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Evaluation of varicella-zoster antiviral drugs by a nucleic acid hybridization assay

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Summary

A simple nucleic acid hybridization assay was developed for the evaluation of antiviral compounds with activity against varicella-zoster virus. Antiviral-induced reduction in varicella-zoster virus DNA, as measured by hybridization with ^{32}P-labeled probes, correlated with drug-induced inhibition of viral cytopathic effect in cell culture. Three compounds were shown to be more potent than acyclovir at inhibiting varicella-zoster virus in vitro.

Varicella-zoster virus; DNA hybridization

Introduction

Although a self-limited childhood exanthem, varicella may be a devastating infection in the adult or the immunocompromised individual. Herpes zoster, a consequence of reactivation of latent varicella-zoster virus (VZV) occurs commonly among the immunocompromised and in the elderly with potential for dissemination and post-herpetic neuralgia. The need for treatment of patients with VZV infection has prompted evaluation of compounds with potential anti-VZV activity.

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Evaluation of anti-VZV compounds by plaque reduction or yield reduction assays is difficult. These assays generally require cell-free virus but in culture VZV is cell associated. Limited quantities of cell-free virus can be obtained by disruption of infected monolayers, but such techniques are laborious and produce only variable and low titered virus stocks (Brunnel, 1967; Schmidt and Lennette, 1976). Additionally, VZV induces plaques that are difficult to accurately enumerate, generally requiring microscopic evaluation.

DNA–DNA hybridization using defined viral DNA probes is a sensitive and specific technique for the detection and quantification of viral DNA in tissues and infected cell monolayers (Brandsma and Miller, 1980; Redfield et al., 1983). We report here the development of a dot-blot DNA hybridization assay providing a simple and reproducible method for evaluation of anti-VZV compounds.

Materials and Methods

Cell cultures

Human foreskin fibroblast (HFF) and fetal guinea pig (FGP) cells were maintained on Eagle's basal medium (BME) containing penicillin (50 μg/ml), streptomycin (50 μg/ml), and supplemented with 10% heat inactivated (56°C, 30 min) fetal bovine serum (FBS) and 2 mM L-glutamine. Cells were prepared and maintained as described previously (Myers et al., 1985).

Virus strain

Three VZV strains were used: strain 1294, propagated in HFF (Myers, 1979), gpVZV, a guinea pig adapted strain derived from 1294 (Myers et al., 1985) and maintained in FGP, and Oka strain (ATCC No. VR 795) grown in HFF (Takahashi et al., 1975). Virus strains were maintained as infected cells stored in liquid nitrogen as described previously (Myers et al., 1985).

Antiviral drugs

The antiviral drugs were provided under code by the Wellcome Research Laboratories, Burroughs Wellcome Co., Research Triangle Park, NC. The drugs were labeled, A, B, C, and D. Drug C was acyclovir.

Antiviral assays

A 150 cm^2 flask containing approximately 3×10^7 confluent cells was trypsinized and resuspended in 130 ml BME containing 10% FBS and antibiotics. One ml aliquots were dispensed into the top four wells of five 24-well polystyrene plates (Corning Glass Works, Corning, NY). VZV infected cells (7×10^3 to 6×10^4 PFU) stored in liquid nitrogen were rapidly thawed and added to the remaining cell suspension. One ml aliquots of the infected cell suspension were dispensed to each of the remaining wells in the 24-well plates, providing from 64 to 545 PFU per well. The plates were incubated for 1 h at 37°C and 1 ml aliquots of BME containing from 0 to 100 μM drug were added to the wells providing a final concentration of

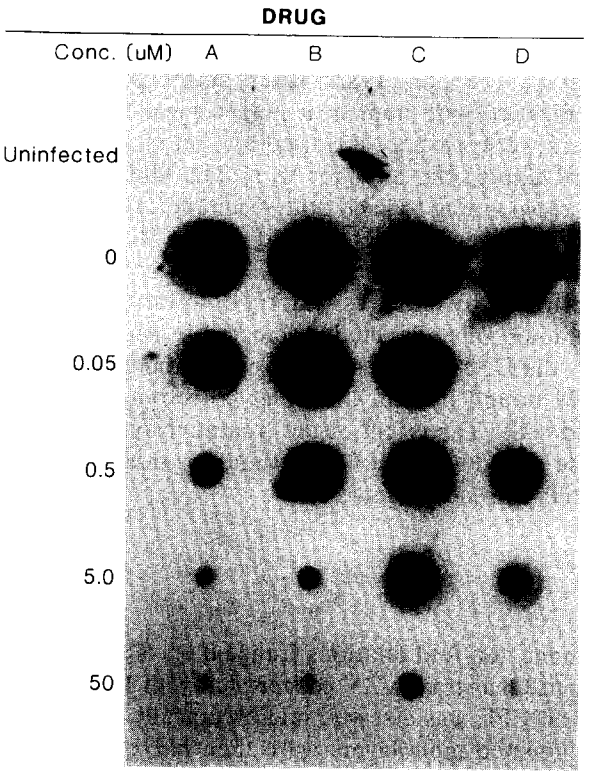

Fig. 1. Effect of antiviral compounds on VZV DNA synthesis. VZV-infected cells were cocultivated with HFF cells in the presence of antiviral for four days. The infected cell monolayer was harvested and hybridized with ^{32}P-radiolabeled VZV DNA probes. The autoradiogram was developed after an 18 h exposure.

0 to 50 μM drug. Cells were incubated for 4–5 days until evidence of viral cytopathic effect (CPE) could be detected by microscopic examination (magnification 40 ×). The media were gently aspirated and 50 μl of SDS (1.2%) – protease K (60 μg) were added to each well. The plates were incubated for 60 min at 37°C and then 5 μl 5 N NaOH was added to each well. The plates were gently shaken, incubated at room temperature for an additional 5 min, 5 μl 5 N HCl was added and the plates were again gently shaken. The contents of each well were transferred to a nylon filter (Genescreen Plus, New England Nuclear Products, Boston, MA) by means of a dot-blot manifold. Each well was washed with 100 μl 1.2% SDS solution and the wash solution was added to the appropriate well of the dot-blot manifold. The filters were then baked at 80°C for 1 h in a vacuum oven. The filter was pre-hybridized overnight at 42°C in 5 × SSC, 10 × Denhardt's, 0.05 M $NaPO_4$, 5% dextran sulfate, 50% formamide, 50 μg/ml salmon sperm DNA, and 0.1% SDS. The filter was hybridized against a ^{32}P-labeled nick-translated VZV DNA probe under highly stringent conditions for 16–24 h at 42°C in 50% formamide (Myers

et al., 1985). The probes consisted of an equal molar mixture of *Hin*dIII fragments A, B, and C, provided by Dr. Richard Hyman (Pennsylvania State University, Hershey, PA) (Ecker and Hyman, 1982). These VZV DNA fragments demonstrate no cross hybridization with human or guinea pig DNA (unpublished data, see Fig. 1). The filters were washed, air-dried and autoradiographs prepared by exposing Kodak XAR film to the filter for 18 h. The filter was subsequently cut into small squares corresponding to each dotted sample. The radioactive emission (CPM) for each filter specimen was determined using a Beckman LS-1800 scintillation counter (Beckman Instruments, Irvine, CA). The relative concentration of VZV DNA was determined by either estimation of autoradiogram dot area or by comparison of the amount of radioactivity detected in each sample dot. The average CPM bound by the DNA samples from wells containing uninfected cells was subtracted from CPM bound by DNA samples corresponding to the wells containing VZV infected cells. The effect of the treatment on VZV in vitro was expressed as the percent of reduction of CPM bound for drug treated samples compared to VZV infected untreated controls.

Results

In the first experiment, ten-fold dilutions (range 0.05–50 μM) of the four coded antivirals were added to Oka strain VZV cocultivated with HFF cells. After 4 days, the drug effect on viral CPE was assessed microscopically at 40 power magnification (Table 1). The infected monolayers were then harvested for DNA hybridization and autoradiography (Fig. 1). Radiolabeled probe demonstrated no binding to uninfected HFF cells. The area of each autoradiogram dot was calculated and the amount of radiolabeled probe bound to the filter determined (Table 1). Comparison of the three methods of evaluating antiviral activity: estimation of CPE; hybridization autoradiogram; and radiolabeled probe binding showed a high degree of correlation between the methods ($P < 0.001$) (Table 1). All methods demonstrated the four antivirals were effective in inhibiting the replication of VZV in vitro with similar patterns of sensitivity (A < B < D < C) established by each method. The data in Table 1 showing the amount of radioactive probe bound to the filter were used to calculate the dose of drug which produced a 50% reduction in VZV DNA (ID_{50}) (Table 2).

In order to confirm the ID_{50} for each antiviral a second experiment was conducted. Two-fold rather than ten-fold dilutions of each antiviral were used over the effective range of concentrations determined in experiment 1. The DNA hybridization autoradiogram shown in Fig. 2 confirmed that all drugs were effective in reducing the amount of VZV DNA present in the samples. Estimation of the amount of radiolabeled probe bound to the filter permitted calculation of the percent of reduction of viral DNA produced by antiviral treatment (Fig. 3). Calculation of the ID_{50} for each antiviral based on data in Fig. 3 is shown in Table 2. The ID_{50} values determined in experiment 2 were similar to those determined in the first experiment (Table 2). Similar inhibitory activities of drugs A and C were

TABLE 1

Comparison between three methods of evaluating antiviral effect on VZV in vitro

Drug												
A				B			C			D		
Drug Conc (μMl)	CPE score[a]	Area of dot[b]	CPM bound[c]	CPE score	Area of dot	CPM bound	CPE score	Area of dot	CPM bound	CPE score	Area of dot	CPM bound
0	3+	147.8	1173	3+	153.3	2432	3+	148.9	1307	3+	148.9	909
0.05	3+	122.7	823	3+	147.2	2189	3+	137.1	1369	3+	ND	ND
0.50	3+	42.7	133	2.25+	98.2	388	3+	116.0	782	2.5+	82.7	595
5.00	0.25+	12.5	97	0	15.6	2	3+	98.2	609	0.5+	71.3	156
50.0	0	7.9	0	0	8.6	0	2+	16.6	25	0	7.3	0

[a] CPE score (average of 2 wells) determined by microscopic examination (40×), 0 = no foci, 1+ = 1–4 foci, 2+ = 5–10 foci, 3+ = 10–20 foci, 4+ = 100% CPE. The value is the average of 2 blinded observers.

[b] Area of dot (mm^2) determined by measuring diameter of dot on autoradiogram and calculating area by formula: area = πr^2.

[c] Counts per minute (CPM) of ^{32}P-labeled probe bound to filter.

Correlation between methods determined by Statpro statistical program (Penton Software Inc. New York, New York): CPE vs. dot: $r = 0.86$, $P < 0.001$; CPE vs. CPM: $r = 0.68$, $P < 0.001$; dot vs. CPM: $r = 0.86$, P 0.0001.

TABLE 2

The 50% inhibitory dose of four antiviral drugs for varicella zoster virus[a]

Experiment number	Virus strain[b]	Drug (μM)			
		A	B	C	D
1[c]	Oka	0.11	0.18	5.3	1.0
2[d]	Oka	0.50	0.26	5.6	1.4
3[e]	Oka	0.10	ND[g]	ND	ND
	1294	1.0	ND	ND	ND
4[f]	gpVZV	1.8	ND	ND	ND

[a] Endpoint determined by reduction of radioactive probe bound to filter in VZV DNA hybridization assay.
[b] All strains cocultivated with HFF cells except gpVZV which was cocultivated with FGP cells.
[c] Data from Table 1.
[d] Data from Fig. 3.
[e] Data from Fig. 4a.
[f] Data from Fig. 4b.
[g] Not done.

also determined by inhibition of microfoci in tube cultures (data not shown).

Two additional experiments were conducted to determine whether this method of evaluating antiviral drugs could be used with other strains of VZV and with other cell lines. In the third experiment, VZV strain Oka or strain 1294 were co-cultivated with HFF cells in the presence of drug A. Estimation of the percent reduction in VZV DNA calculated from amount of radiolabeled probe bound to filters is shown in Fig. 4A. Similarly, in a fourth experiment, FGP cells were co-cultivated with guinea pig adapted VZV in the presence of drug A. The effect of drug A on reducing viral DNA as calculated from the amount of radiolabeled probe bound to the filter is shown in Fig. 4B. Estimations of the ID_{50} for the antivirals based on the percent reduction in viral DNA concentrations are shown in Table 2. These data demonstrate that this DNA hybridization assay can be used to evaluate putative antiviral drugs using a variety of VZV strains grown in either human or guinea pig cell lines.

Discussion

Nucleic acid hybridization as a technique for screening antiviral drugs has been used for human cytomegalovirus (HCMV) (Gadler, 1983) and herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) (Gadler et al., 1984; Swierkosz et al., 1987). This study demonstrates that DNA hybridization as a method to screen anti-VZV compounds is simple, reproducible, and corresponds to the reduction of microfoci.

The preparation of cell-free VZV is time consuming and inefficient, resulting in perhaps a ten-fold increase over input inoculum (Brunnel, 1967; Schmidt and Lennette, 1976). The DNA hybridization technique described in this study used cell-associated virus, hence avoiding one disadvantage of traditional VZV plaque re-

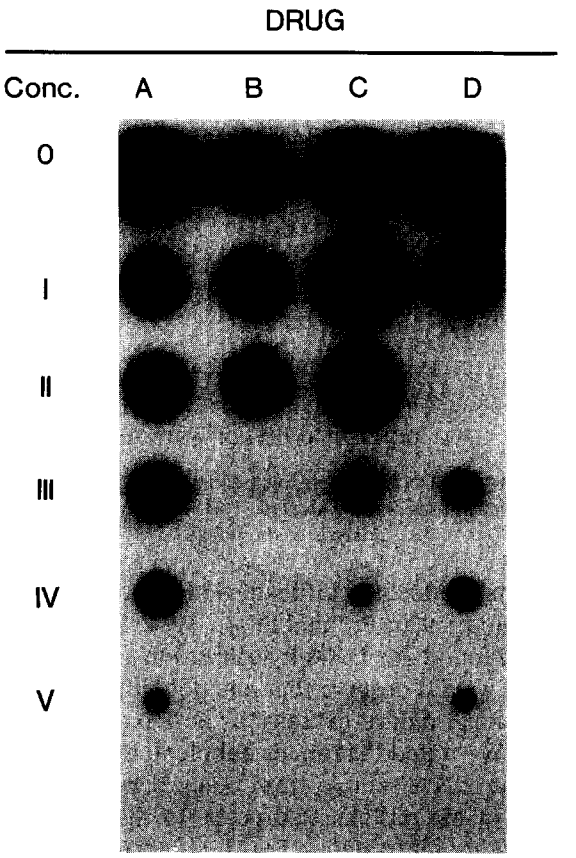

Fig. 2. Effect of antiviral compounds on VZV DNA synthesis. VZV infected cells were cocultivated with HFF cells in the presence of drug for five days. The infected cell monolayers were harvested and hybridized with ^{32}P-radiolabeled VZV DNA probes. The audioradiogram was developed after an 18 h exposure. The concentrations of drugs A and B were as follows: I, 0.31 μM; II, 0.63 μM; III, 1.25 μM; IV, 2.5 μM; V, 5.0 μM. The concentrations of drug C used were as follows: I, 3.13 μM; II, 6.25 μM; III, 12.5 μM; IV, 25.0 μM; V, 50.0 μM. The concentrations of drug D used were as follows: I, 0.63 μM; II, 1.25 μM; III, 2.5 μM; IV, 5.0 μM, V, 10.0 μM.

duction assays. In addition, DNA hybridization screening of antivirals avoids the necessity of counting plaques microscopically (Biron and Elion, 1982; Machida, 1986; Preblud et al., 1984; Shigeta et al., 1983; Shiraki et al., 1984). Antiviral compounds affect plaque size prior to reducing plaque number (Bryson and Hebblewaite, 1981) suggesting that antiviral drugs may affect VZV replication in a manner not accurately reflected by plaque reduction. However, the concentration of DNA directly correlates with virus concentration (Redfield et al., 1983). Thus, this assay determined an ID_{50} for acyclovir (drug C) for the Oka strain of 5.3 to 5.6 μM whereas ID_{50} values for acyclovir from 4.0 to 44.4 μM have been reported by plaque reduction assays. The more than ten-fold differences in results obtained

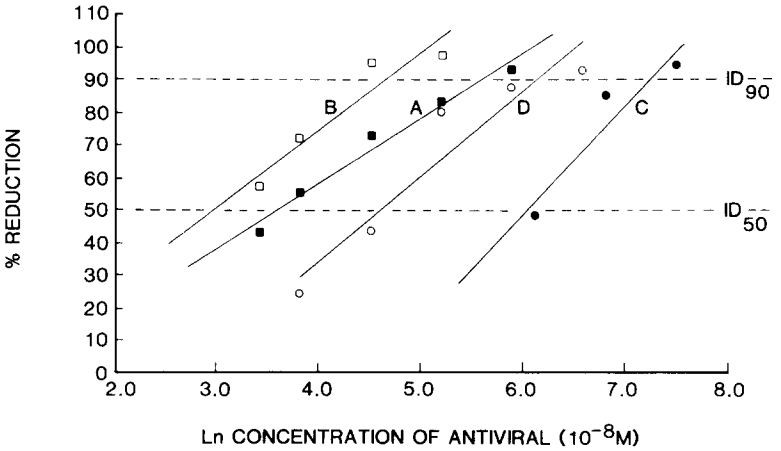

Fig. 3. Percent reduction of VZV DNA synthesis by antiviral compounds. Virus strain, Oka; cell line, HFF. Two-fold dilutions of each drug were evaluated. The lines were constructed by linear regression analysis of the points.

by plaque reduction may reflect the difficulties in performing plaque reduction assays with VZV (Machida, 1986; Preblud et al., 1984; Shigeta et al., 1983; Shiraki et al., 1984). While we found our DNA hybridization assay easier to interpret than reduction in microfoci we did observe that one potentially confusing aspect of this method was the occasional failure to transfer the DNA successfully from the culture dish to the filter. We noted that an air bubble in the well of the dot-blot manifold could prevent the DNA sample from being deposited on the filter, hence, there was no binding of the radiolabeled probe to that portion of the filter. Examples of this problem are illustrated in Fig. 1 with drug D at 0.05 μM and in Fig. 2 with drug D at 1.25 μM (concentration II). These results could be misinterpreted as indicating a complete inhibition of viral replication at these concentrations.

The increased potency of acyclovir against VZV when measured by DNA hybridization is similar to antiviral studies with HCMV and HSV-1 when evaluated by nucleic acid hybridization. Gadler reported the ID_{50} value for vidarabine against HCMV by DNA hybridization to be 44 μM (Gadler, 1983) compared to a value of $\geqslant$ 750 μM noted by Gephart and Lerner using a 50% plaque reduction assay (Gephart and Lerner, 1981). Swierkosz et al. (1987), using a hybridization method, reported ID_{50} values for acyclovir against HSV-1 and HSV-2 isolates of 0.2 and 0.7 μM, respectively, compared to 0.8 and 1.0 μM ID_{50} values determined by plaque assay (Swierkosz et al., 1987). Likewise, Gadler and associates reported an ID_{50} for acyclovir against HSV-1 of 0.2 μM by DNA hybridization versus 0.5 μM by plaque reduction (Gadler et al., 1984).

It is difficult to assess the clinical significance of differences in antimicrobial inhibition measured by differing in vitro assay methods, whether 1 or 10 μM ID_{50} determinations by hybridization or plaque reduction reflect concentrations required for effective therapeutic applications. However, both assay methods allow

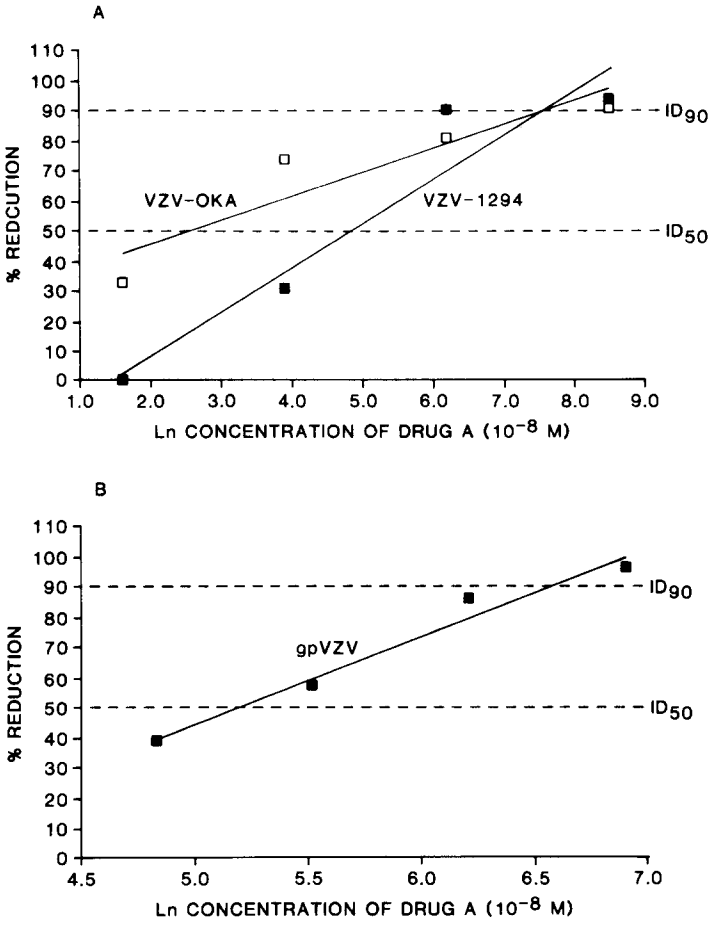

Fig. 4. Percent reduction of VZV DNA synthesis by drug A. (A) Virus strain, Oka and 1294; cell line, HFF. Ten-fold dilutions of drug A were evaluated. The lines were constructed by linear regression analysis of the points. (B) Virus strain, guinea pig adapted VZV, cell line, FGP. Two-fold dilutions of drug A were evaluated. The line was constructed by linear regression analysis of the points.

comparison of the relative inhibition of viral replication by different compounds and differences between strains of virus. Because of simplicity and reduced assay variability, the hybridization assay would seem to lend itself to such comparison, especially when evaluating cell-associated viruses, slow growing viruses, viruses that produce little, atypical, or no cytopathic effect, or when investigating the effect of antivirals on virus replication in organ cultures (Brandsma and Miller, 1980; Flores et al., 1983; Pulliam et al., 1986; Taichman et al., 1984; Weller, 1983).

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