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Concentrations of Idoxuridine in Serum, Urine, and Cerebrospinal Fluid of Patients with Suspected Diagnoses of *Herpesvirus hominis* Encephalitis

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Research Article

A reproducible microbiologic assay of microgram quantities of idoxuridine (IDU) in serum, urine, or cerebrospinal fluid is presented. The antiviral assay is not interfered with by type-specific antibody or interferon. During slow intravenous infusions of idox-uridine (4 mg/min) in patients with suspected diagnoses of *Herpesvirus hominis* encephalitis, the rate of inactivation and/or removal of drug exceeded its administration. During several rapid infusions of idoxuridine (50 mg/min) significant quantities of the drug were found in serum, urine, and cerebrospinal fluid. Idoxuridine is not significantly bound to serum proteins and is not deiodinated in fresh serum or urine in vitro to inactive products (iodouracil, uracil, iodide). It is rapidly excreted into the urine. Inactivation of IDU occurs in tissues. This antiviral assay of IDU in body fluids should be applicable to other viruses and potential antiviral agents.

Minimal inhibitory concentrations of IDU for fresh isolates of *Herpesvirus hominis* (type 1 or 2) were determined. Type 1 herpesviruses' microplaques in baby hamster kidney cell (BHK 21) tissue cultures were sensitive to 2.5-10 μg/0.4 ml. Type 2 macroplaques required 25-50 μg/0.4 ml. This latter characteristic may be an additional biologic marker which may be useful in suggesting type-specificity of herpesvirus isolates.

Concentrations of Idoxuridine in Serum, Urine, and Cerebrospinal Fluid of Patients with Suspected Diagnoses of *Herpesvirus hominis* Encephalitis

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Abstract A reproducible microbiologic assay of microgram quantities of idoxuridine (IDU) in serum, urine, or cerebrospinal fluid is presented. The antiviral assay is not interfered with by type-specific antibody or interferon. During slow intravenous infusions of idoxuridine (4 mg/min) in patients with suspected diagnoses of *Herpesvirus hominis* encephalitis, the rate of inactivation and/or removal of drug exceeded its administration. During several rapid infusions of idoxuridine (50 mg/min) significant quantities of the drug were found in serum, urine, and cerebrospinal fluid. Idoxuridine is not significantly bound to serum proteins and is not deiodinated in fresh serum or urine in vitro to inactive products (iodouracil, uracil, iodide). It is rapidly excreted into the urine. Inactivation of IDU occurs in tissues. This antiviral assay of IDU in body fluids should be applicable to other viruses and potential antiviral agents.

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INTRODUCTION

The effective use of an antibacterial agent versus bacterial infection requires information relating to its in vitro effectiveness and pharmacologic properties in vivo. Particularly, the latter information, when coupled with careful clinical observation, allows assessment of the toxic to therapeutic concentrations. In turn, limits of usefulness of the substance in question are defined.

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In vitro methods of assay of antiviral activity have been reported (1). After administration of potentially useful antiviral drugs to man, quantitative measures of activity in body fluids, to our knowledge, have not been reported. Antiviral properties in specimens from patients attributable to drugs must be clearly separated from similar activities of interferon or type-specific antibody.

We report: (*a*) a quantitative microbiologic assay for 5-iodo-2′-deoxyuridine (idoxuridine, IDU[1]) in body fluids; (*b*) data relating to the stability of IDU in serum, urine, and cerebrospinal fluid; (*c*) minimal inhibitory concentrations (MIC) of IDU for 11 recent isolates of herpesvirus hominis type 1, 3 isolates of type 2, and 1 isolate with intermediate properties; and (*d*) pharmacologic data from nine patients receiving IDU for presumed diagnoses of *Herpesvirus hominis* encephalitis (2–8).

METHODS

Cells. A single vial of baby hamster kidney cells (BHK-21) was obtained from the American Type Culture Collection.[2] Cells were maintained in our laboratory, and used throughout. Tissue cultures were grown in 32-ounce monolayer bottles with Eagle's medium (EM) in Earle's solution (BSS) and twice normal concentrations of amino acids supplemented with 10% tryptose phosphate broth, 10% calf serum, 0.005% phenol red, and 50 μg/ml of penicillin and streptomycin. For virus or antiviral assays, cells were grown in screw-capped test tubes or 30-ml plastic tissue culture flasks, respectively. Test tubes were seeded with 1 ml, tissue culture flasks with 4 ml of growth medium containing 1.2×10^5 cells/ml. Overlay medium for plaque assay contained equal volumes of twice-concentrated medium 199 in BSS and 10% fetal calf serum with 0.6%

[1] *Abbreviations used in this paper:* BHK, baby hamster kidney cells; BSS, Earle's solution; CSF, cerebrospinal fluid; EM, Eagle's medium; IDU, idoxuridine; MIC, minimal inhibitory concentration; pfu, plaque-forming units.

[2] American Type Culture Collection, Rockville, Md.

TABLE I

Adsorption of Herpesvirus; Stability of IDU in Serum, and to Freezing and Thawing

IDU	pfu, *Herpesvirus hominis* (Ket., Am., WI 38, Am., BHK 21$_6$) Time	No.	Mean
μg/0.4 ml	*hr*		
None, in BSS	—	72, 61	67
None, in fresh serum	—	53, 70	62
None, in 6/100 ml g albumin, BSS	—	72, 59	66
10, fresh serum, 36°C	—	8, 4	6
10, fresh serum, 36°C	1	8, 5	6
10, fresh serum, 36°C	2	7, 7	7
10, fresh serum, 36°C	24	5, 4	5
20, fresh serum, 4°C	168	7, 8	8
10, BSS, freeze-thaw X0	—	4, 6	5
10, BSS, freeze-thaw X2	—	8, 6	7
10, BSS, freeze-thaw X5	—	7, 8	8

agarose in distilled water (9). Tube cultures were maintained in 98% medium 199 and 2% heat inactivated fetal calf serum.

Viruses. The Ket strain of *Herpesvirus hominis* (Am., WI38, Am., BHK21$_6$[3]) was used in assays of antiviral activity in serum, urine, or cerebrospinal fluid. We identified it as a type 1 herpesvirus by neutralization tests in tissue culture versus hyperimmune unitypic rabbit sera (10). Ket virus was isolated in human amnion cells at biopsy from the left temporal lobe of a 57 yr old woman with a fatal case of encephalitis (11). After further passages in WI38 diploid cells, human amnion cells, and BHK21 tissue cultures, pools of Ket virus were prepared in BHK21 cells. Cultures were maintained in medium 199 with 5% fetal calf serum. After cytopathic effects were complete, cultures were frozen and thawed three times, and tissue debris was removed by centrifugation (15 min at 2,000 rpm, 4°C, in an International refrigerated centrifuge, model PR-2[4]). 1 ml amounts of virus were stored at −50°C in screw-capped vials. After thawing, the virus was used immediately. Virus was titered using a modified liquid overlay method (12). Ket virus pools contained approximately 5.4×10^7 plaque-forming units (pfu) per milliliter.

Other recently isolated strains of *Herpesvirus hominis* were titered and immunotyped using similar techniques. Type 1 strains were found among isolates from brains of patients with encephalitis in adults, from Kaposi's varicelliform eruptions, and from herpes labialis. It is of interest that a type 1 strain was also isolated from a neonate with generalized vesicular lesions. Type 2 strains were recovered from the penis or cervix. The prototype strain of type 2 *Herpesvirus hominis* (E 304) had been recovered from a vesicle on the thigh. One strain (Zerb.) isolated from a vesicle of a fatal congenital infection, was intermediate in typing tests. It was well neutralized (1/40) by type 2 hyperimmune rabbit serum (E 304), and poorly (1/10) by similar type 1 serum (Con.). The infant's serum, however, contained complement-requiring neutralizing antibodies to Con., type 1 herpesvirus. Previously, we showed that complement-requiring neutralizing antibodies follow type 1, but not type 2 infections in rabbits and man (10).

Type 1 strains produce small (micro) plaques, while the Zerb. and type 2 herpesviruses produced larger plaques in baby hamster tissue cultures. Noteworthy is the fact that by the time type 1 Ket herpesvirus had been passaged nine times, plaques were macroscopic. A similar transformation after passages in tissue culture has been described (13, 14).

Virus titrations. Tube cultures were inoculated with 0.1 ml of logarithmic dilutions of herpesviruses. Two tubes per dilution were used. Cultures were incubated at 35°C for 24 hr when 1 ml of fresh maintenance medium was added. After a further 24 hr of incubation, medium was decanted, and cultures were flooded with paragon-formalin (12). On the following day tubes were washed with cold tap water and inverted to dry. Individual microplaques were easily counted using an AO cycloptic stereoscopic microscope.[5]

Antiviral assay. Complete BHK21 monolayers in 30-ml tissue culture flasks were prepared. Growth medium was decanted, and 0.2 ml of virus containing 50–100 pfu of herpesvirus was added. In preliminary experiments virus was allowed to adsorb (36°C) with intermittent rocking for 90, 120, 180, and 240 min before the additions of hyperimmune anti-Ket rabbit serum. Adsorption of herpesvirus was complete, and virus was nonneutralizable by type-specific antibody at 120 min. In subsequent assays of antiviral activity a 120 min period of adsorption was used.

After virus adsorption, flasks were washed twice with EM (36°C), and 0.4 ml of twofold dilutions of serum, cerebrospinal fluid, or urine to be assayed was added. Specimens were allowed a further adsorption period of 60 min, 36°C. Thereafter, 4 ml of overlay medium at 45°C was added to each tissue culture, and flasks were finally incubated for 96 hr at 35°C. Cultures were then fixed with 2 ml of 10% formalin. Agar overlays were decanted, flasks washed with tap water, and subsequently stained with 2 ml of a stain containing paragon-formalin.

The following day the flasks were washed again with tap water. Areas were measured with a marking pencil, and the total number of plaques in four rectangular areas between the double lines counted (e.g. 40–69 mm zone) using the stereoscopic microscope. Plaques of herpesvirus usually were visible grossly, but were readily enumerated under the stereoscopic microscope. With each set of determinations, controls and specimens obtained before or after administrations of idoxuridine, as well as others containing 0.4 ml of 6 g/100 ml albumin with or without varying concentrations of idoxuridine were included. Final concentrations of IDU were assessed by extrapolation from plaque inhibition curves.

Adsorption of herpesvirus is unaffected in BSS, fresh serum, or 6 g/100 ml albumin in BSS (Table I). Idoxuridine is stable after incubations in serum at 36°C for from 1 through 168 hr. In vivo, IDU is metabolized to iodouracil, uracil, and iodide—products which have no antiherpesvirus activity (15, 16). Therefore, deiodination does not occur in serum. In serum IDU is also unaltered by repeated cycles of freezing and thawing (Table I). IDU is not significantly bound to serum proteins. Repetitive assays of varying

[3] Passages in tissue culture: Am., primary human amnion; WI38, human diploid line; BHK21, baby hamster kidney line.

[4] International Equipment Company, Needham Heights, Mass.

[5] American Optical Corp., Buffalo, N. Y.

amounts of IDU in urine, cerebrospinal fluid, or sera confirm original determinations. Idoxuridine, therefore, is also not deiodinated in urine.

In this assay, addition of specimens after adsorption of virus eliminates antiviral activity of neutralizing antibody. Likewise, use of baby hamster kidney cells utilizes species' specificity of human interferons, and eliminates antiviral activity of these natural intracellular inhibitors of virus (17).

For determination of concentration of IDU (serum, urine, CSF) and MIC's of recently isolated herpesvirus strains, end points were read as that dilution producing a 50% reduction in plaque-forming units. In these assays 50–150 plaque-forming units were used as the herpesvirus challenge.

Intravenous administration of idoxuridine. Patients, with a single exception, received IDU as a constant infusion in 5% glucose in water over a 24 hr period. A total dosage of 300-400 mg/kg divided over a 5 day period was given. During slow intravenous infusions, IDU was diluted in several liters of 5% glucose in water and administered over a 24 hr period. The rate of infusion of IDU was approximately 4 mg/min. One patient received IDU as a rapid infusion, 3 g delivered over 60 min (50 mg/min). Our current practice is to omit the last day of treatment if marked suppression of the bone marrow is evident after the 4th day of therapy. Blood, urines, and cerebrospinal fluids were collected at intervals before, during, and after infusions of IDU.

RESULTS

Repetitive determinations of 0.67–20 μg of IDU per 0.4 ml in antiviral assays give almost identical numbers of pfu of Ket virus. Data from five such standard curves are shown in Fig. 1.

During infusions of idoxuridine 22 samples of serum, 11 urines, and 6 cerebrospinal fluids were tested for antiviral activities (Table II). Six sera, six urines, and one cerebrospinal fluid contained measurable idoxuridine. Values in sera ranged from 9 to 36 μg/ml; urines 45 to 1,040 μg/ml; and the single CSF with activity had 833 μg/ml. No specimen of serum, urine, or cerebrospinal fluid collected after cessation of IDU contained antiviral activity. The data are striking in that most specimens obtained during slow infusions of idox-

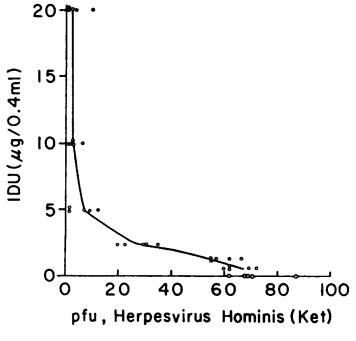

FIGURE 1 Repetitive antiviral assays of varying amounts of IDU describe a similar standard curve.

TABLE II

Concentrations of IDU in Serum, Urine, and CSF

Patient	Day of treatment with IDU	IDU		
		Serum	Urine	CSF
			μg/ml	
A. Received slow intravenous infusions of IDU, *4 mg/min*				
1	1		55	
	+6*		≦2.5	
2	−4	≦2.5		
	4	≦2.5		
	5	≦2.5		
	+1	≦2.5		
	+17	≦2.5		
3	−4	≦2.5		
	1	≦2.5		
	2	≦2.5		
	3	≦2.5		
	4			≦2.5
	+5	≦2.5		
4	−1	≦2.5		
	1	≦2.5	≦2.5	
	2	≦2.5		
	3	33	≦2.5	≦2.5
	4	N.D.		≦2.5
	+2	≦2.5		≦2.5
5	−1	≦2.5	≦2.5	
	1	≦2.5	N.D.	833†
	2	≦2.5		
	3	≦2.5	45	≦2.5
	4	N.D.	≦2.5	
	+3	≦2.5		
6	−1	≦2.5	N.D.	
	3	N.D.	80	
	4	N.D.	N.D.	≦2.5
	5	≦2.5	16, ≦2.5§	
	+4	≦2.5	≦2.5	
7	−2	≦2.5		
	−1			≦2.5
	1	9		
	3	≦2.5		
	4	≦2.5	64, ≦2.5	
8	−5	≦2.5		
	1	≦2.5		
	4	≦2.5		
	5	≦2.5		
	+1	≦2.5		
B. Received rapid intravenous infusion of IDU, *50 mg/min*				
9	−1	≦2.5		
	1 (15 min)‖	10		
	1 (30 min)	10		
	1 (45 min)	20	1040	
	1 (60 min)	36		
	+1		≦2.5	

* +, day after cessation of intravenous IDU; −, day before beginning intravenous IDU; N.D., not done.

† CSF was obtained for assay immediately after beginning a 3 g infusion of IDU.

§ Represents collection of two random samples of urine during day.

‖ Time after start of intravenous infusion of 3 g of IDU, delivered over 60 min.

TABLE III

MIC of Idoxuridine versus Strains of Herpesvirus hominis*

Type of *H. hominis*	Source/Diagnosis	Passages in tissue culture	MIC per 0.4 ml of idoxuridine
			μg
I.			
Ket.	Brain/Encephalitis	Am., BHK 21	5
Con.	Brain/Encephalitis	BHK 21_2	5
Cut.	Brain/Encephalitis	BHK 21_2	2.5
Fran.	Brain/Encephalitis	BHK 21_2	10
Wal.	Brain/Encephalitis	MK,‡ BHK 21	5
Pric (newborn)	Vesicle (chest)/Neonatal infection	WI 38, BHK 21	5
Jah.	Vesicle/*Herpes labialis*	BHK 21_3	5
Nol.	Vesicle/*Herpes labialis*	BHK 21_4	5
Scrus.	Vesicle/*Herpes labialis*	BHK 21_2	5
Ric.	Vesicle/Kaposi's varicelliform eruption	WI 38_2, BHK 21	10
Ford.	Vesicle/Kaposi's varicelliform eruption	Am., BHK 21	5
Intermediate			
Zerb§	Vesicle/Congenital generalized infection	BHK 21	25
II.			
E 304 (prototype)‖	Vesicle (thigh)	RK_3‡ BHK 21	50
Brod.	Vesicle (penis)/*Herpes progenitalis*	BHK 21, MK, BHK 21_3	50
Tom.	Cervix/Cervicitis	BHK 21_2	50

* MIC, minimum inhibitory concentration.

‡ MK, African green monkey kidney (cercopithecus aethiops); RK, rabbit kidney.

§ Ket., Con., Cut., Fran., Wal., and Zerb. strains of herpesviruses are from fatal cases.

‖ Obtained from the American Type Culture Collection, Rockville, Md.

uridine contain no measurable activity. They confirm previous pharmacologic experiments using ^{131}I labeled 5-iodo-2′-deoxyuridine indicating that rapid, and here, almost instantaneous transfer of idoxuridine into tissues and urine occurs (15, 16). Since IDU is not metabolized in serum or urine, inactivation must occur in tissues. The exact site of inactivation is not yet known.

In one patient idoxuridine was administered as a rapid infusion by vein. After 3 g of IDU given over an hour, sera at 15, 30, 45, and 60 min contained 10, 10, 20, and 36 μg/ml, respectively. In this case, in marked contrast to the majority of these data, the rate of administration of drug exceeded its degradation. Likewise, in patient 5, 833 μg/ml of IDU was found soon after initiating a rapid infusion of a liter of 5% glucose in water containing 2 g of IDU. Further study of relatively rapid intravenous infusions may be indicated.

Minimal inhibitory concentrations of idoxuridine vs. recent isolates of *Herpesvirus hominis* are shown in Table III. Minimal inhibitory concentrations for all of the type 1 strains ranged from 2.5 to 10 μg/0.4 ml, while the three strains of type 2 and the intermediate herpesvirus required 25–50 μg/0.4 ml. Every isolate whose MIC was determined had been passaged in tissue cultures for only two to five passages.

DISCUSSION

The use of specific properties of neutralizing antibody and human interferon has allowed the development of a simple quantitative assay of antiviral activity due to idoxuridine. Biologic properties utilized are: (*a*) virus and its specific antibody interact only extracellularly, and (*b*) human interferons function in primate, but not in nonprimate cells. *Herpesvirus hominis* adsorption to BHK 21 tissue cultures is complete within 2 hr, and subsequent addition of serum, urine, or cerebrospinal fluid containing these immunoglobulins does not inhibit replication of virus. Likewise, human antiherpesvirus interferons in these body fluids have no antiviral activity when tested in hamster kidney cells. The antiviral assay has been developed for strains of *Herpesvirus hominis* to test idoxuridine. Sensitivity of the method is about 2.5 μg, and is reproducible. The principles utilized here have applications to other systems.

In serum idoxuridine is stable at 37°C, 4°C, and to freezing and thawing. Since repetitive assays of urines are consistent, IDU is also stable in urine. Therefore, body fluids from patients receiving IDU, which are stored at − 20°C and later assayed reflect original content of drug. Idoxuridine presumably rapidly reaches

tissues. It can enter the CSF in significant concentrations. It is quickly excreted in the urine. Inactivation and deiodinization probably occurs in the tissues. When IDU is given by slow intravenous infusion (approximately 4 mg/min), inactivation regularly exceeds the rate of administration. Under these conditions serum, urine, or CSF only sporadically contain measurable IDU. When given intravenously by more rapid infusions (50 mg/min), IDU can readily be found in serum, urine, and CSF.

Minimal inhibitory concentrations of IDU versus recently isolated strains of *Herpesvirus hominis* from patients with encephalitis, neonatal generalized infections, Kaposi's varicelliform eruption, herpes labialis, or herpes progenitalis were determined. Type 1 strains were uniformly sensitive to 2.5–10 μg/0.4 ml, while type 2 herpesviruses and the single intermediate strain had MIC's of 25–50 μg/0.4 ml. This quantitative difference in sensitivity to idoxuridine may be added to (*a*) larger pocks on the chorioallantoic membrane of embryonated eggs (4), (*b*) greater virulence for female mice which have been infected by the genital route (4), (*c*) greater tendency toward formation of giant cells in tissue cultures (6), (*d*) difference of the density and base composition of their deoxyribonucleic acid (8), and (*e*) antigenic distinctiveness of herpesvirus genital strains.

The actual concentration of active compound which is present after optimal administrations in the brain and other organs is critical information which remains to be determined. The data presented do not show that idoxuridine is effective therapy of herpesvirus infections, but they do offer basic pharmacologic information which need to be considered in possible formulations concerning treatment. Such a formulation for a 70 kg man might be 2.4 g of idoxuridine in 250 cc of 5% glucose in water as an intravenous infusion given over a 60 min period every 12 hr for 4 (or possibly 5) consecutive days. The final day of therapy would be dependent upon the findings at a bone marrow examination obtained on the morning of the 5th day. It has been our experience that infants and children sustain larger total dosages with lesser signs of toxicity.

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