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Comparison of two methods in the determination of the sensitivity of 84 herpes simplex virus (HSV) type 1 and 2 clinical isolates to acyclovir and alpha-interferon

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Summary

Two methods, the colorimetric method (neutral red dye uptake), and DNA hybridization using a HSV thymidine kinase gene probe (TK) have been used to examine the sensitivity of 84 herpes simplex virus (HSV) type 1 and 2 clinical isolates to two antiviral drugs, acyclovir (ACV) and alpha-interferon (α-IFN). Using the colorimetric method, HSV isolates had ED_{50}s ranging from 0.03 ± 0.02 μg/ml to 0.164 ± 0.03 μg/ml for ACV and 6.3 ± 5.2 IU/ml to 55.0 ± 11.4 IU/ml for α-IFN. With the DNA hybridization method, ED_{50}s ranged from 0.033 ± 0.012 μg/ml to 0.190 ± 0.031 μg/ml for ACV and 8.5 ± 5.0 IU/ml to 43.5 ± 6.0 IU/ml for α-IFN. Two strains of HSV-1 were found to be resistant to very high concentrations of ACV (>50.0 μg/ml). The values obtained by the two methods showed good correlation (r=0.724, P=0.002). Furthermore, our results demonstrate that the two methods are reproducible, reliable and the dye uptake assay is suitable for use in a diagnostic virology laboratory.

Herpes simplex virus; Acyclovir; α-Interferon; Sensitivity

Introduction

Herpes Simplex Virus (HSV) causes a broad spectrum of diseases, either as a primary infection or as the result of reactivation of the virus. HSV infections are

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most often asymptomatic but may take several clinical forms, as severe as keratitis and encephalitis. With the advent of selective anti-HSV agents such as ACV, the clinical management of HSV infections has become more effective. However, recent reports describing the isolation of ACV-resistant strains of HSV-1 and HSV-2 from patients undergoing therapy suggest that the emergence of drug-resistant strains of HSV may cause problems for future treatment (Burns et al., 1982; Crumpacker et al., 1982; McLaren et al., 1982; Parris and Harrington, 1983). Acycloguanosine or 9-[(2-hydroxyethoxy)methyl]guanine (Acyclovir or Zovirax, Burroughs Wellcome Co.) is an effective agent for the treatment and prophylaxis of HSV infections (Gold and Corey, 1987). This nucleoside analogue, phosphorylated preferentially by the viral HSV thymidine kinase (TK), is incorporated into DNA chains at the terminal 3′-nucleotide but effects chain termination because of the lack of a 3′-hydroxyl group on the acyclovir molecule (Burns et al., 1982). Alpha-Interferons are host-coded secretory proteins and glycoproteins modulating a variety of cellular functions, including the inhibition of virus replication by mechanisms not fully understood. Since the emergence of drug-resistant strains of HSV, several methods for testing the sensitivity of HSV to different antiviral substances have been reported (Gadler et al., 1984; Langlois et al., 1986; Rabalais et al., 1987; Swierkosz et al., 1987). Here, we report the comparison of two of these methods: a colorimetric method (neutral red dye uptake), one of the most sensitive reference methods for antiviral susceptibility testing (Ellis et al., 1988) and DNA hybridization for determining the sensitivity of clinical isolates of HSV to ACV and α-IFN. The results showed an excellent correlation between the two methods.

Materials and Methods

Human embryo lung (HEL) cells were grown in 50% Medium 199 and 50% Medium-B_2 Eagle (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (25000 U/ml Penicillin, 0.1 μg/ml Gentamycin, 25 μg/ml Neomycin and 2.5 U/ml Bacitracin). Confluent monolayers of HEL cells were maintained in MEM-B_2 Eagle containing 2% heat-inactivated FBS and antibiotics.

Herpes simplex virus type 1 and 2 clinical isolates were obtained from the Diagnostic Virology Laboratory, Hôpital Sainte-Justine, Montreal, Quebec, Canada. Virus antigenic type was determined by indirect immunofluorescence using monoclonal antibodies (Herpes ID Kit, I.A.F. Products, Laval, Quebec, Canada).

Sodium acyclovir (Zovirax, Burroughs Wellcome Co.) was diluted in sterile deionized water to the following concentrations (μg/ml): 50, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.0156 and 0.0078 and was stored frozen at −70°C until use. Then, 200 μl of the cell suspension (10^5 cells/ml) were put in rows of seven wells of 96-well flat-bottom culture plates (Linbro, Flow Laboratories). After a 24 h incubation (confluent cell monolayers), medium was removed and 25 μl of medium containing 30 $TCID_{50}$ of the virus to be investigated were added in each well and 25 μl of each ACV concentration were added in rows of seven wells of the culture plates. Plates were incubated with 5% CO_2 for 90 min at 37°C to allow adsorption of the virus

onto the cell monolayers. Then, 200 µl of maintenance medium were added in each well and plates were incubated for 72 h at 37°C in a 5% CO_2 atmosphere. In this assay, cells were not preincubated 24 h with ACV before infection with the virus since our results clearly demonstrated (data not shown) that preincubation does not affect neither adsorption of the virus, nor results expressed in ED_{50} (µg/ml). The control cultures contained tissue culture medium but no ACV nor virus. Resistance to ACV can be detected directly by the cytopathic effect or by the dye uptake assay (ED_{50} in µg/ml).

Alpha-2 Interferon (Intron-A, Schering Canada Inc.) was diluted in sterile deionized water to the following concentrations (IU/ml): 10^3, 10^2, 10^1, 10^0, 10^{-1}, 10^{-2}. The procedure is identical to the one using ACV, except that for α-IFN, the cells were preincubated 24 h in the presence of α-IFN before infection with 30 $TCID_{50}$ of the virus.

Neutral red dye (Fisher Scientific Co.) was ground with alcohol to a 20% (w/v) solution, diluted in sterile deionized water to 1%, filtered through paper and autoclaved for 20 min. Different dilutions of this stock solution were put in a 96-well multi-plate and optical density at 540 nm was monitored by spectrophotometry. The dilution showing an optical density of 0.84 at 540 nm was used in the dye uptake assay (Finter, 1969).

Then, 25 µl of neutral red dye were added to confluent monolayers of HEL cells previously infected and incubated with HSV for 72 h in the presence of ACV or α-IFN. After a 3 h incubation period, the dye was removed and the monolayers were drained by inversion, washed twice with PBS and drained by inversion again. To elute the neutral red dye from the cells into the wells, 100 µl of the elution buffer were added (50% citrate buffer pH=5.2, and 50% ethanol). The amount of neutral red dye into the well (or the dye uptake per well) was measured by absorbance at 540 nm using a spectrophotometer (Langlois et al., 1986).

The concentration of ACV or α-IFN that protected 50% of the cells (ED_{50}) compared to untreated virus controls (0%) and cell controls (100%) was calculated by the method of Reed and Muench (1938).

The hybridization technique was performed using as a probe the HSV thymidine kinase (TK) gene inserted into plasmid p601, kindly donated by Dr. José Campione-Piccardo (Laboratory Center for Disease Control, Ottawa, Canada).

The HSV-TK gene fragment inserted into plasmid p601 was labelled by the oligonucleotide method (Feinberg and Vogelstein, 1983) as follows: 33 µl of the reaction pool (10 µl 0.2 M KPO_4, 1 µl [^{32}P]dAPT, 1 µl [^{32}P]dCPT, 2 µl 1 mM dGTP, 2 µl 1 mM dTTP, 10 µl H_2O, 4 µl 50 mM $MgCl_2$) were mixed with 5 µl of DNA (100 ng DNA/5 µl) in an Eppendorf tube. The mix was boiled for 5 min, then put on melting ice. One microliter of the Klenow fragment of *E. coli* DNA polymerase and 2 µl of 0.2M DTT were added. The tube was incubated at room temperature for 2 h. Then, 59 µl of H_2O were added and radioactivity counted in a β-counter. After precipitation (3M NaAc, tRNA, H_2O and ethanol, 15 min −70°C, 15 min centrifugation at 7500 rpm, 4°C) the radioactive probe was lyophilized and resuspended in Denhardt's solution to obtain 10^6 cpm per 5 µl.

The cells were taken off the wells using a pipette tip, aspirated off from the wells

and filtered through a 0.22 μm nitrocellulose filter using a filtration apparatus ('Hybri-dot Manifold', 96-well filtration apparatus, Bethesda Research Laboratories) to concentrate the cells in a small spot. The cells were then lysed, thus exposing their DNA which, once denatured, was hybridized with our radioactively labelled probe (Brandsma and Miller, 1980).

The radioactive probe (specific activity approximately 2×10^8 cpm/μg probe DNA) previously denatured by boiling 5 min was added to fresh prehybridization solution (Salmon sperm DNA, Denhardt's solution, NaH_2PO_4, 20 × SSC, 50% formamide and water) (Maniatis et al., 1982) and transferred in a polyethylene bag (Fisher Scientific Co.) containing the filters previously wetted for 3 h at room temperature in a few ml of prehybridization solution. The bags were sealed and incubated overnight in a 41°C water bath. The following morning, the filters were washed consecutively in three solutions: (1) 20 min in 2 × SSC at 25°C with sporadic shaking, (2) 1 h in 0.1 × SSC/0.1% SDS at 60°C with constant shaking, (3) 3 rinses of 1 min each in 0.1 × SSC at room temperature. The filters were allowed to dry in air and processed for autoradiography after a 24 h exposure at −70°C without intensifying screen.

Results

The colorimetric method is based on the fact that neutral red dye, a vital dye, is actively taken up by healthy cells but is excluded from damaged and dead cells. The uptake and subsequent elution of the neutral red dye is measured by spectrophotometry. The principle of the second method is that viral DNA synthesis is inhibited by the drug (ACV or α-IFN). Then, by hybridization with a ^{32}P-labelled HSV thymidine kinase gene probe, the inhibition of viral DNA synthesis can be quantitated in loss of count per minute (cpm) using a β-counter, comparing virus-drug mixture with control virus alone and control cells alone.

Excluding two HSV-1 strains found resistant to acyclovir, eighty-two HSV isolates were sensitive to ACV by the colorimetric method (HSV-1: 69 isolates, HSV-2: 13 isolates) as well as 20 of them assayed by DNA hybridization (HSV-1: 16 isolates, HSV-2: 4 isolates) (Table 1). Results were expressed in means of ED_{50} in μg/ml (range in parentheses), or the amount of drug that can protect 50% of

TABLE 1

Sensitivity of HSV clinical isolates to ACV

	Colorimetric method (82 isolates)	Hybridization (20 isolates)
HSV-1	0.097 (69 isolates)	0.090 (16 isolates)
	(0.030 ± 0.02 to 0.164 ± 0.03)*	(0.033 ± 0.12 to 0.190 ± 0.031)*
HSV-2	0.103 (13 isolates)	0.109 (4 isolates)
	(0.038 ± 0.03 to 0.174 ± 0.071)*	(0.040 ± 0.051 to 0.190 ± 0.050)*

Means of the ED_{50} in μg/ml, range in parentheses (excluding two resistant HSV-1 isolates).
*Based on at least four different determinations.

TABLE 2

Sensitivity of HSV clinical isolates to α-IFN

	Colorimetric method (84 isolates)	Hybridization (20 isolates)
HSV-1	37.8 (71 isolates) (6.3 ± 5.2 to 54.0 ± 9.0)*	31.5 (16 isolates) (8.5 ± 5.0 to 50.0 ± 10.0)*
HSV-2	35.0 (13 isolates) (10.0 ± 5.0 to 55.0 ± 11.4)*	34.0 (4 isolates) (15.2 ± 5.1 to 43.5 ± 6.0)*

Means of the ED_{50} in IU/ml, range in parentheses.
*Based on at least four different determinations.

the cells. With the colorimetric method, HSV-1 isolates had ED_{50}s ranging from 0.030 ± 0.02 μg/ml to 0.164 ± 0.03 μg/ml. HSV-2 isolates had ED_{50}s ranging from 0.038 ± 0.03 μg/ml to 0.174 ± 0.071 μg/ml. Using the DNA hybridization method, values of ED_{50} were similar ranging from 0.033 ± 0.012 μg/ml to 0.190 ± 0.031 μg/ml for HSV-1 isolates and from 0.040 ± 0.051 μg/ml to 0.190 ± 0.050 μg/ml for HSV-2 isolates. Two HSV-1 clinical isolates that were found resistant to 50 μg/ml of ACV were excluded from the group and will be discussed later.

Eighty-four HSV isolates were tested for their sensitivity to α-IFN, by the colorimetric method (71 HSV-1 isolates, 13 HSV-2 isolates) and 20 of them were assayed by DNA hybridization (16 HSV-1 isolates, 4 HSV-2 isolates) (Table 2). The range of sensitivity to α-IFN of the 84 isolates was relatively wide. Using the col-

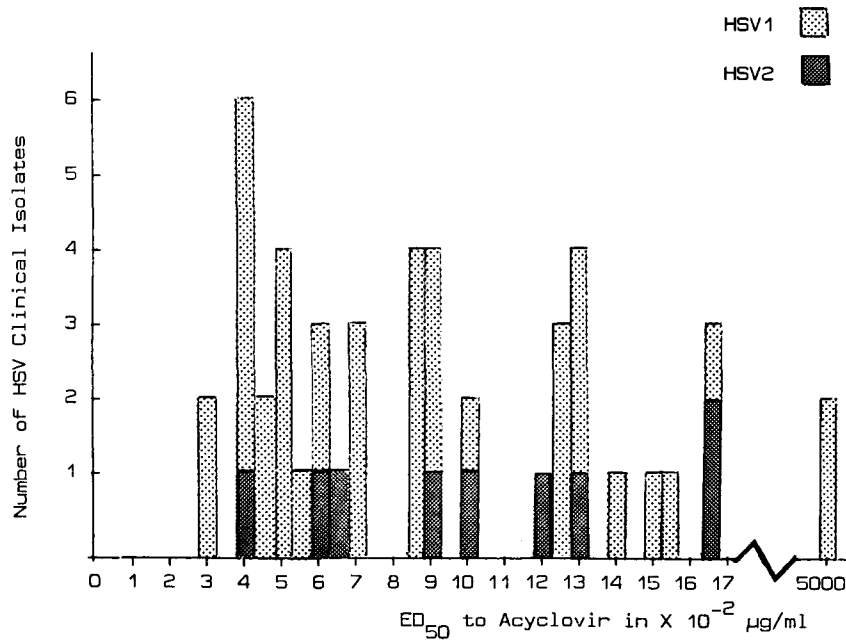

Fig. 1. Range of sensitivity to acyclovir of HSV-1 and HSV-2 isolates.

orimetric method, values of ED_{50} ranged from 6.3 ± 5.2 IU/ml to 54.0 ± 9.0 IU/ml for HSV-1 isolates and 10.0 ± 5.0 IU/ml to 55.0 ± 11.4 IU/ml for HSV-2 isolates. With the DNA hybridization method, ED_{50}s ranged from 8.5 ± 5.0 IU/ml to 50.0 ± 10.0 IU/ml for HSV-1 isolates and 15.2 ± 5.1 IU/ml to 43.5 ± 6.0 IU/ml for HSV-2 isolates.

A statistical analysis demonstrated that the two methods showed a good correlation, with a correlation factor of $P=0.002$ and $r=0.724$.

A schematic representation (Fig. 1) of the distribution of the ED_{50} of 84 HSV clinical isolates (71 HSV-1 isolates, 13 HSV-2 isolates) demonstrated that the sensitivity of these isolates ranged between 0.03 μg/ml and 0.164 μg/ml of acyclovir. The two HSV-1 isolates to the right of this figure were exceptions since their ED_{50} value was 50 μg/ml.

These two HSV-1 isolates and a third one, resistant to very high concentrations of ACV were recovered from a bone marrow transplant patient who received ACV therapy over a period of two months. Fig. 2 showed the development of resistance to ACV in this patient in relation to cycles of treatment with i.v. ACV (intravenous ACV 500 mg/8 h).

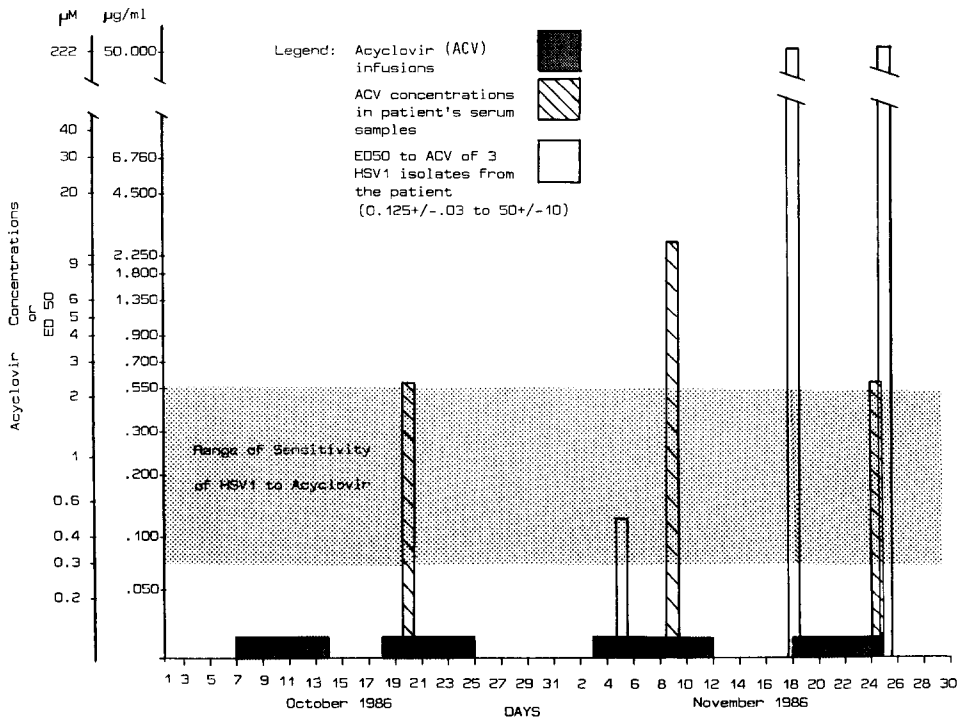

Fig. 2. Development of resistance to acyclovir in a bone marrow transplant patient in relation to cycles of treatment with i.v. acyclovir.

Discussion

The colorimetric method used in this study was simple to perform, did not require special, sophisticated and expensive equipment or material. Results were reproducible since neutral red dye uptake was monitored by spectrophotometry, thus leading to objective results. Titration of the virus has to be done before testing.

The DNA hybridization assay using the HSV thymidine kinase gene probe was also simple. Objectivity of the results was assured both by evaluating count per minute (cpm) with a β-counter and by reading the results after autoradiography. As for the colorimetric method, titration of the virus had to be done before testing. However, the use of radioactive ^{32}P implied manipulation of radioactive products and problems associated with isotope storage and short half-life.

With the widespread use of anti-HSV drugs (the most commonly used being ACV) and the growing availability of additional antiviral substances, reliable susceptibility tests should be available routinely in a diagnostic virology laboratory, to monitor the emergence of resistant strains of herpes simplex virus type 1 and 2. In this study, we demonstrated that the colorimetric method gave reliable and reproducible results, with high sensitivity and specificity. Both assays can be used for laboratory investigations of viral susceptibility and the neutral red dye uptake is suitable for routine use in most virology laboratories with low cost since this technique does not require radioactive material, as for DNA hybridization. Furthermore, our results demonstrated the selection of resistant strains of HSV-1 as a consequence of repeated treatment with intravenous acyclovir in an immunocompromised host.

Previous reports have demonstrated a range of in vitro sensitivities to ACV for HSV isolates. McLaren et al. (1982), using the colorimetric method, demonstrated wide ranges of ED_{50} for HSV-1 and HSV-2 with a greater range for HSV-2; HSV-2 ED_{50}s were therefore not significantly higher. Means of ED_{50} were 0.125 μg/ml for HSV-1 isolates and 0.215 μg/ml for HSV-2 isolates. Our results compare with theirs and means of ED_{50} for HSV-1 and HSV-2 were relatively similar. Parris and Harrington (1982) also showed greater variation in ED_{50}s (results expressed in μM of ACV): 0.11 μM ACV to 1.8 μM ACV (approximately 0.02 μg ACV to 0.45 μg ACV), using the plaque reduction assay. Gadler (1983) reported the use of DNA hybridization to measure antiviral susceptibility of HCMV but a study comparing this method to a standard assay was not available. Recently, Swierkosz et al. (1987), using a new DNA hybridization method, reported similar results with ED_{50}s ranging from 0.03 μg/ml to >8.0 μg/ml (results expressed in μg of ACV/ml). However, in their study, isolates were not submitted to concentrations of ACV higher than 8.0 μg/ml. Thus, the range of susceptibility of their isolates to ACV is difficult to evaluate and very resistant strains could not be detected. Their study showed a good correlation between DNA hybridization and plaque reduction assay. As mentioned earlier, we compared the DNA hybridization method with the neutral red dye uptake method. A recent study of Ellis et al. (1988) (Burroughs Wellcome Co.) demonstrated that the neutral red dye uptake assay appeared slightly more sensitive than the plaque reduction assay.

Our results are in general agreement with all previously published reports of HSV sensitivity to ACV. We have found however that neither range of sensitivity nor median sensitivity to ACV or α-IFN were significantly higher for one of the two antigenic types of HSV. The reason for this minor discrepancy is not readily apparent except possibly that most of our strains were isolated from children.

We demonstrated that results obtained with the DNA hybridization technique were very similar to those obtained by the neutral red dye uptake method for antiviral sensitivity testing of HSV, a fully cytopathic virus ideally suitable for the neutral red dye uptake method. The DNA hybridization method can be applied to viruses with delayed cytopathic effect (CPE) such as HCMV and VZV and to other viruses for which DNA hybridization is the only method available for susceptibility testing, such as the Epstein-Barr virus (EBV) which cannot be tested by a technique relying on CPE or cell viability such as the dye uptake. DNA hybridization applied to these other viruses could be expected to give equivalent results in sensitivity to the neutral red dye uptake assay as well as to the plaque reduction assay which was also compared with the hybridization technique (Swierkosz et al., 1987). A virus such as EBV might even to a certain extent be compared to HSV for its relative sensitivity to acyclovir when the former is tested by DNA hybridization and the latter by the neutral red dye uptake assay as a result of our direct comparison of the two methods for HSV.

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