^- virus of less than 25%. The presence of low levels of ACV-resistant virus, able to produce plaques in the presence of 10 μmol ACV, in clinical isolates has also been reported by Smith et al. [13], with similar findings by Parris and Harrington [10].

The dye-uptake assay has proven to be a valuable and labor-saving technique for the routine determination of the sensitivity of herpes simplex isolates to inhibition by acyclovir and other antiviral drugs. The technique can also be used for screening compounds for activity against other viruses. The method has distinct advantages

over the standard plaque reduction assay but many of these advantages are the result of our use of automatic or semi-automatic equipment which may not be available to all laboratories. Using this technique we have detected a small number of patients whose isolates have shown a significant decrease in sensitivity to acyclovir during therapy [1,7,12,14]. We are, however, unable at this stage to correlate the ID_{50} values observed in our clinical isolates with the eventual clinical outcome in these treated patients. In some instances the patients responded successfully to therapy although their viruses were TK^- and were significantly less sensitive to acyclovir in vitro. These findings underline our present lack of knowledge of the significance of in vitro assays and their correlation with clinical outcome.

Acknowledgement

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