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ISOLATION OF BROMOVINYLDEOXYURIDINE-RESISTANT STRAINS OF HERPES SIMPLEX VIRUS AND SUCCESSFUL CHEMOTHERAPY OF MICE INFECTED WITH ONE SUCH STRAIN BY USING ACYCLOVIR

HUGH J. FIELD and JOHN NEDEN

Division of Virology, Department of Pathology, University of Cambridge, Cambridge, U.K.

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Several strains of herpes simplex virus which were resistant to bromovinyldeoxyuridine were isolated by passaging the virus in the presence of the drug in tissue culture. The resistance of the majority of isolates was accounted for by their reduced ability to induce the enzyme thymidine kinase. These strains were co-resistant to acyclovir, but showed reduced pathogenicity in mice. However, another type of bromovinyldeoxyuridine-resistant virus was isolated which induced normal levels of thymidine kinase and retained virulence for mice. This resistant virus was sensitive to acyclovir and was successfully treated using oral acyclovir therapy.

herpes simplex antiviral chemotherapy bromovinyl deoxyuridine nucleoside analogue chemotherapy Acyclovir

INTRODUCTION

The nucleoside analogue bromovinyldeoxyuridine (BVDU) is a potent inhibitor of herpes viruses [8] which promises to be effective for the chemotherapy of herpes simplex type 1 (HSV-1) and varicella-zoster infections in man [7,24]. BVDU has a potential for systemic use and thus resembles acyclovir (ACV) which is already undergoing clinical trials [1]. Both drugs owe their selectivity to the fact that they are initially phosphorylated by the virus-induced thymidine-kinase (TK); the nucleoside triphosphates subsequently interact with the virus DNA polymerase and interfere with DNA synthesis.

One factor which may influence the success of nucleoside analogues such as BVDU and ACV is the development of resistance in the virus [13]. For example, strains of HSV which are resistant to ACV may be isolated with ease by selection for drug-resistance in vitro [14]. The present study demonstrates that BVDU-resistant strains also arise readily in infected tissue cultures.

The majority of ACV-resistant strains examined to date have turned out to be defective in TK induction (TK^-), so they tend to be co-resistant to all the nucleoside analogue inhibitors which depend on HSV-TK for their 'activation' [27]. However, TK^- ACV-resistant viruses appear to have reduced pathogenicity as judged by infections produced in mice [12] and this would suggest that they will not arise readily in vivo.

Another way in which resistance can occur is through changes in the substrate spe-

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cificities of the virus-induced TK or DNA polymerase enzymes. A particular BVDU-resistant mutant of this type will be described. This mutant attracted interest principally because it retained virulence in mice and showed co-resistance to the thymidine analogues 1-β-D-arabinofuranosylthymine (ara-T) and idoxuridine (IDU). However, in contrast to the TK^- mutants tested, this strain was sensitive to ACV in an in vitro test showing that ACV and BVDU are sufficiently different that co-resistance is not inevitable. The effective chemotherapy, using ACV, of mice infected with the BVDU-resistant virus demonstrates the value of having alternative chemotherapy available should such strains be encountered in clinical practice.

MATERIALS AND METHODS

Virus and cells

The virus used was the oral isolate of HSV-1 strain SC16 [21]. This virus has been used previously to generate mutants resistant to ACV [14]. The virus was propagated in BHK-21 cells using Glasgow-modified Eagle's medium (EMEM-10) supplemented with 10% tryptose phosphate broth and 10% calf serum. For experiments in which nucleoside inhibitors were added, the tryptose phosphate broth was excluded from the medium and the concentration of calf serum reduced to 1% (EMEM-1). For the determination of thymidine kinase (TK) cultures of BUDR-resistant BHK cells (BU-BHK) were used. This is a line derived from BHK-21 by passage in the presence of BUDR and which fails to express TK activity.

Inhibitory compounds and ED_{50} determinations

(*E*)-5-(2-Bromovinyl)-2′-deoxyuridine; bromovinyl deoxyuridine (BVDU) was a gift from Dr. E. de Clercq (Rega Institute, University of Leuven, Belgium). 9-(2-Hydroxyethoxymethyl)guanine, acyclovir (ACV) was a gift from Dr. G.B. Elion (Burroughs Wellcome Ltd., Research Triangle Park, NC) and 1-β-D-arabinofuranosylthymine (ara-T) was also a gift from Dr. G. Gentry (Department of Microbiology, Mississippi Medical Center, Jackson, MS). 5-Iodo-2′-deoxyuridine was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

To determine the 50% effective dose (ED_{50}) of the compounds the test strain of virus was inoculated in 0.2 ml EMEM-1 onto preformed monolayers of BHK cells in 5 cm plastic Petri dishes to give approximately 200 plaques per dish. After allowing the virus to adsorb to the cells for 1 h, the cultures were washed with phosphate-buffered saline, then EMEM-1 containing carboxymethyl cellulose, and various concentrations of the test compound were added. After 48 h incubation at 37°C the cultures were formalin-fixed, stained with 0.1% trypan blue and the plaques counted. The number of plaques in the inhibitor-containing cultures was plotted as % versus $\log_{10}$ (drug concentration) and the ED_{50} determined directly from the graph.

Thymidine kinase determination

TK was measured using extracts of BU-BHK cells 18 h after infection at a multiplicity of 5 plaque-forming units (p.f.u.)/cell. The method used [14] was a modification of that described by Klemperer et al. [23].

Mouse infections

LD_{50} by intracerebral inoculation. To determine the 'neurovirulence' of virus strains by i.c. injection, 0.02 ml of various concentrations of the test virus suspended in EMEM-1 were inoculated into the left cerebral hemisphere of 3-week-old anaesthetized BALB/c mice. Deaths were scored between the 2nd and 14th day post-infection (p.i.) and the LD_{50} determined by the method of Spearman-Karber [19].

Mouse ear infection. Mice were also inoculated intradermally into the left ear pinna with a dose of 10^4 p.f.u. The progress of the disease was monitored 1) by measuring ear thickness using an Engineer's micrometer), and 2) by amputating the pinnae from groups of mice, homogenizing the tissue and treating with ultrasonic vibration to release infectious virus. The amounts of virus in the ear tissue were then determined independently for each mouse by plaque titration in BHK cells. The measurement of ear thickness has been found by us to be a reliable and reproducible method for determining the amount of cell-mediated inflammation in the infected ear [25] and thus provides a convenient method for determining the clinical severity of the infection; if necessary repeated measurements can be made on the same mice.

Chemotherapy of mice. ACV or BVDU was dissolved in the drinking water. This was shown to be an effective and reliable method for administering both drugs to mice [15]. The concentration of the drug was 1 mg/ml in both cases. The mice showed some distaste for BVDU which was lessened by adjusting the pH to 3 using HCl. The average daily consumption of the drugs was approximately 170 mg/kg/day for ACV and 140 mg/kg/day for BVDU. In neither case were any signs of toxicity observed.

Detection of latent infections

Four to 6 weeks p.i. mice were killed and the 2nd, 3rd and 4th cervical dorsal root ganglia explanted into small culture tubes containing 0.5 ml EMEM-1. The ganglia were incubated for 6 days at 37°C, then the tissue was homogenized and infectious virus titrated in BHK cells in the normal way. This technique has been found to be an effective method for reactivating latent HSV [18].

RESULTS

The ready isolation of resistant virus in vitro

The development of resistance to BVDU was readily demonstrated in tissue culture. The inoculum virus, strain SC16, was sensitive to BVDU when tested in BHK monolayers (ED_{50}, 0.005 μg/ml). In a standard plaque-reduction assay in which an inoculum of approximately 200 p.f.u. was subjected to increasing concentrations of BVDU, a steep curve was obtained (Fig. 1) with no plaques visible at drug concentrations greater than 1.0 μg/ml. However, when much larger numbers of p.f.u. were inoculated, plaques were visible at higher concentrations of BVDU (Table 1). A similar pattern of plaque numbers

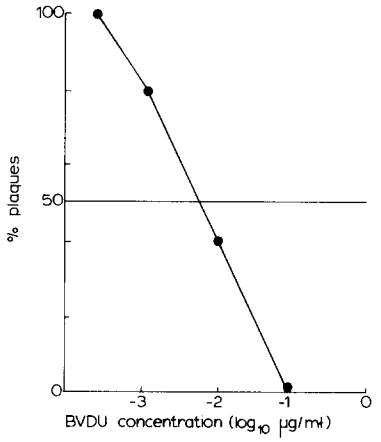

Fig. 1. Inhibition of HSV-1 SC16 by BVDU in vitro. ED_{50} measured in BHK-cell monolayer cultures by the plaque-reduction test.

TABLE 1

The numbers of plaques observed to develop in the presence of either ACV or BVDU using various amounts of inoculum virus

Virus inoculum[a] (p.f.u./well)	BVDU concentration (μg/ml)				ACV concentration (μg/ml)			
	0	0.1	1.0	10	0	0.1	1.0	10
10^4	UC[b]	UC	UC	10	UC	UC	UC	>10
10^3	UC	40	10	0	UC	UC	6	2
10^2	UC	5	1	0	UC	9	0	0
10^1	14	1	0	0	9	1	0	0

[a] HSV-1 SC16 inoculated onto preformed monolayers of BHK cells in a multiwell culture tray.
[b] UC, plaques too numerous to count.

was obtained with ACV (Table 1) showing that the phenomenon was not peculiar to BVDU. Several such plaques which developed in the presence of $\geqslant 1$ μg/ml BVDU were investigated by removing a few infected cells from the plaque by means of a Pasteur pipette before staining. These isolates were subsequently confirmed to be resistant to the inhibitory drug used in the test.

Further resistant viruses were obtained by infecting BHK cells at a multiplicity of infection of 0.1 p.f.u./cell in the presence of 1 μg/ml BVDU. The yield virus was plaque-purified by inoculation at limiting dilution onto BHK cells seeded in 96-well dishes. The infected cells from a well containing a single plaque were used to infect further cultured BHK cells to produce a working stock of virus. The latter passage was carried out in the absence of BVDU.

Two examples of resistant viruses obtained in this way were SC16 A1Cl and SC16 A2Cl. These viruses were shown to be respectively 100-fold and 1000-fold more resistant to BVDU than the parental strain (Table 2).

Yields of virus obtained from the tissues of SCl6-inoculated mice following BVDU treatment for 5 days (experiments described below) were also tested for BVDU resistance. In contrast with the observation of rapid development of resistance to BVDU in tissue culture no marked change in ED_{50} concentration was observed in virus isolated from mice (data not shown).

BVDU resistance accounted for by defective TK induction

Several resistant viruses obtained by passage in BVDU-treated BHK cells were tested for their ability to induce TK activity in infected BU-BHK cells. In all cases the enzyme activity was markedly reduced when compared with SC16. The levels of TK compared with SC16, using a multiplicity of infection of 5 p.f.u./cell, ranged from $<1\%$ to 5% the activity of parental strain SC16. The resistance to BVDU of these strains ranged from 0.5 to >5 μg/ml (Table 2). Thus the defective TK induction appeared to account at least in part for the resistance of the strains but they were not all identical. The TK-defective strain SC16 A2C1 was tested for virulence in mice. Previous work has shown that mutants selected for resistance to BUDR, ACV [12], and ara-T [26,29,30] were attenuated in experimentally infected animals. As shown (Table 2) SC16 A2C1 resembled the previously described TK^- strains, showing reduced lethality following i.c. inoculation, reduced disease in the skin and no evidence of the establishment of latent infections in the dorsal root ganglia related to the sensory nerve supply to the inoculation site (the ear pinna).

The isolation of TK-inducing BVDU-resistant mutants of HSV

Normally, ED_{50} determinations were performed by infecting preformed monolayers of BHK cells with the test virus and incubating the infected cells in medium containing the inhibitor. It was observed that when BHK cells were infected in suspension, then

TABLE 2

Properties of several BVDU-resistant mutants of HSV-1 in vitro and in mice

Virus	In vitro		Ear inoculation[a]			I.c. inoculation (p.f.u./LD_{50})
	ED_{50}[b] (μg/ml BVDU)	TK induction[c] (% SC16)	Max. inflammation[d] (% SC16)	Max. virus[e]	Latency detected	
SC16	0.005	100	100	5.1 ± 0.6	6/9	7
SC16 A1C1	0.5	5	ND	ND	ND	ND
SC16 A2C1	5.0	<1	30	ND	0/9	$>10^3$
SC16 B1	5.0	<1	ND	ND	ND	$>10^3$
SC16 B3	>10	⩾100	69	4.3 ± 0.9	5/9	24

[a] 10^4 p.f.u. inoculated into left ear pinna.

[b] ED_{50} determined by the plaque-reduction test in BHK cell monolayers.

[c] TK activity expressed as % SC16, the parental strain, using extracts of BU-BHK cells measured 18 h after inoculation at a multiplicity of 5 p.f.u./cell.

[d] Increased ear thickness (left−right × 10^{-2}) expressed as % SC16.

[e] Virus titre in ear (log_{10} p.f.u./mouse) geometric mean titre and standard deviation obtained from three mice.

[f] Number of mice from which virus isolated from explant cultures of dorsal root ganglia (2nd, 3rd and 4th cervical pooled) over number of mice tested.

allowed to form monolayers in the presence of BVDU a different result was sometimes obtained in that TK^--resistant strains became fully sensitive to BVDU. These results, however, were not reproducible and despite efforts to make conditions identical widely differing results for the degree of sensitivity of TK^- strains were obtained from separate tests using cells infected in suspension, whereas infected monolayers yielded consistent results. Although this phenomenon was not elucidated, the same infection system (in suspension) was used to select for resistance to BVDU. Four resistant clones were isolated independently and of these two were low TK^- inducers (<1% SC16) but two isolates induced normal levels of TK despite their being highly resistant to BVDU. These TK^+ resistant strains were of particular interest because TK^+ ACV-resistant strains had been found previously to retain virulence in mice.

One mutant of each of the two TK phenotypes was selected for more detailed investigation. The viruses were again cloned by single plaque isolation and were designated SC16 B1 and SC16 B3. Three subclones of SC16 B3 were all found to induce similar levels of TK showing that the enzyme induced by the mutant resulted from a homogeneous virus population and could not be accounted for by a mixture of TK^+ and TK^- strains as sometimes occurs [11].

The mutants were tested in vitro for sensitivity to ACV, ara-T, and IDU; all three compounds depend on being phosphorylated by HSV-TK. As expected, the TK^- strain (SC16 B1) was co-resistant to the three alternative drugs (Table 3). The TK^+ mutant (SC16 B3) was also found to have acquired resistance to ara-T and IDU, but in contrast to TK^- mutant, SC16 B3 was equally sensitive to ACV as the parental virus, SC16, from which it was derived.

When SC16 B3 was injected into mice using a peripheral inoculation site (the ear pinna) the infection closely resembled that produced by SC16, producing approximately 70% maximum inflammation, as judged by ear thickness, and only slightly less virus replication in the ear (Table 2 and Fig. 2). Furthermore, 6 weeks after the ear inoculation, mice were

TABLE 3

TK induction and in vitro sensitivity to several nucleoside analogues of two mutants of SC16 selected for resistance to BVDU

	ED_{50}[a] concentration (μg/ml)				
	TK induction[b]	BVDU	ACV	Ara-T	IDU
SC16	100	0.005	0.02	1.0	0.2
SC16 B1	<1	5.0	1.3	>10	>10
SC16 B3	⩾100	>10	0.01	8.0	60

[a] ED_{50} measured by the plaque-reduction test in monolayers of BHK cells.

[b] TK induction (% SC16) measured in extracts of infected BU-BHK 18 h after inoculation at a multiplicity of infection of 5 p.f.u./cell.

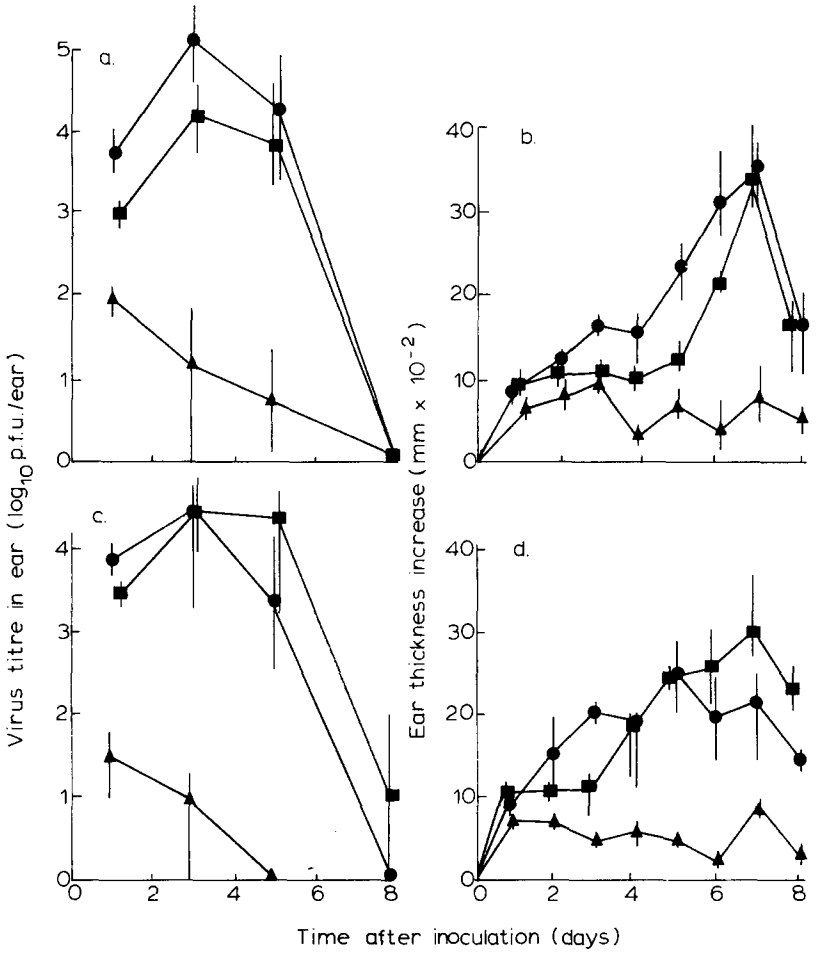

Fig. 2. The comparative growth of SC16 and SC16 B3 in the mouse's ear and effects of ACV or BVDU therapy. a, b) Inoculated with SC16. c, d) Inoculated with SC16 B3. Untreated (●); BVDU-treated (1 mg/ml in the drinking water) (■); ACV-treated (1 mg/ml in the drinking water) (▲). Mice were inoculated with 10^4 p.f.u. of virus into the skin of the left ear pinnae. Virus titres are the geometric mean and range obtained from three mice tested at each time. Ear thickness is the swelling (i.e. the increased thickness of the left compared with the right, uninoculated) ear and is an indication of cell-mediated inflammation. Each point is the arithmetic mean value and range obtained for three mice tested at each time.

killed and the cervical dorsal root ganglia explanted. Virus was reactivated in vitro from 5/9 mice tested (Table 2) and these re-isolated viruses were shown to retain the BVDU-resistant phenotype.

Mice were also inoculated i.c. and the LD_{50} determined. The p.f.u./LD_{50} ratio for SC16 B3 was found to be 24 corresponding to a 3-fold increase compared with SC16. This contrasts with TK^- strains derived from SC16 which typically showed increases in p.f.u./LD_{50} of $>10^3$-fold [12].

The successful chemotherapy of a BVDU-resistant infection using oral ACV

When the mutant SC16 B3 was inoculated into the ear pinnae of mice an infection was established which showed similar characteristics to that produced by the parental strain (see above and Fig. 2). The infection produced by SC16 responded to oral BVDU therapy; inflammation was less marked (Fig. 2b) and virus replication in the ear reduced (Fig. 2a). However, chemotherapy with BVDU was less effective than a similar dose of ACV. That ACV is more effective than BVDU in BALB/c mice infected with HSV SC16 has been reported previously [15]. In contrast to the parental strain, the infection produced by the mutant SC16 B3 was completely resistant to BVDU therapy, but, in mice treated with ACV, therapy was fully effective (Fig. 2c,d) thus confirming in mice the sensitivity of this strain to ACV which had been shown in tissue culture.

DISCUSSION

Four important points emerged from this study: i) strains of HSV-1 resistant to BVDU can readily be isolated in tissue cultures by passaging virus in the presence of the drug; ii) the majority of resistant strains isolated are TK-defective and these tend to be less virulent in mice; iii) conditions can be contrived which result in the isolation of TK^+, BVDU-resistant strains that tend to retain virulence in mice; and iv) one type of BVDU-resistant virus is sensitive to ACV, an alternative chemotherapeutic agent.

The ready isolation of strains of virus resistant to BVDU was not unexpected since the loss of the ability to induce TK confers resistance on the virus, while it does not impair the ability of the virus to replicate in actively growing tissue culture cells [18, 22]. Similar rapid emergence of TK^--resistant viruses has been demonstrated previously by passage of HSV in BUDR [10], IDU, ara-T and ACV [14,28]. The mutant strains are co-resistant to the four drugs but not to trifluorothymidine or ara-A, both of which are activated independently of the HSV-TK [4]. The biological properties of the BVDU-selected TK^- virus tested in the present study resembled previously described TK^- viruses in that the virulence in an animal model was reduced using several different criteria. The fact that these viruses grow less well in skin and have a reduced ability to invade the nervous system would explain why they do not emerge readily during the chemotherapy of HSV infections. That is not to say that such TK^- viruses will not have a role in human infections. Indeed, recently there have been two separate accounts of the development of resistance to ACV reported in man [2,5] and in these cases the virus isolates were shown to induce low levels of TK. Similar results have been obtained in this laboratory in mice infected with HSV-1 SC16 undergoing oral therapy with ACV and repeated re-inoculation into further mice undergoing continuous therapy [11]. In this case an ACV-resistant infection emerged comprising a mixture of TK^+ and TK^- strains.

On the other hand, the clinical potential of TK^+-resistant viruses would, in theory, be much greater since several strains of this type have already been shown to be virulent, at least in animal models [6,12]. For this reason much attention was focused on a TK^+ BVDU-resistant strain of SC16.

The biochemical nature of the mutant is not yet fully elucidated but preliminary results show that the strain (SC16 B3) induces a TK which has an altered substrate specificity (G. Darby and B.A. Larder, personal communication) such that the affinity of the mutant enzyme for BVDU is greatly reduced compared with the wild-type TK. However, this may not alone account for the high resistance of this strain and together with the observation that resistance to BVDU of SC16 B3 is retained when measured in transformed BU-BHK cells, which express HSV-TK, suggests that the mutant has a second site of resistance, possibly in the DNA polymerase. These observations will be published fully in the near future.

From a practical point of view SC16 B3 was shown to be co-resistant to IDU (which is in current clinical use) and the experimental drug ara-T. However, the mutant was completely sensitive to ACV which is currently emerging as a new and effective agent for the chemotherapy of HSV [1].

The lack of cross-resistance between ACV and BVDU of this strain is an important observation, since it demonstrates that, although both drugs are initially phosphorylated by the HSV-TK [9,20] (and hence TK^- viruses are cross-resistant) when resistance is mediated by qualitative changes in TK [6] or DNA polymerase [3,28], cross-resistance is not inevitable. (However, it should be pointed out that one of two TK^+ ACV-selected resistant mutants that we described previously showed co-resistance to BVDU [16,17].

This study further emphasises the need for virological monitoring of herpes infections undergoing chemotherapy so that virus isolates can be tested in vitro for sensitivity to a range of inhibitors. Should resistance become a problem there is at present insufficient information to predict the pattern of cross-resistance to different compounds, but there clearly is a potential for a rational approach to alternative chemotherapy. Thus the availability of several different effective compounds active against HSV may prove valuable in counteracting the tendency of HSV to subvert successful chemotherapy by the acquisition of resistance to nucleoside analogues.

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