Effects of Oral Treatment with Acyclovir and Bromovinyldeoxyuridine on the Establishment and Maintenance of Latent Herpes Simplex Virus Infection in Mice

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SUMMARY

Mice infected with herpes simplex virus (HSV) were treated (separately) with the nucleoside analogues acyclovir or bromovinyldeoxyuridine by incorporating the drugs in the drinking water. This method of treatment was found to be effective for both drugs and compared favourably with intraperitoneal injection. Prompt treatment with either compound could prevent the establishment of latent infections but latent infections once established were intractable using prolonged courses of oral administration.

INTRODUCTION

Several new nucleoside analogues have been developed which are potent inhibitors of herpes simplex virus (HSV) with low toxicity in vivo. Two of these, acyclovir or 9-(2-hydroxyethoxymethyl)guanine (ACV) and E-5-(bromovinyl-2'-deoxyuridine) (BVDU) behave as analogues of thymidine and are selectively phosphorylated by the HSV-specified pyrimidine deoxynucleoside kinase (thymidine kinase or TK). Both drugs are then metabolized within the infected cells to their respective nucleoside triphosphates which then interfere with HSV-specified DNA polymerase and inhibit virus replication (Elion et al., 1977; Fyfe et al., 1978; De Clercq et al., 1979a).

Both ACV and BVDU have been shown to suppress acute HSV infections in mice, rabbits and guinea-pigs (De Clercq et al., 1979a; Field et al., 1979; Schaeffer et al., 1977; Kaufman et al., 1978; Maudgal et al., 1980). Trials with ACV and BVDU for the treatment of HSV and varicella-zoster infections in man are now underway. However, there are complex interactions between HSV and the peripheral nervous system involving latency and recurrent disease. The effects of chemotherapy on these aspects of the disease will be very difficult to determine in man and, therefore, the study of animal models is particularly valuable in developing a rational approach to chemotherapy.

There have been several reports on the effects of ACV on latent infections in animals but none to date with BVDU. Our own previous studies with ACV showed that prompt treatment [twice daily doses intraperitoneally (i.p.) totalling 50 mg/kg/day from the time of virus inoculation for 10 days] prevented or at least reduced the establishment of latency (Field et al., 1979). Similar results have been reported by others (Klein et al., 1979; Park et al., 1979). In a different study Pavan-Langston et al. (1979) showed that already-established latent infections in mice could be cured by 15 days systemic treatment with ACV; however, Field et al. (1979), Klein et al. (1979) and Blyth et al. (1980) all reported that latent infections once established were not reduced by subsequent treatment with ACV.
The systemic treatments described in all the above studies employed repeated injections of ACV and had the disadvantage that the tissue half-life of ACV is relatively short. This was borne out by the recent study of Boulter et al. (1980) who showed that intravenous (i.v.) dosing at 6-h intervals was essential in order to prevent progression of the highly neurotropic herpes virus B (which also has a lower susceptibility to ACV) infection in rabbits.

In our previously described treatment schedule (Field et al., 1979) there was a 15 h gap between i.p. doses (i.e. 6 p.m. to 9 a.m.) and this presumably led to periods of relatively low drug levels in the tissue (Schaeffer et al., 1977). In order to treat a fairly large number of mice over lengthy periods the drug was given ad lib. in the drinking water. This proved to be a successful method of administration of both ACV and BVDU.

This paper compares the effects of oral and i.p. administration of the two nucleoside analogues and describes their effects on the establishment of latent infections in mice.

METHODS

Virus infection. Acute HSV infections were produced in 4-week-old Balb/c mice by inoculating HSV type 1 strain SC16 (Hill et al., 1975) into the skin of the left pinna using a dose of $10^4$ or $10^5$ p.f.u, suspended in 0.02 ml Glasgow-modified Eagle’s medium. Latent infections became established in the cervical dorsal root ganglia relating to the sensory nerve supply of the ear.

Measurement of virus infectivity, inflammation and detection of latent infections. Virus titres were determined by plaque titration in BHK cells of dilutions of homogenized ear tissues using standard virological techniques. Three mice were sampled at each time point; the tissues were titrated independently and the mean virus titre obtained. As a measure of the severity of the disease, inflammation produced during the infection was assessed by the determination of ear swelling. The ear thickness was measured using an engineer’s micrometer screw gauge on groups of three to five mice at each time point. The swelling was taken to be the thickness of the left minus the right (uninoculated) pinna. This technique has been characterized in detail in an identical mouse model (Nash et al., 1980). For the detection of latent infections, the 2nd, 3rd and 4th cervical ganglia were explanted into 0.5 ml Glasgow-modified Eagle’s medium containing 1% calf serum. The three ganglia from each mouse were pooled. The cultures were incubated for 6 days at 37 °C, then homogenized and titrated using BHK cells as above.

Drugs. ACV was a gift from Dr G. B. Elon, Burroughs Wellcome Ltd., Research Triangle Park, North Carolina, U.S.A. BVDU was synthesized by R. Busson and H. Vanderhaeghe (Rega Institute, Leuven, Belgium). ACV was dissolved in water at pH 6-8 using a concentration of 1 mg/ml and the drug-containing water was substituted for the normal water supply. In the case of BVDU, the pH was adjusted to 3 using HCl. ACV is tasteless and mice drank it normally, but BVDU was found to be more palatable at the lower pH. Both drugs are stable at room temperature under these conditions. Naturally, the water intake of mice varies with environmental conditions. However, observations over several weeks indicated a surprisingly constant rate of drinking. The in vitro activity of the two drugs was determined by a plaque reduction test in monolayer cultures of Vero and BHK cells as described by Field et al. (1980).

RESULTS

Administration of ACV and BVDU by means of drinking water

The daily intake of drinking water by groups of mice was observed over a 10 day period. The daily consumption (approx. 3 ml/day/mouse) remained surprisingly constant. There was no reduction in the intake of water containing 1 mg/ml ACV compared to controls but a slight distaste for BVDU-containing water was noted. The consumption of BVDU was
Improved slightly by acidifying the water to pH 3. The average daily consumption of the drugs was ACV 167 ± 56 mg/kg/day, BVDU (pH 5-8) 137 ± 28 mg/kg/day and BVDU (pH 3) 151 ± 27 mg/kg/day. After 10 days continuous treatment the mice showed no obvious signs of toxicity in either case and there was no difference between the weight of treated mice and the untreated controls.

An attempt was made to combine drinking water treatment with ACV with an additional dose of a further 50 mg/kg/day given i.p. This combined dose appeared to exceed the toxic dose for this drug and the mice ruffled and died after 7 to 10 days treatment.

In vitro sensitivity to ACV and BVDU

HSV strain SC16 was tested in BHK, Vero and primary mouse embryo fibroblast cells for sensitivity to ACV and BVDU by a plaque reduction test. In all cases BVDU appeared to be more active than ACV. However, while ACV showed similar activity in BHK and mouse embryo fibroblast cells (ED₅₀ = 0.05 μg/ml) BVDU was found to be somewhat less active in the latter cell type (BVDU ED₅₀ for BHK cells = 0.002 μg/ml and for primary mouse fibroblasts = 0.01 μg/ml).

Comparison of oral with i.p. treatment of acute HSV infection

Mice were inoculated with the type 1 strain of HSV, SC 16, into the skin of the left ear. A virus inoculum of 10⁴ p.f.u. was employed and treatment either by incorporating 1 mg/ml ACV in drinking water or by two daily i.p. injections totalling 50 mg/kg/day was commenced from the time of inoculation. The effects of treatment were assessed by examining the amount of virus replication in the ear pinna and by measuring the inflammation as judged by increased skin thickness. Fig. 1 shows that treatment with ACV in the drinking water reduced virus titres in the ear by 2 to 3 log₁₀ with a concomitant reduction in inflammation. In this experiment the conventional i.p. dosing route was less effective.

The experiment shown in Fig. 2 was similar to that described above except that BVDU was substituted in the drinking water and for i.p. administration a larger dose of BVDU was...
Effects of i.p. or oral treatment with BVDU on HSV infection in mice. Mice were inoculated as for Fig. 1. (a) Virus replication in the skin. (b) Inflammation: ear swelling is the thickness of the left minus the right (uninoculated) ear. ○, Untreated; □, BVDU (100 mg/ml/day i.p. in two injections daily); ○, BVDU (1 mg/ml/day in drinking water). Treatment commenced at the time of virus inoculation and continued for 10 days thereafter.

employed (100 mg/kg/day). In this case treatment by the two different routes was indistinguishable. However, it was noted that, while virus titres were reduced by 1 to 2 log₁₀, the drug appeared to be less effective than ACV in either reducing virus replication or inflammation. However, it is recognized that the lower toxicity of BVDU for mice would enable higher doses of BVDU, e.g. 250 to 500 mg/kg/day to be investigated in future studies.

A further experiment was carried out to compare directly BVDU with ACV employing the drinking water treatment. This confirmed that while BVDU certainly influenced the course of the infection when given in the drinking water, in this particular model ACV was clearly more effective (data not shown).

Effects of oral treatment with ACV or BVDU on the establishment of latent infections in mice

Previous studies using i.p. administration of ACV have indicated that, provided treatment is instituted within 24 h of infection and the doses of virus employed are moderate, the establishment of latent infections in the sensory ganglia related to the inoculation site was reduced or prevented.

The mice remaining from the above experiments (Fig. 1 and 2) were later tested for evidence of latent infections. The treated mice had been given ACV or BVDU in the drinking water from the time of inoculation for 10 days thereafter. The cervical dorsal root ganglia were explanted into culture vessels 4 weeks after inoculation (18 days after treatment ceased).

Subsequent culture of the explanted ganglia and testing for the presence of infectious virus indicated (Table 1) that both drugs were effective in preventing the establishment of latent infections which can be detected by this in vitro culture technique. One out of 18 mice from the BVDU-treatment groups was found to be harbouring the infection in the cervical ganglia. All other treated animals yielded negative cultures. The incidence of latent infection in the control, untreated groups were 100% and 55% in two separate experiments.

Effects of oral treatment with ACV and BVDU on established latent infections in mice

Mice which had recovered from the acute phase of an HSV infection in the ear following an inoculum of 10⁴ p.f.u. were treated with ACV in the drinking water commencing 1 month
**HSV in mice: oral nucleoside therapy**

Table 1. *Effect of ACV and BVDU on the establishment of latent HSV* in mice

<table>
<thead>
<tr>
<th>Treatment†</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACV (1 mg/ml in drinking water)</td>
<td>0/7</td>
<td>0/18</td>
</tr>
<tr>
<td>BVDU (1 mg/ml in drinking water)</td>
<td>ND §</td>
<td>1/18 (6%)</td>
</tr>
<tr>
<td>None</td>
<td>7/7</td>
<td>11/20 (55%)</td>
</tr>
</tbody>
</table>

* HSV-1 SC16 inoculated into left pinna (10⁴ p.f.u./mouse).
† Treatment commenced from time of inoculation and continued for 10 days thereafter.
§ Virus isolated by culture of dorsal root ganglia for 6 days (2nd, 3rd and 4th cervical ganglia pooled from each mouse).

Table 2. *Effects of ACV on established latent HSV* in mice

<table>
<thead>
<tr>
<th>Treatment†</th>
<th>Time after terminating treatment (weeks)</th>
<th>No. of mice positive/total tested</th>
<th>Virus titre in positives‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACV (1 month)</td>
<td>0 Control</td>
<td>8/8</td>
<td>5.4 ± 0.6</td>
</tr>
<tr>
<td>Treated</td>
<td>8/8</td>
<td>6.0 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>(1 month)</td>
<td>1 Control</td>
<td>6/7</td>
<td>5.7 ± 1.0</td>
</tr>
<tr>
<td>Treated</td>
<td>6/7</td>
<td>6.1 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>3 Control</td>
<td>3/7</td>
<td>5.1 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>5/7</td>
<td>5.5 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>(1 month)</td>
<td>5 Control</td>
<td>3/6</td>
<td>4.1 ± 2.8</td>
</tr>
<tr>
<td>Treated</td>
<td>5/6</td>
<td>4.9 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>ACV (2 months)</td>
<td>4 Control</td>
<td>ND §</td>
<td>4.3 ± 2.3</td>
</tr>
<tr>
<td>Treated</td>
<td>7/8</td>
<td>4.4 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>BVDU (1 month)</td>
<td>2 Control</td>
<td>6/10</td>
<td>4.3 ± 1.2</td>
</tr>
<tr>
<td>Treated</td>
<td>4/10</td>
<td>5.3 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>(1 month)</td>
<td>4 Control</td>
<td>7/10</td>
<td>5.3 ± 0.9</td>
</tr>
<tr>
<td>Treated</td>
<td>6/10</td>
<td>5.2 ± 0.9</td>
<td></td>
</tr>
</tbody>
</table>

* Virus: HSV SC16 inoculated into left ear pinna (10⁴ p.f.u./mouse).
† Treatment: 1 mg/ml in drinking water for 1 or 2 months starting 1 month after inoculation.
‡ Reactivation by culturing explanted cervical dorsal root ganglia (2nd, 3rd and 4th pooled from each mouse).
§ ND, Not done.

After inoculation; treatment continued for 1 or 2 months. Since it has been suggested that the period which elapses between the time of terminating treatment and explanting ganglia influences the detection of latency (D. Pavan-Langston, personal communication) mice were tested at various times from 0 to 5 weeks after treatment ceased. A total of 28 treated mice were compared with a similar number of untreated controls. There was no significant difference in the number of mice in which latent infection was detected or in the titres of virus in the ganglion explant homogenates (Table 2). A further group of mice was tested after 2 months treatment but, again, these mice showed no evidence of reduced latency. A small number of mice were treated with oral BVDU again for 1 month and again this produced no effect on the latent infection as determined by this method of in vitro reactivation (Table 2).

**DISCUSSION**

Oral administration of the two nucleoside analogues under investigation proved to be an effective method of treatment which compared favourably with twice daily i.p. injections. The results of pharmokinetic studies of BVDU in mice following oral treatment have been reported previously (De Clercq *et al.*, 1979b) but no data were obtained in the present study on the serum or tissue levels obtained during the course of treatment. Oral administration also suffers from the disadvantage that the quantity and regularity of drug intake are unknown. Despite this uncertainty, in practice, oral therapy may still be an extremely valuable method
for treating mice experimentally since large numbers of animals can be given the drug for extended periods without the need for regular handling of individual mice.

It was noted that BVDU was less effective than ACV in controlling the acute phase of the infection. This was true when BVDU was given orally or by i.p. injection (using a larger dose than that employed for ACV). The reasons for this are not yet clear since the in vitro activity of BVDU appears to be greater than that of ACV and this was confirmed to be true for the particular strain of HSV used in the present study. One possibility is that BVDU may be inactivated or eliminated more readily than ACV. However, the extremely low toxicity of BVDU for mice means that much higher dose levels can be examined. This was not possible in the present study because of the limited quantity of BVDU available.

Both drugs were found to prevent the establishment of latent infections as detected by an in vitro reactivation technique. This phenomenon has previously been described for ACV (Field et al., 1979; Park et al., 1979) and for other drugs, e.g. phosphonoacetic acid (Klein et al., 1978). It is assumed that the failure to establish latency results from reduced replication in the skin. Lower levels of virus replication in the skin resulting from smaller virus inocula in untreated mice were found to induce latency but less reproducibly (Field et al., 1979). A previous study has also shown that direct inoculation of virus into the nervous system does result in the establishment of latent infection despite continuous therapy (Field et al., 1979).

However, the effective suppression of cutaneous virus replication using either ACV or BVDU may mean that both drugs will have a role in suppression of recurrent infections in man and may reduce the subsequent colonization of further neurons during such recurrences.

Finally, oral treatment of mice by means of the drinking water enabled the effects of long-term continuous treatment on established latent infections to be investigated. Using explant culture of dorsal root ganglia to reactivate latent virus in vitro, no reduction in the latent infection was observed following treatment for up to 2 months. Similar negative results have been previously reported by ourselves (Field et al., 1979) using daily i.p. administration of ACV. These data would be consistent with the failure to demonstrate specific virus products including TK in latently infected tissue (Fong & Scriba, 1980). An earlier report described the detection of TK in mouse dorsal root ganglia using a sensitive technique (Yamamoto et al., 1977), although even in this case the levels of TK became undetectable after 60 days although the mice remained latently infected. The failure to eliminate latent virus would be consistent with our own in vitro observations on the effects of incubating explanted latently infected ganglia in the presence of inhibitory levels of ACV. These studies indicated that in vitro reactivation could be suppressed for several days in this way, but when the drug was removed reactivation occurred with normal kinetics (J. Rajcani & H. J. Field, unpublished observations). It might be argued, however, that if virus expression occurs in neurons during a recurrent infection, then the infected cell might become amenable to chemotherapy. It has been shown (Darby et al., 1980; Nishiyama & Rapp, 1979; Furman et al., 1980) that the growth of TK-transformed cells which express the HSV TK gene are inhibited by low concentrations of ACV (<1 μg/ml). However, these are dividing cells and would not necessarily reflect the situation in the neuron which harbours the latent virus, this being a non-dividing cell.

While the interactions of effective nucleoside analogues with HSV during reactivation events in vitro remain to be elucidated, the clear indications from the work described in this paper are that these drugs should only be used to suppress the active phase of primary or recurrent infections. This should not only ameliorate the disease but would be expected to reduce the establishment of latent infections in further neurons during a recurrence and the decrease in virus titres would lessen the chance of auto-inoculation or transmission. However, the attempted treatment of the infection during the latent phase appears fruitless and
widespread use of the drugs in this way may only increase the risk of developing strains of HSV which are resistant to the drugs concerned (Field & Darby, 1980).

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REFERENCES


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