Antiviral Research, 5 (1985) 157–168
Elsevier

AVR 00164

Chemotherapy of Aujeszky's disease (pseudorabies) in the mouse by means of nucleoside analogues: bromovinyldeoxyuridine, acyclovir, and dihydroxypropoxymethylguanine

H.J. Field*

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

(Received 16 July 1984; accepted 4 October 1984)

Summary

Pseudorabies virus (PRV) infection was established in mice by means of inoculating the ear flap. The infection was universally fatal once clinical signs appeared. Bromovinyldeoxyuridine (BVDU) was a potent inhibitor of PRV in vitro, but this drug failed to protect mice and produced only marginal reductions in virus titre and slight prolongation of survival. Acyclovir (ACV) and dihydroxypropoxymethylguanine (DHPG) were both less active than BVDU when tested against the virus in BHK cells, yet DHPG therapy was extremely effective in mice; it reduced virus titres markedly and resulted in the long-term survival of mice given a potentially lethal infection. When ACV and DHPG were tested in vitro using murine rather than hamster cells, these compounds, especially DHPG, were shown to be much more active against PRV.

pseudorabies; Aujeszky's disease; acyclovir; bromovinyldeoxyuridine; dihydroxypropoxymethylguanine; chemotherapy; herpes

Introduction

Pseudorabies virus (PRV) is a natural herpes virus infection of pigs. It has received increasing attention as a disease of considerable economic importance. Some pro-

* *Present address*: Department of Clinical Veterinary Medicine, University of Cambridge, U.K.

gress has been made with the development of vaccines [20,28] but so far there has been relatively little interest in the possibility of effective chemotherapy.

PRV is analogous to herpes simplex virus (HSV) in man in that virus becomes established in a latent form within peripheral nerve ganglia from which it has been reactivated by explant techniques [16,24]. The virus may be transmitted to a range of other domestic animals and rodents in which it produces an extremely neuropathogenic and usually fatal infection. Rodents are a particularly useful model for studying the pathogenesis of PRV and in previous papers [13,14] the progression of the infection in mice inoculated in the footpad with the N1A-2 strain of PRV is described in detail. The disease progressed rapidly and was universally fatal. Virus was found to spread through the peripheral nervous system to the CNS via the dorsal root ganglia. Late in the infection virus was present in skin proximal to the inoculation site, probably as a result of centrifugal spread of virus in peripheral nerves. There was no evidence of hematogenous spread and no non-neural organs were affected except the kidney which appeared to be infected via the adrenal gland. In the present study a similar model was employed except that virus was inoculated by means of the ear flap. This had been found recently to produce a useful murine model for the study of potential chemotherapeutic agents against HSV in mice [10].

Similar to other neurotropic herpes viruses, PRV induces a virus-specific pyrimidine deoxynucleoside kinase (thymidine kinase or TK) in the cells which it infects [17]. This enzyme has the potential to phosphorylate nucleoside analogues and thus accumulate antiviral nucleotides within the infected cells. The TK of PRV has been much less studied than the counterpart enzyme expressed by HSV but some evidence suggests that the former is less 'promiscuous' having a narrower range of substrate specificity. For example, under normal conditions, the enzyme does not seem to phosphorylate deoxycytidine [4] (Field, unpublished observations) and also PRV has been found to be relatively resistant to ACV, although the virus was sensitive to this drug when ACV was tested in transformed cells which themselves expressed an HSV TK [2]. However, several of the new nucleoside analogues have been reported to be active against PRV in normal cell cultures, particularly BVDU [3,4,22]. In the present study the mouse model revealed that this compound was poorly effective in treating the disease but the relatively new nucleoside analogue, dihydroxypropoxymethylguanine (DHPG), while much less active in vitro in BHK cells, was very effective in mice and could protect them from a potentially lethal infection. However, results obtained in vitro using murine cells indicated that DHPG may be particularly active in mice. These data suggest that much care is needed when extrapolating from animal models for chemotherapy of herpes virus infections to the situation in the natural host.

Methods

Viruses and mouse inoculation

The strain of PRV used was 'N1A-2' originally isolated from pig brain by Dr. J.B. McFerran, Belfast. The virus had been passaged twice in pig kidney cells, twice in

Vero cells and subsequently, twice in BHK-21 cells to produce a working stock. A TK-defective variant was isolated by passaging the virus in the presence of 0.01 μg/ml BVDU; the yield virus was plaque-purified and then shown to be TK-defective by an enzyme assay similar to that used to estimate the TK activity of HSV [18].

The pathogenicity of PRV strain N1A-2 for mice inoculated in the footpad is described in detail in earlier papers [13,14]. In the present study 3-week old female BALB/c mice were inoculated via the ear flap. Virus was suspended in Glasgow-modified Eagle's minimal essential medium (MEM) and inoculated in a volume of 0.2 ml into the skin of the left pinna. The LD_{50} dose by this means of inoculation was approximately 10^3 p.f.u./mouse. Pruritis (intense irritation of the skin) was the first and most obvious clinical sign and once this appeared it led eventually to death in *all* cases; the mice scratching their ears with increasing intensity until they became comatose and died within 24–48 h of onset. In all subsequent experiments, when severe pruritis developed the mice were killed to prevent unnecessary suffering in the experimental animals. These mice were scored as if they had died 24 h later.

Virus titrations

Tissue specimens were stored dry at -70°C. For measuring the amount of infectious virus present, the specimens were minced with scissors, then ground in MEM in small conical glass grinders. BHK cells were added to dilutions of the tissue homogenates and allowed to form monolayers in 5 cm diameter plastic Petri dishes. The cultures were incubated at 37°C for 2 days, then formalin-fixed, stained with 0.1% toluidine blue and the plaques counted. For the attempted reactivation of latent virus from peripheral nervous tissue, whole dorsal root ganglia (the 2nd, 3rd and 4th cervical) were incubated in MEM containing 1% bovine serum for 5 or 6 days, then homogenized and tested for the presence of infectious virus as above. This method had been found previously to be effective for reactivation of latent HSV from cervical dorsal root ganglia subsequent to ear inoculation [10].

Antiviral compounds and plaque reduction assay

Acyclovir, 9-(2-hydroxyethoxymethyl)guanine (ACV), and 9-1,3-dihydroxypropoxymethylguanine (DHPG) (also known as 2'-nor-2'-deoxyguanosine, or BW759) were supplied by Dr. P. Collins, The Wellcome Foundation, Beckenham, Kent. *E*-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) was a gift from Dr. E. De Clercq, Rega Institute, Leuven, Belgium.

The compounds were dissolved in water, this being facilitated by brief ultra-sonic vibration and warming to 50°C. The drugs were administered by i.p. inoculation using volumes of between 0.1 and 0.5 ml/dose. BVDU was also given in the drinking water at a concentration of 1 mg/ml. Mice showed a distaste for BVDU which was overcome by adding a small quantity (5%) of blackcurrant juice. The average intake of BVDU by means of the drinking water was approximately 150 mg/kg per day.

To test the antiviral activity of the compounds in vitro, approximately 100 p.f.u. of PRV were used to infect pre-formed monolayer cultures of BHK cells or primary mouse embryo fibroblasts (at the 3rd or 4th passage). These cells were obtained from embryos from the same mouse stock that were used for the in vivo chemotherapy

experiments. The methods for preparing the murine cell monolayers have been described in detail previously [11]. Following adsorption of the virus onto the monolayers for 1 h the cells were overlaid with MEM containing various concentrations of the test drug. The medium was also supplemented with 2% bovine serum (fetal calf serum for the mouse cells) and carboxymethylcellulose was added to thicken the medium and prevent the formation of secondary plaques. The number of plaques was plotted as (%) the number without drug versus $\log_{10}$ (drug concentration). That concentration of drug giving a 50% reduction in plaques (ED_{50}) was obtained directly from the graph.

Results

Relative activities three nucleoside analogues in vitro

The three nucleotides BVDU, ACV and DHPG were compared in BHK cells by the method of inhibition of plaque reduction. BVDU was the most active (Table 1) giving an ED_{50} value of 0.05 μg/ml (≈ 0.2 μM). ACV and DHPG were much less active yielding values of 32 and 20 μg/ml, respectively. However, when the activities of the same compounds were compared in primary mouse embryo fibroblasts, ACV and DHPG appeared to be markedly more effective (ED_{50} values down to 6 and 1.5 μg/ml, respectively). It was notable that a BVDU-selected, TK-defective variant of PRV was also relatively sensitive, particularly to DHPG, when tested in mouse embryo cells (Table 1).

Effects of BVDU on PRV-infected mice

Forty mice were inoculated with 10^3 p.f.u. PRV in the skin of the left ear flap. Half the mice were left untreated, and half were supplied with drinking water containing 1 mg/ml BVDU from the time of virus inoculation. In each group 10 mice were kept under observation and 10 were used to provide tissue samples at intervals after inoculation. Clinical signs appeared in about half the mice. In control, untreated, mice pruritis began to develop first in the inoculated ear on the 4th day after

TABLE 1

The activity of PRV inhibitors measured by plaque-reduction in tissue culture

Nucleoside analogue	Virus	ED_{50} (μg/ml)	
		BHK cells	Mouse embryo fibroblasts mean (range)
BVDU	PRV	0.05	0.03
ACV	PRV	32	6.0 (4.2–9.4)
DHPG	PRV	20	1.5 (0.3–3.2)
ACV	PRVTK⁻	>50	7.3 (1.9–15.8)
DHPG	PRVTK⁻	>50	2.8 (1.3–4.5)

inoculation. BVDU-treated mice showed a delay in the onset of disease, by approximately 1 day (Fig. 1A). Ear tissue samples were obtained by removing the pinnae from mice on days 1, 3 and 5 post-infection (p.i.). The results (Table 2) showed a small but consistent reduction in virus present in the ear tissue, both in the proportion of mice from which virus was isolated and in the virus titres in the positive samples.

A similar experiment was carried out using a 5-fold higher virus inoculum to give increased mortality. Surprisingly, the onset of disease was slightly slower, despite the increased inoculum, but as expected a greater proportion of untreated mice eventually developed pruritis. Again a delay in the appearance of pruritis was observed in BVDU-treated mice and a lower proportion of mice became ill (Fig. 1B). However, results of extensive virus titration on a range of neural tissues at various times after inoculation failed to show a significant reduction in virus titres (Table 3) in the samples which yielded virus.

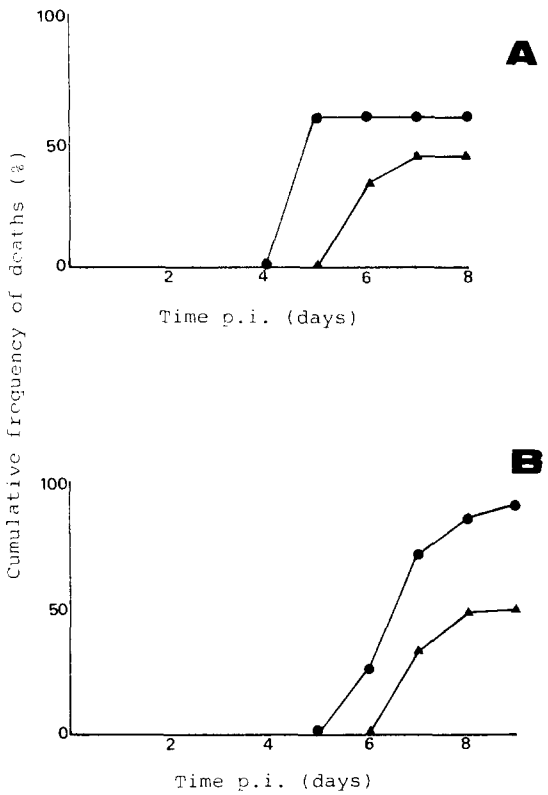

Fig. 1. The cumulative frequency of death in mice infected with pseudorabies and treated with BVDU. (A) Virus dose 10^3 p.f.u./mouse into the left ear pinna. BVDU therapy by means of the drinking water from the time of virus inoculation. ●, Untreated; ▲, BVDU (1 mg/ml) in the drinking water. (B) Virus dose 5×10^3 p.f.u./mouse, therapy as for (A).

TABLE 2

Virus titres in ears of mice inoculated with 10^3 p.f.u. PRV with or without BVDU therapy

Days post-infection	Therapy[a]	Virus present in ear tissue[b]	
		Proportion of mice from which virus was isolated from the ear	Geometric mean titre (range)
1	Untreated	3/3	1.1 (0.6–1.9)
	BVDU	2/3	0.0 (0.0)
3	Untreated	3/3	2.5 (2.3–2.6)
	BVDU	2/3	1.2 (0.2–2.3)
5	Untreated	2/2	0.3 (0.0–0.5)
	BVDU	1/3	0.3 (0.3)

[a] Therapy, BVDU at 1 mg/ml in the drinking water from the time of virus inoculation.
[b] Virus titres are $\log_{10}$ p.f.u./sample.

TABLE 3

Virus titres in tissues of mice inoculated with 5×10^3 p.f.u. with and without BVDU therapy

Days post-infection	Therapy[a]	Virus in tissue[b]			
		Proportion samples positive, geometric mean titre (range)			
		Ear	d.r.g.[c]	Spinal cord	Brain
3	Control	3/3, 4.0 (3.7–4.6)	2/3, 5.0 (5.0–5.0)	2/3, 2.7 (2.5–2.8)	2/3, 0.8 (0.0–1.5)
	BVDU	3/3, 4.4 (4.0–4.8)	3/3, 1.3 (0.5–2.0)	2/3, 2.6 (2.5–2.6)	2/3, 1.3 (1.0–1.5)
4	Control	4/4, 4.0 (3.3–4.5)	2/3, 1.7 (0.5–2.9)	2/3, 4.3 (3.5–5.0)	2/4, 3.9 (3.8–4.0)
	BVDU	4/4, 3.8 (3.5–4.0)	1/3, 2.0 (2.0)	3/3, 1.9 (0.7–2.6)	3/4, 3.2 (3.0–3.5)

[a] Therapy, BVDU 1 mg/ml in the drinking water from the time of virus inoculation.
[b] Virus titres are $\log_{10}$ p.f.u./tissue sample.
[c] d.r.g. are left 2nd, 3rd and 4th cervical ganglia combined for each mouse.

Since mice suffering from a neurological infection are likely to reduce their intake of drinking water, a further experiment was carried out in which the drinking water therapy was supplemented with i.p. injections (50 mg/kg per day given twice daily) in order to insure a more continuous supply of drug to the mice. Combined therapy (i.p. and by drinking water) was commenced 1 day before virus inoculation to ensure maximum advantage to the drug. The results (Table 4) for virus levels in the ears, cervical dorsal root ganglia, spinal cord and brain measured 4 days after virus

TABLE 4

Virus titres in the tissues of mice inoculated with 10^4 p.f.u. PRV, with or without BVDU therapy

Therapy[a]	Virus in tissue[b]		
	Ear	d.r.g.[c]	Spinal cord
Untreated	4/4, 4.0 (3.4–4.4)	2/4, 3.9 (3.5–4.2)	2/4, 4.5 (4.2–4.7)
BVDU	4/4, 3.8 (3.5–4.0)	4/4, 3.5 (2.0–4.2)	4/4, 3.4 (1.7– 4.5)

[a] Therapy, BVDU in the drinking water (1 mg/ml) combined with BVDU i.p. (100 mg/kg) in 2 doses daily starting from day 1 before virus inoculation.

[b] Virus in tissue is $\log_{10}$ p.f.u./sample 4 days after inoculation, for each tissue the values are proportion of samples which yielded virus, geometric mean virus titre in p.f.u./sample and (range).

[c] d.r.g. are 2nd, 3rd, and 4th cervical ganglia combined.

inoculation again failed to show any consistent reduction in virus in BVDU-treated mice compared with controls.

Effects of ACV and DHPG on PRV in the mouse

Mice were inoculated with 10^4 p.f.u. PRV into the skin of the left ear in the usual way. ACV and DHPG were each tested at a dose of 100 mg/kg per day given by i.p. injection twice daily starting from the time of virus inoculation. This regimen had been found earlier to be an extremely effective method for chemotherapy of HSV in mice [9]. Survival was scored and virus titres were measured in ear tissue 3 days after virus inoculation. With both the acyclic nucleosides there was a marked reduction in virus titre in all mice receiving chemotherapy (Table 5, experiment 1). Groups of 10 mice were observed for clinical signs; among these no control mice survived beyond the 4th day and the onset of disease was delayed only slightly in the ACV-treated mice (Fig. 2A); however, the majority of the DHPG-treated mice survived till the 5th day when therapy was terminated.

TABLE 5

Effect of ACV or DHPG on mice infected with 10^4 p.f.u. PRV in the ear pinna

Expt. No.	Therapy[a]	Virus in ear tissue[b] (proportion positive, geometric mean titre (range))
1	Untreated	4/4, 4.6 (3.9–2.8)
	ACV	3/3, 2.8 (2.7–2.8)
	DHPG	4/5, 1.9 (0.0–3.6)
2	Untreated	4/4, 5.5 (5.5–5.6)
	DHPG	4/4, 1.8 (1.5–2.1)

[a] Therapy is i.p. inoculation, in 2 daily doses totalling 100 mg/kg per day from the time of virus inoculation.

[b] Virus titre is p.f.u./sample 3 days p.i.

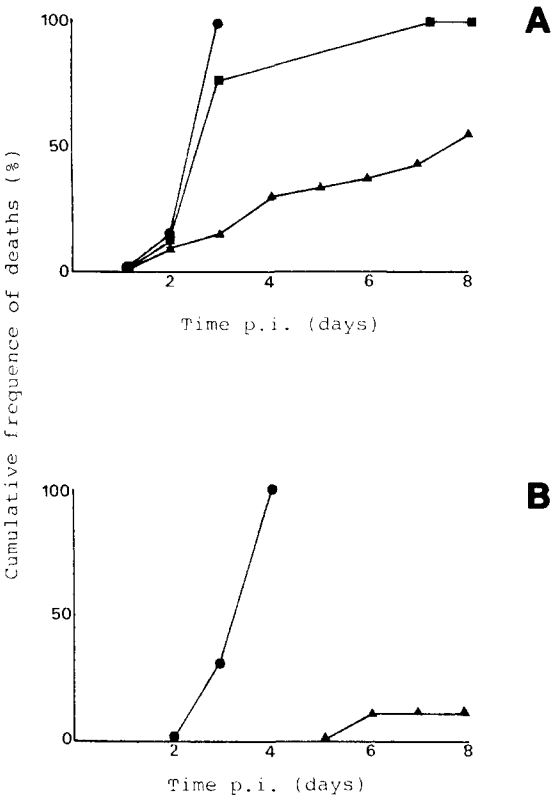

Fig. 2. The cumulative frequency of death in mice inoculated with pseudorabies and treated with ACV or DHPG. (A) Virus dose 10^4 p.f.u./mouse into the left ear pinna. Nucleoside therapy by means of i.p. administration from time of virus inoculation. (See Table 5, experiment 1.) ●, Untreated; ■, ACV (100 mg/kg per day) given twice daily; ▲, DHPG (100 mg/kg per day) given twice daily. (B) Virus dose and therapy as for (A). (See Table 5, Expt. 2.) ●, Untreated; ▲, DHPG (100 mg/kg per day) given twice daily.

The above experiment was repeated (omitting the ACV-treated group) and chemotherapy was continued for 7 days. Similar protection was observed in the DHPG-treated group in which only 1/10 mice developed the disease (Fig. 2b). The 9 remaining mice were all long-term survivors. All 10 untreated mice developed severe pruritis but in mice set aside for virus estimation the titres in ear tissue were about 3 $\log_{10}$ lower in the treated mice compared with controls (Table 5, Expt. 2).

Several weeks later the surviving DHPG-treated mice were killed. It was noted that all these mice had greatly enlarged cervical draining lymph nodes which was thought to be indicative of a recent active virus infection. The contralateral lymph nodes were overtly normal. The cervical dorsal root ganglia were explanted and cultured in vitro in an attempt to reactivate latent virus. Similar methods had previously been used successfully to reactivate HSV from ear flap-inoculated mice. However, none of the ganglia explanted from the 9 PRV-inoculated mice yielded virus by these methods.

Discussion

The results of these studies emphasize the general point that there is much difficulty in using the results from in vitro measurements of antiviral activity in order to predict efficacy in vivo. Discrepancy is most likely to occur when cells are obtained from a different species from the animal model which itself may not be the natural host for the virus. Thus, the high in vitro activity of BVDU against simian varicella virus measured in African green monkey cells was reported to correlate well with the good activity observed in monkeys when the compound was tested against the same infection in vivo [25]. Confirming the published results of Reefschläger et al. [22] and De Clercq [3], BVDU was found to be an extremely active inhibitor of PRV in vitro; measured in the present study by the plaque reduction test in both hamster and murine cells, however, the compound was poorly effective in the mouse model for PRV infection. Disappointing results with BVDU have been previously observed in a similar mouse infection model for HSV. While the compound was quite effective in reducing a skin infection [12,15] it was not effective in controlling encephalitis following an intranasal inoculation [9]. (It should be mentioned that others have reported good activity of BVDU against HSV encephalitis in mice [5,21]; one factor may be the use of higher doses by these workers.) There may be several reasons underlying the discrepancy between results obtained with BVDU in tissue culture and in mice. First, the compound may not readily pass the blood–brain barrier [6]; the presence of inhibitory concentrations of the drug within the nervous system must be essential for the control of a highly neurotropic infection such as PRV. Second, the action of BVDU is readily reversed by thymidine (and its triphosphate) which compete with the analogue for the virus-induced TK (and DNA-polymerase [19]). As the disease progresses, mononuclear cells rapidly migrate into the infected tissue and may secrete thymidine into the focus of infected cells [26] thus reducing the effectiveness of the drug. Finally, and most important, BVDU is readily cleaved by pyrimidine nucleoside phosphorylases to bromovinyluracil [7] (although BVDU can be generated again from bromovinyluracil even in vivo [8]). Clearly, the particular species of animal in which the drug is being tested will have a great effect on the relative importance of these factors mentioned above. Indeed preliminary data on the efficacy of BVDU in the therapy of PRV in its natural host, the pig, has been quite encouraging. For example, oral BVDU treatment at 25 mg/kg per day for 5 days starting at 1 day after infection was reported to reduce mortality, reduce fever, and lessen the decrease in body weight caused by the disease (P. Bironf and E. De Clercq, personal communication, 1981).

In contrast to BVDU, both acyclic nucleosides were relatively effective in mice although, in BHK cells, their activity appeared to be below that expected to produce a therapeutic effect. The explanation in these cases seems to be that both compounds were markedly more effective inhibitors of PRV when tested in murine cells. A similar enhanced activity of DHPG in mouse embryo fibroblasts has also been noted against HSV (H.J. Field, to be published) and against HSV in murine cell lines [1]. The most likely explanation for this is that murine cellular kinases are better able to phosphorylate both analogues, especially DHPG. This idea was further supported by the observation that a TK-defective variant of PRV was fairly susceptible and this would

be explained if high levels of nucleoside analogue triphosphate were achieved in the infected cells as a result of action by host cellular enzymes. However, it was notable that during the experimental work no obvious signs of toxicity such as weight loss were observed in the drug-treated animals following up to 10 days of continuous therapy.

The use of DHPG produced long-term survival in mice given a potentially lethal dose of PRV by means of skin inoculation. Attempts to reactivate virus from the ganglia of such survivors were not successful. Furthermore, PRV could not be reactivated from cervical ganglia explanted from untreated or treated survivors of a sublethal infection (Fig. 1). In the natural infection PRV has been shown to establish a latent infection in porcine spinal ganglia [16,24]; whether or not such a latent relationship between the virus and murine neurons can be established is an interesting question and if so, this could be an extremely useful model for the study of PRV latency mechanisms. It may be that in the present study virus multiplication was reduced in the skin such that minimal spread of infection to the ganglia occurred and foci of latently infected cells were not established. Similar results were obtained in HSV-infected mice when ACV or BVDU therapy was commenced within 24 h of virus inoculation [10,12]. Alternatively, in the present case it may be that latency is established but the techniques employed for reactivation were inappropriate. It is notable that Tenser et al. [27] also failed to reactivate PRV from the spinal ganglia of mice inoculated with sublethal doses of the virus. The use in the future of molecular probes to detect the presence of residual PRV DNA, for example, by means of Southern blot analysis of digested ganglia DNA and hybridization to a suitable PRV probe, may indicate that virus does remain and that further development of reactivation techniques would be worthwhile.

The results of this study in mice confirm and extend the data reported previously by Rollinson and White [23] and suggest that DHPG may have useful potential for the treatment of natural PRV. Even in mice, though, the disease was difficult to control and, despite the application of relatively large doses of drug at regular intervals from early in the infection, some mice succumbed to the disease. However, it is unlikely that the optimal dosing schedule was used in this study since earlier work on the therapy of mouse encephalitis showed that 6-hourly dosing was superior to administering the same total dose twice daily [9]; this would be consistent with the relatively short half life of the nucleoside analogues in tissue. For the reasons discussed above, great caution must be exercised before extrapolating from results obtained in experimental models to the natural infection in different species, but these results encourage the view that intervention in 'veterinary herpesvirus' infections will be possible using appropriate nucleoside analogues. This may be useful, at least for controlling neurotropic herpes infections in valuable animals such as breeding stock.

Acknowledgements

I wish to thank Mrs Elizabeth Lay for her expert technical help. This work was supported, in part, by a Program Grant from the Medical Research Council of Great Britain.

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