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A rapid and reliable assay for testing acyclovir sensitivity of clinical herpes simplex virus isolates independent of virus dose and reading time

J.P. Kruppenbacher*, R. Kläss and H.J. Eggers

Institut für Virologie der Universität zu Köln, Fürst-Pücklerstr. 56, 50935 Köln 41, Germany

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Summary

The determination of acyclovir (ACV) sensitivity of clinical herpes simplex virus (HSV) isolates was studied by means of a cytopathic effect (CPE) inhibitory assay (CIA). Medium of HSV-infected Vero cells was supplemented with different ACV concentrations. The CPE was read quantitatively by light microscopy. The inhibitory dose for 50% CPE reduction (ID_{50}) was calculated by applying 1st or 3rd degree regression lines, and aspects of different methods for calculation are discussed. The CIA proved highly reproducible. Surprisingly, the obtained ACV-ID_{50} values were independent of reading time and virus dose used in the test. In comparison to dye-uptake assay, CIA could be evaluated earlier. Therefore, CIA provides simple, rapid, and precise determination of ACV sensitivity of clinical HSV-isolates which can be achieved within 1 or 2 days after the virus has been isolated out of a clinical specimen.

Acyclovir sensitivity; Herpes simplex virus; Antiviral sensitivity testing

Introduction

Various tests have been designed to determine acyclovir (ACV) sensitivity of clinical herpes simplex virus (HSV) isolates: plaque reduction assays (PRA), virus yield inhibition assays (YIA) and assays measuring reduction of viral cytopathic effects (CPE) (Eggers, 1988). In principle, with all these tests the

*Corresponding author. Fax: +49 0221 407490.

inhibition of HSV replication was scored in the presence of different ACV concentrations. Reduction of virus replication was monitored by quantification of viral antigens by ELISA (Wahren et al., 1983; Harmenberg et al., 1986; André et al., 1988; Agut et al., 1990), quantitative hybridization of HSV-DNA (Gadler et al., 1984; Harmenberg et al., 1986; Brisebois et al., 1989; Englund et al., 1990), titration of yields of infectious virus (Field et al., 1980; Svennerholm et al., 1985; Harmenberg et al., 1986), measurement of dye-uptake of surviving cells (McLaren et al., 1983, based upon the method of Finter, 1969), plaque assay with ACV in the agar or carboxymethyl cellulose overlay (Field and Darby 1980; Crumpacker et al., 1982), or by light-microscopically-observed inhibition of CPE (De Clercq et al., 1980).

However, some HSV strains are poor plaque producers in permanent cell lines (Munk and Donner, 1963), and consequently do not permit a reliable PRA. PRA and especially YIA, as well as ELISAs, DNA hybridizations, and virus titrations are rather time-, labor- and material-consuming. Additionally, the dye-uptake assay (DUA) seems to be time- and virus dose-dependent (McLaren et al., 1983), and can be read only once. Therefore, all these assays are hardly practical for routine test programs. Thus, we wanted to establish a rapid, simple and reliable method for determination of ACV sensitivity of clinical HSV isolates. Our assay is based on readings of CPE inhibition (CPE inhibition assay (CIA)). We have shown that the concentration of ACV for 50% inhibition (ACV-ID_{50}) of CPE is essentially independent of virus dose and reading time.

Materials and Methods

Cells

An African green monkey kidney (Vero) cell line was grown in minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS). Human embryonic lung cells (HEL) and human foreskin fibroblasts (HFF) were established in our laboratory and maintained in MEM containing 10% FBS. The permanent human colon carcinoma (CaCo-2) cell line (kindly provided by Dr. Reigel, Basel) was cultured in MEM with 5% FBS. All media were supplemented with penicillin (100 I.U./ml) and streptomycin (0.5 mg/ml).

Compound

Acyclovir (Lot. 18183) was a gift of Deutsche Wellcome GmbH. A stock solution of 1 mM was prepared in MEM, and aliquots of 1 ml were stored at $-20°C$. ACV concentrations used for assays were monitored by photometric measurement of the solutions in 0.1 M hydrochloric acid at 255 nm, as described by the manufacturer.

Virus

Herpes simplex viruses were isolated either on CaCo-2, HEL or HFF cells

from patients suffering from the following diseases: HSV-1 strains: 18104/1 and 36637/1 stomatitis aphthosa; 32244/1 herpes labialis/endogeneous eczema; 35585/2 vulvitis; 26538/2 perianal ulcer (anti-HIV positive); 15728/7 immunosuppressed kidney recipient; 3788/ 13 nasal ulcer (anti-HIV positive); Ka/2 clinical data not available. HSV-2 strains: 7091/14, 12498/13, 19017/3 and 26802/3 perianal ulcer (anti-HIV positive); 4621/11 scrotal ulcer (anti-HIV positive); 44792/2 clinical symptoms not reported (anti-HIV positive).

Isolates were passaged in Vero cells one to three times to prepare stocks, if necessary. Virus was harvested by freezing and thawing three times. Cell debris were pelleted by centrifugation (1700 × *g*, 4°C) for 30 min. Supernatants were stored in aliquots of 1 ml at −70°C. Strains were typed using an HSV ELISA Kit (Dakopatts, Denmark, Code No. K 223). Virus titrations were performed using the limiting dilution method in microtiter plates (8 replicate wells with 100 µl Vero cell suspension (5×10^4 cells) and 100 µl virus dilution).

CPE inhibition assay (CIA)

50 µl ACV (usually serial 2-fold dilutions in MEM to obtain the desired final concentrations of 0.025 to 1.6 µM), 50 µl virus dilution and 100 µl Vero cell suspension (10^5 cells/ml in MEM with 5% FBS) were distributed into wells of a 96-well microtiter plate (Falcon No. 3075, Becton Dickinson, Lincoln Park, NJ, USA); 12 replicate wells were used for each drug dilution. Control wells containing neither drug nor virus (cell control), the highest ACV concentration used without virus (toxicity control), or virus without antiviral compound (virus control) were included in each assay. All control wells were filled with MEM 5% FBS in order to achieve a final volume of 200 µl per well. After an incubation period for at least 20 h at 37°C in a humidified atmosphere of 5% CO_2 in air, the development of CPE was examined by light microscopy.

Examinations for CPE were performed several times at intervals of 4 to 12 h. Each well was scored on a scale ranging from 0 to 4 (0 = no CPE; 0.5 = < 10% CPE; 1 = 25% CPE; 2 = 50% CPE; 3 = 75% CPE; 4 = > 90% CPE). The sum of scores of wells of the same ACV concentration (maximum of 12 × 4 = 48) was divided by the sum of scores of virus control wells (without ACV). These ratios (CPE scores with ACV/CPE scores without ACV) were plotted against $\log_2$ ACV-concentration and the data entered into a computer for further analysis.

Dye-uptake assay (DUA)

Virus, cells and compound were prepared as described for the CIA. After appropriate incubation times (see Table 2), 100 µl of neutral red dye (0.15% in phosphate-buffered saline (PBS), pH 5.5) was placed into each well. The plates were then incubated for a further 30 min at 37°C, after which medium and residual stain were removed and the wells rinsed twice with PBS (pH 6.5). Thereafter, 200 µl phosphate ethanol buffer (PBS and ethanol 1:1 (v/v), pH 4.2) was distributed into each well for elution of dye incorporated by supposedly viable cells. The optical density (OD) of the solutions was read at 550 nm using an automatic spectrophotometer (easy-reader 400 ATC, SLT-Labinstruments, Salzburg,

Austria). The mean OD for each ACV concentration was calculated, and computer analysis (as described below) was used to determine the ACV concentration exhibiting a dye-uptake of 50% (as measure for reduction of virus-induced cytopathic alterations) in comparison to virus (=0%) and cell control (= 100%).

Polynomial regression lines to determine ACV-ID_{50} values

The effects in the presence of ACV (reduction of CPE, increase of dye-uptake) were plotted as ratios of the scores determined for the appropriate ACV concentrations vs. virus control. The data served for calculation of 1st and 3rd degree polynomial regression lines which best fit the experimental values. Data analysis is based upon a least square method to calculate the coefficients of the orthogonal polynom using the algorithm described by Wood (1987). With aid of the obtained coefficients (a, b, c, d) of the equations $y = a + bx$ and $y = a + bx + cx^2 + dx^3$, respectively, the ACV concentration (x) required for 50% inhibition of viral CPE ($y = 0.5$) as compared to the virus control can be computed (using a BASIC program written by A. Ritzkowsky, Institute of Virology, University of Cologne) and yields the ACV-ID_{50} value.

Application of calculation for ACV-ID_{50}

Some comments about the selection of CPE score ratios and the degree of regression analysis are necessary.

First, the CPE score in the virus control should amount to at least 10 (10/48 (21%)). A CPE score 5 is then expected for the ACV concentration yielding ACV-ID_{50}. If lower CPE scores are observed, the accuracy of ACV-ID_{50} values decreases. Nevertheless, the test can be evaluated at an early time.

Second, after the CPE score in the virus control has reached 48 (48/48 (100%)), CPE scores in the ACV groups will still be increasing in contrast to the CPE scores of the virus control. Consequently, increasing ACV-ID_{50} values will be obtained, especially when 1st degree regression analysis including all CPE score ratios is applied (e.g., this occurs with ACV-resistant HSV strains; see Results and Discussion).

Third, the 1st degree regression line based on calculations excluding extreme values (i.e., CPE score ratios which are <0.05 and >0.95) gives a much better fit in the descending part of the curve (note: CPE score ratios should not be confused with CPE scores). This method was preferred by us, since it is easy to perform, and, therefore, all ACV-ID_{50} values were calculated by this procedure, unless stated otherwise.

The calculated 3rd degree curves yielded the best fit, especially in the descending part of the curve, including the point of intersection with the 0.5-line. This is even true when the virus control has reached 100% and/or CPE score ratios (at least of lower ACV concentrations) have approached 1.0. But a 3rd degree curve is cumbersome to calculate. It should only be applied when the just mentioned conditions took effect before the first reading had been done. This might occur, for example, when the HSV isolate is growing very fast, or with ACV-resistant HSV strains.

Results

Effect of virus dose and reading time on ACV-ID_{50} value

When the test was performed with different virus doses of the same isolate (range 50–10000 $TCID_{50}$ HSV-1/18104/1) and CPE scores were determined at several reading times (21–72 h), ACV-ID_{50} values between 0.04 and 0.27 μM were obtained with an average of 0.08 ± 0.04 μM (calculated by 3rd degree regression). Fig. 1 demonstrates that the values within this range of virus dose were distributed randomly, independent of virus dose and reading time.

Eight dose-response curves obtained by 2 tests with 100, and 1000 $TCID_{50}$, respectively, and read at different incubation times are shown in Fig. 2. The curves run in parallel to each other and intersect the 0.5-line within a small

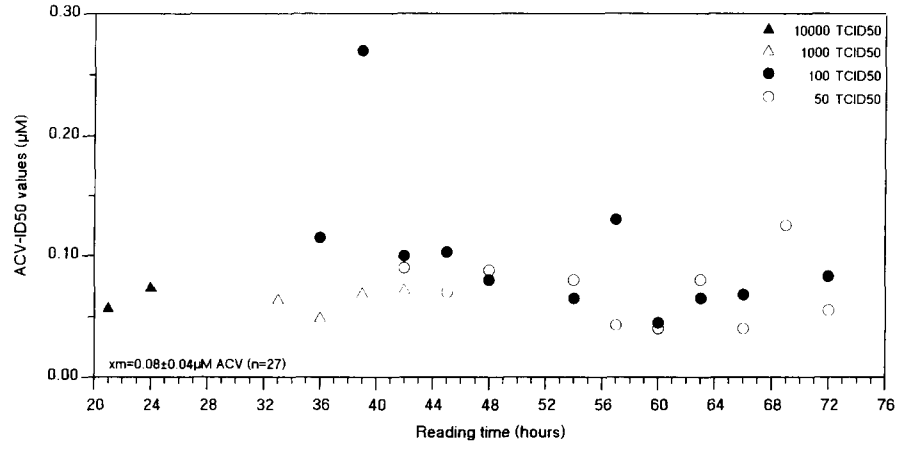
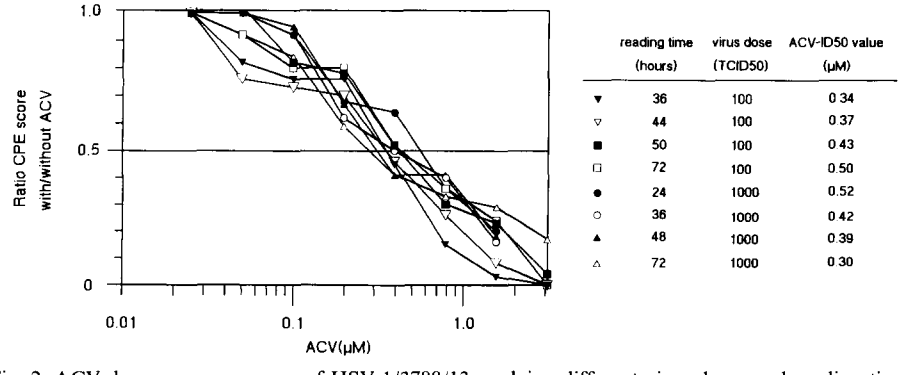


Fig. 1. Effect of challenge dose and reading time on ACV-ID_{50} values in CIA. ACV sensitivity of HSV-1/18104/1 (laboratory reference strain) was determined with 50, 100, 1000, 10 000 $TCID_{50}$ doses. Readings were performed at intervals of 6 or 8 h. When the CPE score in virus controls (without ACV) had reached 100%, tests were terminated. 27 ACV-ID_{50} values were obtained with a mean of 0.08 ± 0.04 μM.



reading time (hours)	virus dose (TCID50)	ACV-ID50 value (µM)
36	100	0.34
44	100	0.37
50	100	0.43
72	100	0.50
24	1000	0.52
36	1000	0.42
48	1000	0.39
72	1000	0.30

Fig. 2. ACV dose-response curves of HSV-1/3788/13 applying different virus doses and reading times.

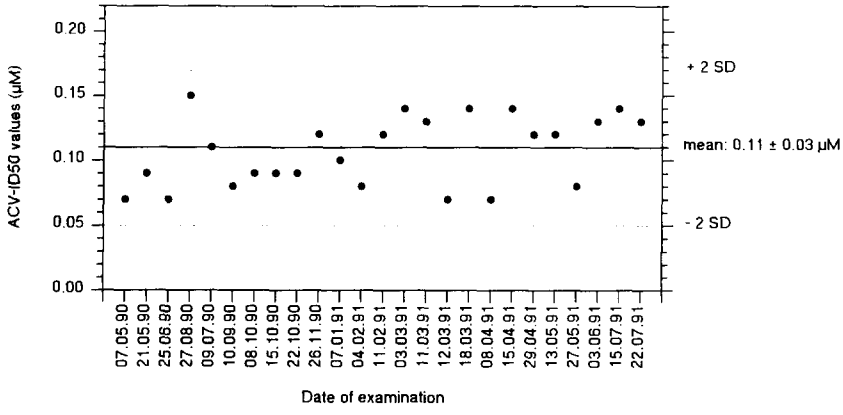


Fig. 3. Reproducibility of ACV-ID_{50} values by CIA. Results of 25 determinations for HSV-1/18104/1 with 100 $TCID_{50}$, which served as controls for routine testing between 07.05.90 and 22.07.91. The mean ACV-ID_{50} value was 0.11 μM, the standard deviation (S.D.) 0.03 μM ACV; no value exceeds ± 2 S.D. limits. Each final ACV-ID_{50} value is the mean of values which has been obtained by several reading times whereby values from regression lines with correlation coefficients $r \leqslant 0.90$ were excluded.

range (ACV-ID_{50}: 0.30–0.52 μM). This demonstrates, too, that the ACV concentration reducing the CPE score to 50% is independent of virus challenge dose and reading time. Accordingly, each curve might serve for calculation of the ACV-ID_{50} value.

Reproducibility and sensitivity of the ACV-ID_{50} value by CIA

Between 7.5.90 and 22.7.91 25 CIA routine tests were performed with our laboratory reference strain. The mean ACV-ID_{50} value was 0.11 ± 0.03 μM; the range was 0.07 to 0.15 μM (Fig. 3). We conclude that ACV-ID_{50} values obtained by CIA are highly reproducible.

Fig. 4 shows ACV dose-response curves of 3 HSV strains with different drug sensitivities. Each point represents an average of CPE score ratios of several determinations (numbers of assays and readings are given in the legend of Fig. 4). The standard deviation is small. Therefore, it can be stated that the dose-response curves of the two HSV strains 18104/1 and 3788/13 with ACV sensitivities of 0.10 μM and 0.39 μM, respectively, were significantly different. We conclude that this test clearly discriminates within this range of ACV sensitivity.

Comparison of 1st and 3rd degree regression analysis

ACV-ID_{50} values by CIA were determined by 1st degree regression analysis choosing two different sets of values: either all CPE score ratios of a definite time point were used, or only ratios ranging from 0.05 to 0.95 were taken for regression analysis. Third degree regression analysis was performed with all CPE score ratios. The respective curves for a sensitive and a resistant HSV strain are shown in Fig. 5. For HSV-1/15728/7 (Fig. 5A) the ACV-ID_{50} values (0.17/0.17/0.16 μM) and the correlation coefficients (0.94/0.93/0.99) were very

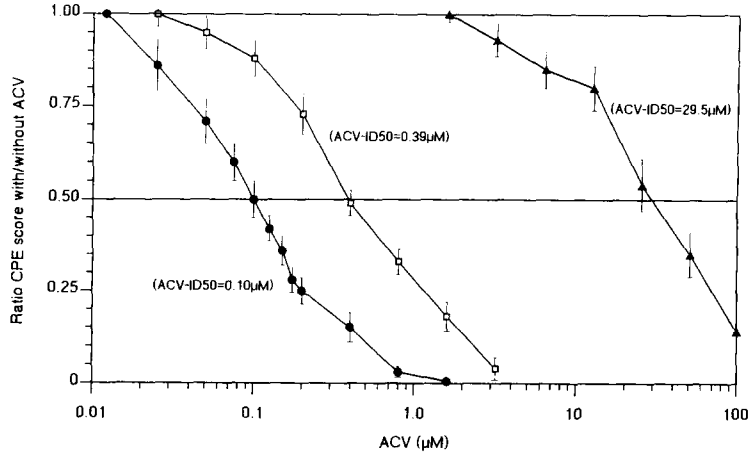


Fig. 4. Dose-response curves obtained by CIA of HSV strains with different ACV sensitivities. The mean ± S.D. for each ACV concentration is indicated. HSV-1/18104/1 (●) was tested 26 times; 21, 3 and 2 tests were performed with 100, 1000 and 10 000 $TCID_{50}$, respectively; 36 readings were executed between 20 and 88 h p.i. HSV-1/3788/13 (□) was tested 7 times; 4 and 3 tests were performed with 100 as well as 1000 $TCID_{50}$; 14 readings were done between 24 and 84 h p.i. HSV-2/7091/14 (▲) was tested 5 times; 3, 1 and 1 tests were performed with 100, 1000 and 10 000 $TCID_{50}$, respectively; 11 readings were done between 20 and 96 h p.i.

similar; for the resistent HSV-2/7091/14 strain (Fig. 5B) ACV-ID_{50} values were 61.7/32.0/41.4 μM, and the correlation coefficients were 0.80/0.95/0.98, respectively.

TABLE 1

ACV-ID_{50} in CIA obtained by different methods of calculation

Virus Type	Isolate	A ID_{50}	r	B ID_{50}	r	C ID_{50}	r
HSV-1	32244/1	0.04	0.93	0.05	0.93	0.04	0.98
	35585/2	0.08	0.87	0.07	0.99	0.07	0.99
	26538/2	0.10	0.91	0.10	0.94	0.09	0.98
	18104/1	0.14	0.92	0.11	0.96	0.11	0.98
	15728/7	0.17	0.94	0.17	0.93	0.16	0.99
	3788/13	0.43	0.96	0.46	0.97	0.45	0.99
	36637/1	0.78	0.93	0.73	0.97	0.79	0.99
	'Ka'/2	6.31	0.90	6.76	0.88	6.40	0.98
HSV-2	26802/3	0.31	0.94	0.32	0.95	0.33	0.97
	4621/11	0.41	0.84	0.34	0.94	0.35	0.96
	19017/3	0.44	0.94	0.46	0.98	0.45	0.99
	12498/13	0.68	0.87	0.64	0.98	0.64	0.99
	44792/2	10.18	0.86	12.28	0.96	13.25	0.99
	7091/14	61.70	0.80	32.00	0.95	41.40	0.98

A linear regression 1st degree (all CPE score ratios included).
B linear regression 1st degree (only CPE score ratios ranging from 0.05–0.95 included).
C linear regression 3rd degree (all CPE score ratios included).
Note: highest and lowest correlation coefficients (r) are underlined.

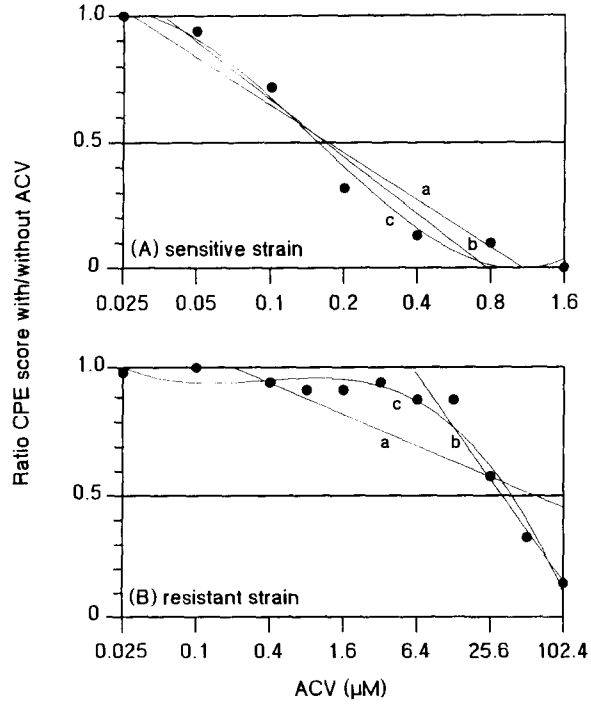


Fig. 5. ACV-ID_{50} values for the CPE readings of isolates no. 15728/7 (HSV-1) after 62 h with 100$TCID_{50}$ (A), and of 7091/14 (HSV-2) after 34 h with 1000$TCID_{50}$ (B) were calculated by 1st degree regression considering (a) all CPE score ratios, (b) ratios between 0.05 and 0.95, and (c) 3rd degree regression analysis including all CPE score ratios. For the sensitive strain ACV-ID_{50} values were obtained (a) 0.17 μM, $r=0.94$; (b) 0.17 μM, $r=0.93$; (c) 0.16 μM, $r=0.99$, and for the resistant strain (a) 61.7 μM, $r=0.80$; (b) 32.0 μM, $r=0.95$; (c) 41.4 μM, $r=0.98$.

The 3 different methods for calculation of ACV-ID_{50} values were applied to various HSV isolates as listed in Table 1. ACV-ID_{50} values do not differ significantly except for isolate 7091. On the average, the correlation coefficients amounted to 0.90 ± 0.05 with 1st degree polynomial considering all values, 0.95 ± 0.03, excluding extreme values, and to 0.98 ± 0.01 with 3rd degree polynomial. The lowest value for a correlation coefficient was 0.80 (isolate 7091, 1st degree polynomial regression). However, it has to be realized that already for mathematical reasons correlation coefficients do increase with regressions of higher degrees.

ACV-ID_{50} values obtained by CIA and DUA

ACV sensitivity tests as determined by CIA and DUA yield comparable values as long as the CPE score in the virus control is below 100% (Table 2). However, as soon as the CPE of virus control had reached 100%, the values became greater. Therefore, with higher virus challenge doses as well as with late

TABLE 2

Influence of virus dose and incubation period on ACV-ID_{50} values assayed by DUA and CIA (HSV 18104/1)

Incubation time (h)	Virus dose ($TCID_{50}$)	CPE virus control (%)	ACV-ID_{50} (μM)	
			DUA	CIA
24	50	2	NA[a]	–[b]
	100	13	NA[a]	–[b]
	1000	25	NA[a]	0.09
	10000	78	NA[a]	0.09
48	50	25	NA[a]	0.12
	100	47	NA[a]	0.08
	1000	78	0.17	0.16
	10000	100	0.53	0.45
72	50	78	0.16	0.13
	100	100	0.09	0.14
	1000	100	0.26	0.47
	10000	100	0.69	0.97
96	50	100	0.13	0.24
	100	100	0.31	0.44
	1000	100	0.79	1.05
	10000	100	1.45	2.46

[a]Not applicable; [b]CPE values in control out of range (20 to $<100\%$) applicable to CIA.

reading times, ACV-ID_{50} values do increase (see Discussion).

Virus yields after isolation on different cell lines

The titer of HSV isolates after the first passage on HFF was $10^{4.0\pm1.8}TCID_{50}/50$ μl ($10^{0.6}$, $10^{1.4}$, $10^{1.9}$, $10^{2.5}$, $10^{3.6}$, $10^{4.2}$, $10^{4.3}$, $10^{4.4}$, $10^{4.8}$, $10^{5.3}$, $10^{5.6}$, $10^{5.6}$, $10^{5.7}$, and $10^{6.2}TCID_{50}/50$ μl), and CPE scores averaged 3.0 ± 1.0 after 3.5 ± 1.6 days ($n=14$ isolates); isolates after a first passage on Caco-2 cells yielded $10^{3.0\pm1.0}TCID_{50}/50$ μl with a CPE score of 1.3 ± 0.5 after 7.0 ± 1.3 days ($n=8$ isolates). After HEL-passage 2/Vero-passage 1 (for stock preparation), the virus titer of isolates averaged $10^{5.0\pm1.0}TCID_{50}/50$ μl ($n=53$ isolates).

Discussion

Severe HSV infections are frequently found in immunosuppressed patients, but sometimes virus-specific ACV therapy fails (Erlich et al., 1989b; Englund et al., 1990). In these cases treatment with another drug such as foscarnet may be successful (Erlich et al., 1989a; Safrin et al., 1990; Sall et al., 1990; Stellbrink et al., 1991). Therefore, it is important for the physician to know the ACV

sensitivity of a clinical HSV isolate, particularly after prolonged treatment with ACV. The multitude of sensitivity tests and some of their shortcomings have been stated in the Introduction. We, therefore, wanted to establish a rapid and reliable assay for testing ACV sensitivity of clinical HSV isolates which is easy to perform. Development of HSV-induced CPE served as a marker for virus replication in the presence and absence of ACV and was used for calculating the ACV sensitivity. Various methods of calculation were compared.

An essential result of our investigation is that the ACV-ID_{50} value, i.e., the drug concentration inhibiting HSV CPE to 50% is clearly independent of the dose of challenge virus and reading time of the CIA (Figs. 1 and 2). Each point of the dose-response curves shown in Fig. 4 represents the mean of determinations obtained with different virus challenge doses and different reading times. The standard deviation is small, and HSV isolates with ACV-ID_{50} values of 0.1 μM and 0.39 μM, respectively, were discerned as significantly different. This implies important practical advantages. ACV-ID_{50} values can be determined at time points convenient to the investigator. (If more than one reading of the same test is available, extreme deviating values become readily apparent and can be eliminated; for example see Fig. 1: 0.27 μM with 100$TCID_{50}$ after 39 h).

HSV isolates with a wide range of ACV-ID_{50} values (0.06–2.50 μM) and nearly all intermediate stages (data not shown) were observed. Thus, the ACV-ID_{50} appears to be a characteristic marker of an individual HSV isolate.

Since CIA is not dependent on virus dose, ACV sensitivity tests can be performed subsequent to HSV isolation without virus titration. Challenge virus, drug dilutions, and cell suspension are given onto the microtiter plate immediately one after another, the CPE scores are evaluated by light microscopy during the next 24–48 h (see Fig. 2), and ACV-ID_{50} values can be calculated. Therefore, an ACV-ID_{50} value can be obtained by CIA within 5 days, provided HSV in a clinical specimen does reach a CPE score of about 3 within 3.5 ± 1.6 days on HFF cell cultures. This is an obvious advantage in comparison to other ACV sensitivity tests.

Occasionally the virus yield of first passage may reach or exceed 10^6 $TCID_{50}$/50 μl (mean titer: $10^{4.0 \pm 1.8}TCID_{50}$/50 μl; see also Results). Then, the undiluted virus suspension may induce complete CPE within less than 24 h in the presence of all ACV concentrations, and consequently the CIA can not be evaluated. Therefore, under certain circumstances it is advisable to dilute the HSV suspension accordingly after primary passage (3.5 ± 1.6 days after inoculation) on HFF. If not enough virus material is available from primary passage, the HSV isolate has to be passaged again.

With DUA (which is also dependent on virus-induced cell damage) decreasing ACV sensitivities are observed with increasing virus doses and reading times; consequently, it has been recommended to read all tests after a constant time period (McLaren et al., 1983). However, higher virus doses necessarily accelerate development of CPE and complete CPE will be reached much earlier in the virus control. Readings *after* this time point (when 100%

CPE has been achieved in the virus control) will result in 'time-dependent' increases of ACV-ID_{50} values. The earlier a 100% CPE in the virus control has been reached the more time will be left for virus replication in the presence of ACV under the condition of a constant reading time, and higher ACV-ID_{50} values will be obtained. We assume that the variations observed by McLaren et al. (1983) do not only reflect the variation of biological assays, but are also dependent on factors resulting from a constant reading time.

With low virus doses, e.g., 50$TCID_{50}$ and 100$TCID_{50}$, no determination of ACV-ID_{50} is possible after 24 h or 48 h by DUA (Table 2). Furthermore, no result could be obtained after 24 h with up to 300 DU_{50} (1 DU_{50} is equivalent to 8$TCID_{50}$ as determined in our laboratory) and after 48 h with 3DU_{50} (McLaren et al., 1983). In contrast, CPE can microscopically be observed at these early time points, i.e., reliable values are obtained by CIA (Table 1).

In conclusion, we established an assay for testing ACV sensitivities of clinical HSV isolates. The test is easy to perform. Results are obtained within a reasonably short period of time and are highly reproducible. During more than 14 months HSV-1 18104/1 was tested 26 times by several technicians, and the mean value of ACV-ID_{50} was 0.11 ± 0.03 μM. This variation is within the range found in many biological assays. Furthermore, the test is rapid and easy to perform, measures the inhibition of HSV replication and, therefore, might be suitable as a reference test (Swierkosz, 1992). Further investigations have to show, in what way ACV-ID_{50} correlates with clinical events and whether the described procedure can be applied to other antiviral drugs.

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