Limited Efficacy of Inhibitors of Herpes Simplex Virus DNA Synthesis in Murine Models of Recrudescent Disease

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SUMMARY

The herpesvirus DNA polymerase inhibitor foscarnet, applied topically, and the anti-herpesvirus guanosine analogue buciclovir, given orally, decreased virus replication and disease development in primary skin infections of mice caused by herpes simplex virus type 1 (HSV-1). If the same tissues were infected via sensory nerves, following zosteriform spread of the virus the same treatments showed strongly decreased efficacy, or were inefficacious, when started before development of clinical signs in the infected tissues. These results were obtained in murine models of zosteriform spread of HSV-1 to the ear (following inoculation of the ventral side of the neck) or to the lower flank (following inoculation of the upper flank). In these models the immune system played a dominant role in virus clearance. The topically applied foscarnet could not prevent disease development in these models of recrudescent disease even when applied before the virus was detected in the skin, but a decrease in virus titre was obtained. Orally administered buciclovir lost efficacy when administered at the time of virus entry into the skin, i.e. 1 or 2 days before development of clinical signs. In the flank model, measuring lesion development, orally administered acyclovir also had a strongly decreased efficacy, when compared with its effect during infections in which lesion development did not involve translocation of virus through nerves. In the presence of developing immunity the inhibitors could not accelerate the clearance of virus from infected tissues. Furthermore, all treatments (topical foscarnet and oral buciclovir or acyclovir) were without effect on disease development when treatment was initiated on appearance of the first clinical signs of disease. As disease development following zosteriform spread of HSV resembles that in recurrent herpes in humans, and as the limited efficacy of the inhibitors observed resembles the poor results obtained with inhibitors of herpesvirus DNA synthesis in clinical studies on the treatment of symptomatic recurrent herpes, we suggest the use of animal models of zosteriform spread for pre-clinical evaluation of new antiherpes drugs.

INTRODUCTION

The herpesvirus DNA polymerase inhibitor foscarnet exerts significant clinical benefit in several experimental herpesvirus skin infections in animals (Öberg, 1983). The drug, applied topically, can also suppress the development of clinical signs of disease and replication of herpes simplex virus (HSV) inoculated into grafted human skin maintained on nude mice (Van Genderen et al., 1987). Indeed, foscarnet can penetrate skin to adequate levels (Helgstrand et al., 1980; Spruance et al., 1986). In a single-centre clinical study on recurrent genital herpes only a limited, but statistically significant, effect of topical 0·3% foscarnet cream on time to healing was observed in men (Wallin et al., 1985). However, in two multi-centre trials on recurrent genital herpes no statistically significant improvements in time to healing or loss of symptoms were observed with foscarnet (Barton et al., 1986; Sacks et al., 1987). Compilation of several
studies showed that patients who initiated treatment in the prevesicular stage had a slightly reduced number of days with sores (Astra Alab AB, unpublished data).

Oral acyclovir (ACV) is beneficial in the treatment of primary episodes of genital herpes or for suppressing recurrent episodes (Mertz et al., 1984; Bryson et al., 1983; Nilsen et al., 1982; Mindel et al., 1984; Douglas et al., 1984; Straus et al., 1984; Halsos et al., 1985), but shows no, or only limited, effect on relief of symptoms in recurrent genital herpes (Nilsen et al., 1982; Reichman et al., 1984). Treatment of recurrent disease with topical acyclovir has also been disappointing (Luby et al., 1984; Reichman et al., 1983; Corey et al., 1982; Spruance & Crumpacker, 1982). These results of the treatment of recurrent herpes are in contrast with the significant clinical benefits obtained with ACV in several experimental skin infection models (Kern, 1982; Alenius et al., 1982; Hsiung et al., 1984). Following an assessment of the drugs' pharmacokinetic properties, we suggested (Datema et al., 1987) that the fundamental differences between the pathogenesis of recurrent genital herpes in humans and the pathogenesis of several primary infection models in animals preclude the use of these animal models for valid prediction of clinical efficacy of viral DNA polymerase inhibitors for recurrent genital herpes. Similar considerations apply to recurrent labial herpes.

Lesion formation following zosteriform spread of HSV in animals is analogous to the formation of recrudescent lesions after reactivation of latent virus (Hill, 1985; Klein, 1985; Stanberry, 1986). Zosteriform spread of HSV infection following primary inoculation of HSV leads to lesion formation that is preceded by spread or replication, or both, of virus in the nervous system; furthermore, the adjacent skin is infected via sensory nerves, and lesions occur in the face of developing immunity (Blyth et al., 1984; Simmons & Nash, 1984; Bernstein & Stanberry, 1986). Such models have, therefore, been advocated for antiviral drug testing (Hill et al., 1984). Two murine models allow easy quantification of disease due to zosteriform spread of HSV-1: first, inoculation of the ventral surface of the neck causes zosteriform spread of the lesion to the ear pinna and swelling of the ear (Blyth et al., 1984), and second, inoculation of the dorsal flank, just lateral to the spine, causes a zosteriform rash on the lower flank (Simmons & Nash, 1984) within the infected dermatome.

Using these zosteriform models we have investigated the effects of topical foscarnet and oral administration of the antiherpes guanosine analogues ACV or buciclovir (BCV) on disease development and virus spread. Buciclovir and ACV are equally effective inhibitors of HSV-1 replication in mouse cells (Larsson et al., 1986a), have similar modes of action (Larsson et al., 1986b; Stenberg et al., 1986), and have similar efficacies in systemic i.e. generalized HSV-1 infections in mice and HSV-1 skin infections in guinea-pigs (Ericson et al., 1985). However, the compounds differ in their pharmacokinetic properties, that is, oral uptake, plasma half-lives, intracellular trapping of the antiviral triphosphates and nervous tissue penetration (Datema et al., 1987). It was therefore of interest to compare three different inhibitors of HSV DNA synthesis in two different models of zosteriform spread of HSV-1.

METHODS

Materials. The acyclic guanosine analogues BCV and ACV were synthesized at Astra Alab AB (Ericson et al., 1985). Foscarnet creams (3% or 1-5%) were prepared as previously described (Alenius et al., 1982). The cream base without drug was used as a placebo.

Inoculation of mice. For infection of the flank, 6- to 8-week-old male NMRI mice (Anticimex) were used. The right flanks were shaved, depilated and inoculated, just lateral to the spine, by scratching in with a hypodermic needle a 10 μl drop (10⁴ p.f.u.) of HSV-1 strain C42. This virus is a recent clinical isolate, and was grown and stored within the infected dermatome.

The mice were examined daily for clinical manifestations of the disease according to the following scale: −4, no symptoms; −3, one vesicle and swelling; −2, some vesicles; −1, local erosion; 0, ulceration of the local lesion; 1, primary lesion plus isolated zosteriform lesions; scores 2 to 4 describe the ulceration of confluent zosteriform lesions from mild to severe (Nagafuchi et al., 1979). Thus, scores −4 to 0 describe the lesion development at the site of inoculation (primary lesion) whereas scores 1 to 4 describe the development of the zosteriform lesion (zoster score). Groups of 10 or 15 animals were used for each experiment, and the mean score for each treatment group was determined.
Denervation of the flank was carried out as described by Simmons & Nash (1984). Ten operated and 10 mock-operated animals were infected with virus as above and viral titres in skin were determined (see below).

For infection of the neck, female BALB/c mice (16 to 18 g) were anaesthetized by intraperitoneal injection of Hypnorm–Valium, shaved and depilated on the right side of the neck. Inoculation was performed as described by Blyth et al. (1984). Scarification was carried out through a 20 μl drop (containing 2 × 10^5 p.f.u. of HSV-1 strain C42), at the right side of the ventral surface of the neck. The occurrence of blisters on the neck and pinna was examined in anaesthetized mice by using a dissecting microscope, and the ear swelling was measured using an engineer's micrometer. The number of animals used for each experiment is indicated below.

For infection of the ear, female BALB/c mice (16 to 18 g) were used. Ten μl of HSV-1 C42 (containing 10^5 p.f.u.) was injected intradermally at the centre of the back of the pinna of each ear or the left ear.

Drug treatment. Oral treatment of mice was by providing the drugs (ACV or BCV) in the drinking water (1 mg/ml) throughout the experiments, but starting at different times post-infection (p.i.) (see Results). Administration via the drinking water leads to even drug intake, and drug concentrations in plasma and certain tissues have been described (Datema et al., 1987). Topical treatments of the flank were done three times daily by putting the cream (approximately 30 mg of 3% foscarnet or placebo cream) on a segment of the skin which included the inoculation site and the whole dermatome to be infected. Treatment started at different times p.i. and continued for 5 days. Ears were treated with approximately 25 mg of cream (1-5% foscarnet or placebo) distributed equally over each side of the ear, every 8 h for a number of days, as indicated below.

Removal of tissues and the test for infectious and latent virus. The flank-skin sample from the inoculation side was 0-5 cm^2 and encompassed the whole inoculation site. The skin sample from the lower flank of the dermatome was a 0-5 cm wide strip between the ventral midline and the inoculation site, leaving out a 0-3 cm section adjacent to the inoculation site. For titration of ear skin, the whole ear ipsilateral to the inoculation site in the neck was clipped off. The pieces of skin were placed in 1 ml of Eagle’s minimal essential medium containing 10% foetal calf serum and stored at −70 °C. To test for infectious virus, skin samples were homogenized in this medium; 10-fold dilutions were tested for plaque production in SIRC cells (Ericson et al., 1985).

Thoracic ganglia (numbers 9 to 13) and the first lumbar ganglion from latently infected mice were removed and placed in 1 ml of Eagle's minimal essential medium containing 10% foetal calf serum and stored at −70 °C. To test for infectious virus, skin samples were homogenized in this medium; 10-fold dilutions were tested for plaque production in SIRC cells.

Statistical analysis. To analyse the results of the flank model, the Mann–Whitney U test was used to compare the lesion score data (the cumulative scores) and the mean day of death for the control and treated animals. Differences in virus titres were compared by using Student's t-test for groups of animals or, when possible, by using statistics on paired observations. The U test was used to analyse the effects of treatment on virus titres and ear swelling in ear- and neck-infected mice.

RESULTS

Flank model

Following infection of the flank with HSV-1 C42, vesicles rapidly developed at the inoculation site, and a lesion was seen 24 h p.i. Zosteriform spread of lesions was not observed until 4 to 5 days p.i. These lesions originated as small blisters in the vicinity of the inoculation site, spread in the dermatome to the lower flank and became confluent 6 to 7 days p.i. (Fig. 1).

High titres of infectious virus were obtained from the skin at the inoculation site from day 1 p.i. Virus isolations from the lower flank were negative until 3 days p.i., and high titres were obtained from day 4 p.i. (Fig. 1). Thus, virus replication in the lower flank of the skin preceded the development of lesions therein by approximately 1 day (Fig. 1). After sensory denervation of the flank, infectious virus could not be isolated from the lower flank on days 4, 7 or 10 p.i. (data not shown). This confirms that an intact nerve supply to the skin was required for virus spread to the lower flank.

Treatment of the whole dermatome with topical 3% foscarnet, starting 12 h p.i. and continuing for 4 days, prevented zosteriform spread of lesions (Fig. 2a) and prevented mortality, which amounted in different experiments to 50 to 90% of the untreated and placebo-treated infected animals (Table 1). Similar results were obtained when treatment was started 24 h p.i. However, treatment of the whole dermatome 48 h or 72 h p.i. and continuing for 4 days could not prevent zosteriform spread of lesions (Fig. 2a). Thus, topical treatment starting 2 or 3 days before the first clinical manifestation of zosteriform spread of HSV to the lower flank had no effect on the development of the disease due to virus entering the skin.

A continuous, systemic treatment using BCV dissolved in the drinking water (1 mg/ml) was efficacious when started 24 or 48 h after infection (Fig. 2b). However, this treatment was not
Fig. 1. Lesion development and virus titres in skin of mice infected with HSV-1 C42. Symbols: △, virus titres at inoculation site; □, virus titres in the lower flank; ○, disease development. Scores -3 to 0 describe lesion development at inoculation site and scores >0 the zoster score. Results are mean values from n = 10.

Fig. 2. Effects of antiviral drugs on the zosteriform rash in the flank model. (a) Treatments with topical 3% foscarnet, initiated 72 h p.i. (×), 24 h p.i. (●) or 12 h p.i. (△), or placebo at 48 h p.i. (○). (b) Treatments with BCV, 1 mg/ml in drinking water, initiated 96 or 72 h p.i. (×), 48 h p.i. (□), 24 h p.i. (●) or 1 h p.i. (△), or untreated (○). (c) Treatments with ACV, 1 mg/ml in drinking water, initiated 96 or 72 h p.i. (×) or 12 h p.i. (△), or untreated (○). Results are mean values from n = 10.
Table 1. Effects of inhibitors on zosteriform rash and mortality in HSV-1 C42-infected animals (flank model)

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Initiation (h p.i.)</th>
<th>Reduction† cumulative zoster score (%)</th>
<th>Mortality‡ Treated (%)</th>
<th>Mortality Not treated (%)</th>
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<tr>
<td>3% foscarnet</td>
<td>12</td>
<td>100</td>
<td>0</td>
<td>70</td>
</tr>
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<td>24</td>
<td>78</td>
<td>20</td>
<td>70</td>
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<td>48</td>
<td>0</td>
<td>100</td>
<td>70</td>
</tr>
<tr>
<td>3% foscarnet</td>
<td>72</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1 mg/ml BCV (dw)</td>
<td>12</td>
<td>100</td>
<td>0</td>
<td>86</td>
</tr>
<tr>
<td>1 mg/ml BCV</td>
<td>48§</td>
<td>83</td>
<td>0</td>
<td>73</td>
</tr>
<tr>
<td>1 mg/ml BCV</td>
<td>72</td>
<td>0</td>
<td>0</td>
<td>50</td>
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<td>57</td>
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<tr>
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<td>1 mg/ml ACV</td>
<td>96</td>
<td>0</td>
<td>10</td>
<td>85</td>
</tr>
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</table>

* See Methods; dw, drinking water.
† Relative to placebo, or untreated controls, and calculated from the lesion score and day area under the curve (Fig. 2) (Kern, 1982), taking the area between 4 and 14 days p.i.
‡ Mortality in the 3 weeks observation period.
§ In an experiment where mortality in the non-treated group was 50%, a 92% reduction in cumulative zoster score was obtained.
¶ In an experiment where mortality in the non-treated group was 50%, a 47% reduction in cumulative zoster score was obtained. The difference between 70% and 100% mortality of animals not treated or treated with 3% foscarnet (48 h p.i.) is not statistically significant.

effective in decreasing zoster formation when postponed to 72 h p.i., when the infected animals still showed no signs of disease associated with zosteriform spread of virus (Fig. 2b). The treatment starting 48 or 72 h p.i. did prevent mortality (Table 1). When treatment initiated 48 h or 72 h p.i. was withdrawn after 2 weeks, and the ganglia were taken from each mouse 7 weeks later, eight out of nine, and seven out of nine mice, respectively had latently infected ganglia, the titres (log_{10} p.f.u./ml) of reactivated virus being 4.6 ± 2.0 and 6.1 ± 0.4. No latent virus was recovered from the ganglia of mice that received BCV starting 24 h p.i. Thus, BCV prevented the spread of HSV-1 C42 to and/or in the nervous system preventing infection of ganglia, and when given 72 h p.i. it prevented mortality. However, BCV treatment started 72 h p.i. did not prevent infectious virus from reaching the skin of the lower flank, or replication of virus therein, or both, until approximately 3 days after the initiation of treatment (Fig. 3). The prophylactic administration of 3% topical foscarnet starting 72 h p.i. to the whole dermatome of infected mice treated with oral BCV decreased virus titres in the skin (Fig. 3), but did not prevent development of disease (data not shown).

Results similar to those with BCV were obtained with ACV supplied in the drinking water (1 mg/ml), as shown in Fig. 2(c). When the first signs of disease were manifest in 50 to 60% of the infected animals (approx. 98 h p.i.) neither oral BCV not oral ACV could prevent the further development of the disease (Fig. 2b, c). However, both regimens did prevent mortality (Table 1).

Ear model

Two or 3 days after inoculation of the skin of the ventral side of the neck with HSV-1 blisters developed at the inoculation site. A zosteriform spread of blisters from the neck to the ear occurred between 5 and 6 days p.i. An increase in the thickness of the ipsilateral (right) ear was observed from 5 to 6 days p.i., whereas high virus titres in the ear were obtained from day 4 p.i. (Fig. 4). Thus, virus replication in the ear preceded development of clinical signs by 2 days. Death occurred in less than 5% of the infected animals during the observation period of 3 weeks.
Starting 4 days p.i., neck-infected mice were provided with drinking water containing BCV (1 mg/ml) or were treated with foscarnet cream (1.5%) applied topically on both sides of the ear every 8 h. Treatment was for 4 days. Control mice were infected and treated with placebo cream. Despite initiation of treatment before development of clinical signs, administration of BCV or foscarnet did not prevent development of clinical disease (ear swelling and lesion development on the ear, see Fig. 5b, c). BCV treatment did not affect virus titres either, but foscarnet treatment prevented virus titres in the ear reaching the values of the placebo-treated control animals on days 5, 6 and 7 p.i. (P < 0.01), as shown in Fig. 5(a).

The lack of effect of BCV is surprising since the concentration of the drug in the ear (2.2 μmol/kg tissue) is sufficient to decrease virus replication in, for example, lungs of intranasally infected mice (Datema et al., 1987). Therefore, mice were inoculated with HSV-1 directly into the pinna of the ear (ear-infected animals), and given BCV (1 mg/ml) in the drinking water. Treatment was started 12 h after inoculation, so that animals were asymptomatic and had high virus titres in the ear (Fig. 5d, e), and continued for 5 days. It is shown in Fig. 5(d, e) that in this experiment BCV decreased virus titres and, presumably as a consequence, ear swelling. It can be seen in Fig. 5(a, d) that in animals with a developing immunity (Fig. 5a), the rate of virus elimination was higher than in non-immune animals (Fig. 5b).
Fig. 5. Effects of foscarnet and BCV on HSV-1 infections in the ear of BABL/c mice. (a to c) Spread from the neck (n = 20); (d and e) virus injected into both ears of previously uninfected mice (n = 5). Treatments: ○, placebo cream; △, topical foscarnet (1.5%); □, BCV, 1 mg/ml in drinking water.
5 d). Furthermore, the inhibitors scarcely, if at all, increased the rate of virus elimination in animals with developing immunity (Fig. 5 a), and an effect of drugs on virus titres was most clearly seen in the ears of non-immune animals (Fig. 5 d). The application of topical foscarnet to ears of ear-infected mice from 12 h to 5 days p.i. decreased viral titres, but did not decrease ear thickness in these non-immune animals (Fig. 5 d, e). This is caused by an inflammatory response to topical foscarnet in infected tissues and is not seen in foscarnet-treated non-infected ears (A. Kristofferson et al., unpublished data).

**DISCUSSION**

Previous results from several laboratories, including ours, have shown a good efficacy of ACV, foscarnet or BCV, inhibitors of HSV DNA synthesis, in the control of primary skin infections of HSV in several experimental animal models (see for example Kern, 1982; Alenius et al., 1982; Ericson et al., 1985). In this study, we have shown that treatment of the initial infections gave beneficial results: decreased viral replication and spread and decreased development of disease. These beneficial effects were obtained despite high initial viral titres and some progression of disease development. Again, this has been described earlier; see for example Kern (1982), Alenius et al. (1982) and Ericson et al. (1985). However, when the same tissues were infected via sensory nerves, in zosteriform spread models, the efficacy of the same treatments was markedly reduced. This limited efficacy, or even lack of effect, was observed when treatment was started before clinical signs appeared in the tissues to be infected.

In the flank model of zosteriform spread such prophylactic treatment (from 48 h p.i.) with topical foscarnet of the dermatome to be infected, was not effective even before the appearance of virus in the lower flank (approximately 70 h p.i.). The same treatment of the initial infection did not result in disease development, and it prevented death. This indicates that the drug can penetrate skin as also shown earlier (Helgstrand et al., 1980; Spruance et al., 1986). Oral treatments with the viral DNA synthesis inhibitors ACV or BCV were still efficacious in preventing zoster development when starting 48 h p.i., but a marked reduction of efficacy was obtained when treatment was initiated 72 h p.i. when no zoster development had occurred but virus had reached the lower flank. These treatments could not prevent the virus in the flank from reaching high titres. This was also observed in the ear model of zosteriform spread. Again, as shown in the experiments where virus infected the pinna of the ear, oral treatment with BCV reduced virus replication when virus was inoculated directly into the ear, but not when the virus infected the tissue via sensory nerves. This indicates that limited availability of BCV to the skin need not be the cause of poor efficacy in zosteriform spread models. This is supported by a study of BCV's pharmacokinetic properties (Datema et al., 1987).

It is therefore possible that there is a phase of virus replication in zosteriform spread models, which is poorly accessible to oral BCV or ACV, and/or occurs before initiation of treatment and symptoms. It is unlikely that this replication occurs in the skin as only low virus titres occur there at the initiation of treatment, and because treatment of skin with foscarnet before infection is not effective. This replication phase is possibly recognized by the immune system, but the action of protective immunity is too slow and peripheral skin damage (disease) develops even when a certain reduction of virus titres can be obtained (Fig. 3, 5).

Circumstantial evidence suggests that replication may occur in nervous tissues. Thus, in an experiment (footnote of Table 1) using treatment with 1 mg of ACV per ml of drinking water, initiated 72 h p.i., some benefit was obtained whereas in the same experiment BCV at the same dose was inefficacious. As analysed elsewhere (Datema et al., 1987), serum concentrations and nervous tissue concentrations of ACV were higher than those of BCV due to a higher oral bioavailability and better nervous tissue penetration of ACV than of BCV. Indeed, when BCV was given subcutaneously in high doses (2 × 400 mg/kg/day) a beneficial effect against zoster development was obtained if treatment was started 72 h p.i. (Lundgren et al., 1986; data not shown). Nevertheless, in these experiments a maximum of 50% reduction in zoster score was obtained, and therefore this treatment was less efficacious than treatment started 48 h p.i., when virus had already reached the sensory ganglia but not yet the skin of the lower flank (see Simmons & Nash, 1984). However, when either treatment (1 mg/ml ACV in drinking water or
800 mg/kg/day BCV subcutaneously) was postponed until the first clinical signs had developed in the flank, these treatments failed to show effect. Therefore, in models of zosteriform spread of HSV, the inhibitors used were much less effective in preventing virus replication and disease development than in models of primary, acute skin disease, where translocation of virus through the peripheral nervous system is not required for involvement of contiguous skin.

In fact, the results obtained here with the zosteriform spread models are reminiscent of the results of clinical studies on treatment of recurrent herpes infections in man with inhibitors of viral DNA synthesis. That is, there was little or no clinical effect despite reductions in the time of virus shedding obtained with topically applied inhibitors (foscarnet, acyclovir), a limited effect of oral ACV in treatment of symptomatic disease or an improved effect in 'suppressive' (i.e. prophylactic) treatment with oral ACV (for references see Introduction). This similarity underlines the analogy in the mechanism of disease development in recurrent herpes and in zosteriform spread models and suggests that inhibitors of herpesvirus DNA synthesis may not be of significant benefit in treatment of symptomatic recurrent herpes infections. However, immunity plays an important role in virus clearance here (Fig. 5) and in humans (Hill, 1985).

Thus, the effects of inhibitors of HSV replication in murine models of zosteriform spread need to be investigated in the presence of a fully developed cellular immune response to the HSV-1 strain used. This work is in progress.

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REFERENCES


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