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The acyclic nucleoside analogue penciclovir is a potent inhibitor of equine herpesvirus type 1 (EHV-1) in tissue culture and in a murine model

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Summary

Equine herpesvirus type 1 (EHV-1) was sensitive to the nucleoside analogue penciclovir (PCV) when tested in tissue culture; the ED_{50} was 1.6 μg/ml. Drug-resistant mutants were selected which were found to be TK-defective and approx. 45-fold less sensitive to PCV compared with the parental strain. PCV was compared with the phosphonyl derivative, HPMPA in mice infected with EHV-1. Both drugs were shown to be effective in vivo, limiting wild-type virus replication in respiratory tissues, and reducing viraemia. The treated mice also showed less clinical signs and reduced histopathology compared with placebo-treated controls. The establishment of latent EHV-1 in the mice, however, was not prevented. The results obtained with mice suggest that antiviral chemotherapy may be practical in the horse and that this possibility is worthy of further investigation in the natural host.

Equine herpesvirus type 1; Resistant mutant; Penciclovir; Nucleoside analog; Pyrophosphate analog; Animal model

Introduction

Equine herpesvirus type 1 (EHV-1) is a major cause of abortion and respiratory disease in horses (Allen and Bryans, 1986), and thus results in considerable economic losses to the thoroughbred industry. The virus is also

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associated with perinatal mortality in foals (Hartley and Dixon, 1979) and neurological disorders (Campbell and Studdert, 1983; Edington et al., 1985). Like other herpesviruses, EHV-1 establishes latent infections and recurrent shedding of virus from asymptomatic carriers may contribute to the spread of the infection in the equine population (Burrows and Goodridge, 1984; Edington et al., 1985).

Natural immunity to infection by EHV-1 appears to be incomplete or short-lived and horses can become reinfected at intervals throughout their lifetimes (Allen and Bryans, 1986; von Steinhagen, 1988). Although vaccines have been developed, they do not provide an adequate spectrum or duration of protection, and require repeated administration (Allen and Bryans, 1986; Burrows et al., 1984). Thus, the availability of chemotherapeutic agents effective against EHV-1 would be of value in the treatment of the disease, as well as in preventing infection or reducing virus shedding in infected animals.

Recently, we described a murine model for EHV-1 infection which mimics several features of the equine diseases including the production of clinical signs, the restriction of primary virus replication to the respiratory tract and the subsequent cell-associated viraemia during the acute disease (Awan et al., 1990). We also demonstrated that infection of pregnant mice resulted in the premature birth of dead or abnormal offspring (Awan et al., 1991). This model, therefore, facilitates the investigation of different aspects of EHV-1 host responses, and has been used successfully to study the antiviral effect of phosphonylmethoxyalkyl derivatives of adenine against EHV-1 infection (Field and Awan, 1990; Field et al., 1991).

The drug penciclovir, 9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine, has been shown to have antiherpes activity in tissue culture (Boyd et al., 1987) and in animals (Boyd et al., 1988). This paper describes the antiviral activity of penciclovir against EHV-1 in cell cultures and in the murine model for EHV-1.

Materials and Methods

Viruses and cell culture

The EHV-1 (strain AB4) was a gift from Dr. Neil Edington, Royal Veterinary College, London, UK. The penciclovir resistant mutants (PR1, PR2 and PR3) derived from AB4 strain, were selected as described below. The HPMPA resistant mutant (HR3) was selected as described previously (Field et al., 1991). The rabbit kidney cell line (RK-13) was used for preparation of virus stocks, for ED_{50} titration, for selection of the penciclovir-resistant mutants and for virus isolation from the murine tissues.

Cells were cultured in Eagle's minimal essential medium (EMEM) supplemented with 10% new-born calf serum. The thymidine kinase (TK) defective cell line M143(TK^-) from Upjohn, USA, was supplied by Dr. W.P.H. Duffus and these cells were used to determine TK activity of the viruses.

Antiviral agents and determination of antiviral activity

Penciclovir (PCV), 9-(4-hydroxy-3-hydroxymethylbut-1-yl) guanine (Boyd et al., 1987, 1988) was a gift from Mr. M. Boyd, SmithKline Beecham Pharmaceuticals, Great Burgh, Epsom, Surrey, UK. (*S*)-9-(3-(hydroxy-2-phosphonylmethoxypropyl)) adenine (HPMPA) (De Clercq et al., 1986) was a gift from Dr. E. De Clercq, Rega Institute, Leuven, Belgium.

The antiviral activity of PCV was determined by plaque-reduction assay as described previously (Field and Awan, 1990). Briefly, approx. 100 PFU of virus were inoculated onto preformed monolayers of RK-13 cells in multiwell dishes. After 1 h adsorption, medium containing various concentrations of PCV was added and incubation continued at 37°C for three days. The monolayers were then fixed, stained and virus plaques enumerated. The percent reduction in plaques in the presence of drug was plotted against the $\log_{10}$ drug concentration, and the 50% effective value was determined directly from the curve. The assays were carried out at least 3 times for each drug.

Selection of resistant mutant

RK-13 cells were inoculated with EHV-1 (AB4) at a multiplicity of infection of 1 PFU/cell in the presence of 10 μg/ml of PCV. After three days, single plaques were aspirated from the infected monolayers and inoculated onto RK-13 cells in the absence of drug. The single plaque isolation was repeated twice in the absence of drug. The latter virus preparation was then inoculated into RK-13 cells in the presence of 25 μg/ml of PCV. Mutants were selected after three further rounds of single plaque purification in the absence of drug. Three EHV-1 mutants were isolated independently, and were termed PR1, PR2 and PR3. Working stocks of these mutants were prepared in RK-13 cells. Mutants were confirmed to be EHV-1 by a virus neutralization test using an EHV-1-specific hyperimmune sera. For convenience the parental virus strain of EHV-1 will be referred to as wild type (EHV-1 w/t).

Determination of thymidine kinase activity

The TK activity was determined by the method of Klemperer et al. (1967) adapted by Mittal and Field, (1989). Briefly, M143 (TK^-) cells were inoculated with the different virus strains at a multiplicity of 0.1–1.0 PFU/cell. The cells were harvested after 48 or 72 h of incubation, and the enzyme activity determined in 25-μl aliquots of infected or uninfected cell extract using [H^3]thymidine (specific activity 20 Ci/mmol at a concentration of 1.6 μCi/ml) as substrate.

Intranasal inoculation of mice

Three-week-old female Balb/c mice were obtained from Bantin and

Kingman Ltd., Hull, U.K. Mice at 4–5 weeks of age were inoculated intranasally (i.n.) under light general anaesthesia by instillation into both nares of 40, or 50 μl of virus suspension containing a total dose of 10^7 PFU of w/t or mutant. When all mice had been inoculated, surplus virus suspension was titrated to confirm the precise dose.

Chemotherapy of mice

PCV and HPMPA were dissolved in sterile distilled water and administered to mice for the duration of the experiment by subcutaneous injection starting from one day before virus inoculation. The drugs were given twice a day to give a total dose of 100 mg/kg per day and 50 mg/kg per day, respectively.

Isolation of virus from murine tissue and infectious centre assay

Previous study showed that the important target organs for virus replication are nasal mucosa, lungs and blood (Awan et al., 1990). Mice were killed at various times after inoculation and the lungs and turbinate bones were minced with scissors and homogenised in an electric blender in a small quantity of EMEM. The suspension was sonicated for 1 min and centrifuged at 500 $\times$ g for 10 min to remove cellular debris. Dilutions of the supernatant were made in EMEM and aliquots inoculated onto confluent RK-13 monolayers. After 45 min adsorption, EMEM containing 2% FCS and 1% carboxymethyl cellulose (CMC) was added, and the cultures incubated at 37°C. Cultures were examined after two or three days and plaques stained with crystal violet for enumeration. Eleven plaques derived from the infected organs of different mice inoculated with the PR3 mutant were checked for sensitivity to PCV in RK-13 monolayers and for TK activity.

To assess viraemia, blood was collected and mixed with anticoagulant (2 mg/ml EDTA). The blood was centrifuged in microfuge tubes and the buffy coat was mixed in distilled water for 1 min to lyse the erythrocytes. The osmotic balance was restored with 10 $\times$ strength phosphate-buffered saline (PBS). Cells were counted in a haemocytometer and a given number of cells added to confluent monolayers of RK-13 cells. The development of plaques was determined as above.

Histology

Mice were killed by lethal injection of pentabarbitone sodium. Small pieces of tissues were carefully excised and immediately fixed in 10% formal saline, and embedded in paraffin wax. Sections were stained by means of H & E, using standard methods.

Results

Sensitivity to PCV and TK activity of EHV-1 strains

PCV was found to inhibit the replication of EHV-1 in RK-13 cells with a 50% effective dose concentration of 1.6 ± 0.2 μg/ml. The three mutants selected in the presence of PCV were shown to have ED_{50} concentrations for PCV at least 40 times higher than the w/t (Table 1). The HPMPA resistant mutant (HR2) was included for comparison, and was found to be as sensitive to PCV compared with w/t virus. Conversely, the three mutant viruses PR1, PR2 and PR3 selected for resistance to PCV, did not differ in their sensitivity to HPMPA in comparison with the w/t (Table 1). The growth characteristics and plaque morphology of all the mutant viruses in RK cells were similar to those of the w/t virus at the normal incubation temperature 37°C. The TK activity of each of the mutants selected in the presence of PCV was less than 1% of that shown by the w/t virus (Table 1), while the TK activity of HR2 mutant did not differ from that of w/t.

Comparison of drugs in mice

Four groups of mice were inoculated with EHV-1 by the i.n. route. Therapy with the different drugs commenced 24 h before virus inoculation. One group was treated with distilled water to act as control.

Subjective differences were observed in the clinical signs in the different groups, with placebo-treated mice showing the most severe signs. The infected mice treated with PCV and HPMPA showed reduced clinical signs with no obvious respiratory distress and less ruffled fur compared with the infected placebo-treated controls. These subjective observations were supported by pathological findings (see later) and by virus titrations on lungs and nasal turbinate bones. At day 3 and 5 after inoculation, the groups treated with PCV

TABLE 1

The antiviral sensitivity and TK activity of EHV-1 strains

Virus	ED_{50}[a]		TK activity
	PCV	HPMPA	
EHV-1 AB4 (w/t)	1.64 ± 0.15	0.1 ± 0.05	+
PR1	73.5 ± 2.5	0.1 ± 0.05	−[b]
PR2	68.6 ± 3.5	0.1 ± 0.05	−[b]
PR3	70.8 ± 3.5	0.1 ± 0.05	
HR2	1.58 ± 0.15	3.8 ± 0.2	+

Antiviral activity was measured by plaque reduction in RK-13 cells and TK activity was measured in M143 (TK^-) cells.

[a] μg/ml ± SD.

[b] <1% w/t.

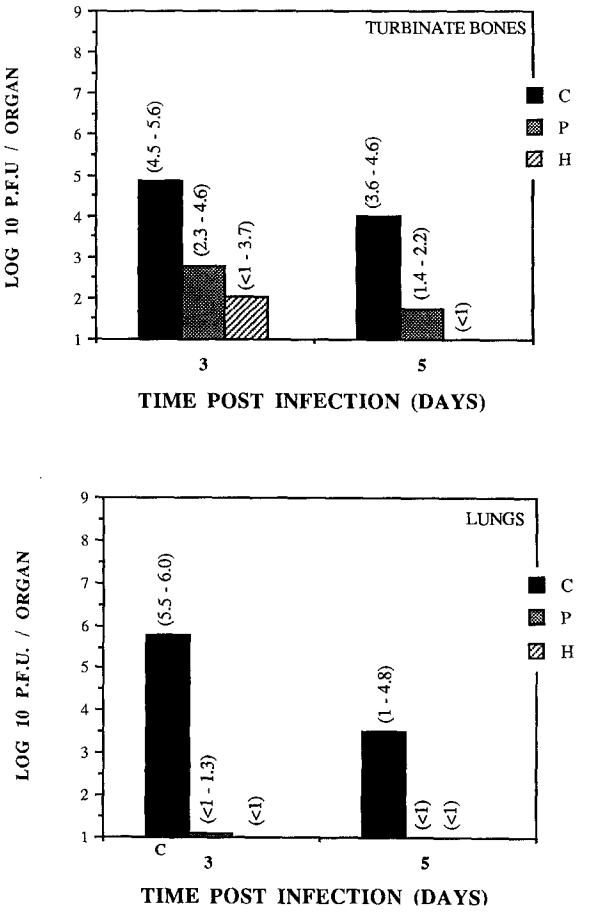
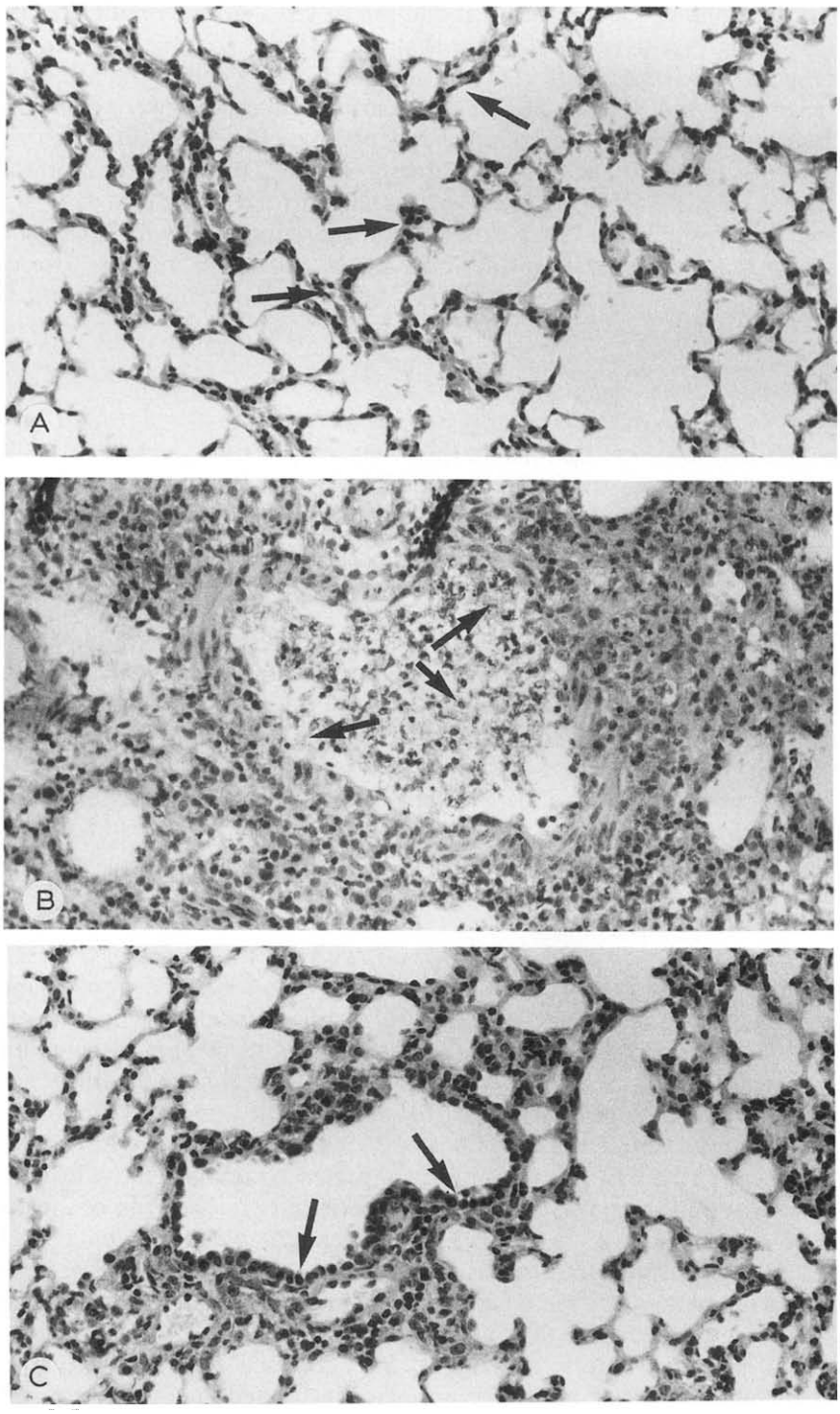


Fig. 1. Histogram showing the virus isolation from the tissues of mice treated with PCV (P), HPMPA (H) or placebo (C). PCV and HPMPA were administered by s.c. route 100 mg/kg per day and 50 mg/kg per day, respectively. Mice were inoculated with 10^7 PFU/mouse and treated for the duration of the experiment and the organs tested on days 3 and 5 post-infection. Bars represent the geometric means with ranges of three mice processed individually from each group.

→

Fig. 2. Histopathological changes in the lungs of mice infected with EHV-1 (w/t) with or without PCV treatment (100 mg/kg per day s.c. for the duration of the experiment). (A) The lung of mock-infected mice showing the normal histological alveolar (arrow) and bronchiolar architecture with intact bronchiolar mucosa. (B) The lung of a placebo-treated mouse 5 days p.i. showing the intense inflammatory changes with almost complete loss of the alveolar and bronchiolar architecture. Many bronchioles are occluded by desquamated bronchiolar epithelial cells together with inflammatory exudate (arrow). (C) The lung of mouse treated with PCV 5 days p.i. shows less histopathological changes than (B). Note that the inflammation is relatively mild with most of the lung parenchyma in a relatively normal state with intact bronchiolar epithelium (arrow). Similar findings were observed in HPMPA-treated mice. (Magnification × 294).


A
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and HPMPA showed a significant reduction in virus titres in both sites tested, HPMPA being slightly more effective than PCV (Fig. 1). These differences were also reflected in clearance of infectious-centre forming cells from the blood.

In mice receiving PCV or HPMPA, no infectious-centres were detectable in blood samples on days 3 or 5 after inoculation. The mortality of infected, untreated mice was about 60%. Treatment with PCV prevented mortality in mice as did HPMPA. When mice were treated with the same total dose of PCV (100 mg/kg/day) given in three doses, 8-hourly, compared with a single daily administration there was little difference in the reduction in virus titre in the organs (data not shown), however the single administration appeared to be slightly less effective.

Histopathological observations

The histopathological findings in the lungs of untreated mice were similar to those described previously (Awan et al., 1990). Briefly, changes were clearly visible by day 3 post-infection. These consisted of accumulation of inflammatory cells among the alveolae and damage in the lining of the bronchioles. By day 5, the lung was typically heavily infiltrated with inflammatory cells with complete loss of alveolar architecture and many bronchioles were occluded by the presence of desquamated mucosal cells together with inflammatory exudate (Fig. 2b). All the histological changes were markedly reduced in the tissue specimens from PCV and HPMPA-treated mice. The cellular infiltration was much less with some preservation of the alveolar architecture (Fig. 2c). Although some changes were visible in the lining to the bronchioles, it was notable that portions of the epithelial surface appeared to remain intact at day 5 p.i.

Comparison between therapy of w/t and PR3 mutant-infected mice

Three groups of mice were inoculated i.n. with 10^7 PFU of the PCV-resistant mutant PR3. One group was treated with PCV as indicated above, another group was used as control (treated with distilled water). A third group of infected, untreated mice was kept separately to observe clinical signs and to check mortality. Three similar groups of mice were infected with w/t virus. Mice from the treated and untreated groups were killed on day 1, 3, and 5 after inoculation to determine the levels of infectious virus in the respiratory organs and to assess viraemia. The mutant virus produced clinical signs in the mice similar to w/t, and there was no obvious difference in the severity of the disease produced by mutant or w/t virus. However, the mortality rate in PR3-inoculated mice without treatment (approx. 50%) was about half that observed in the w/t-inoculated untreated group. No mortality was observed in either group of mice treated with PCV.

Histopathological lesions in lungs of mice infected with PR3 mutant were similar to those described above for w/t placebo-treated mice (data not shown).

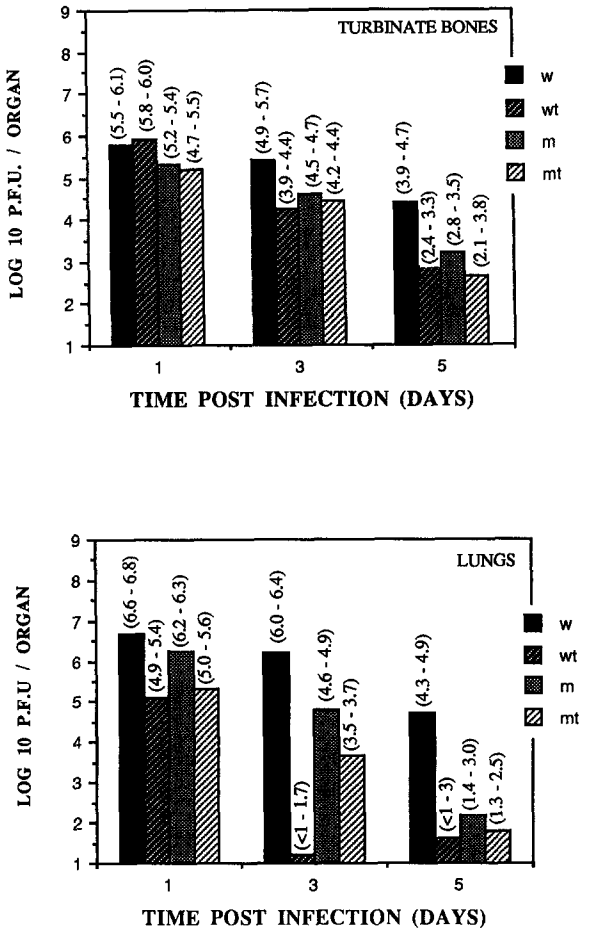

Fig. 3. Isolation of virus from the tissues of mice infected with w/t or PCV mutant (PR3). The infected mice were treated with PCV one day before virus inoculation by s.c. (100 mg/kg per day) for the duration of experiment: (w), w/t untreated; (wt), w/t treated; (m), PR3 untreated; (mt), PR3 treated. Mice were killed on days 1, 3 and 5 post-infection and their organs were tested for infectious virus on RK-13 monolayers. Each bar represents the geometric mean with range of three mice processed individually from each group.

The results of virus titration on lungs and nasal turbinate bones of the different groups of mice are shown (Fig. 3); the treatment with PCV reduced virus titres in the respiratory organs of both w/t- and PR3-infected mice, except in the case of turbinate bones at day 1 p.i. in w/t-infected mice. However, as shown (Table 2), at all times excluding the above-mentioned case the mutant-infected mice showed less reduction of virus titre with therapy in comparison with those inoculated with w/t. In w/t-inoculated mice the reductions ranged from approx. 5.9 to 1.1 $\log_{10}$, while in the PR3 mutant-infected mice they were from 1.2 to 0.1 $\log_{10}$. The differences in reductions of virus titre were much more pronounced

TABLE 2

Log_{10} reduction in virus titre in the organs of w/t or PR3-infected mice following treatment with PCV

Virus inoculated	Days post-infection					
	1		3		5	
	Turbinate	Lung	Turbinate	Lung	Turbinate	Lung
EHV-1 (w/t)	0.1[a]	1.5	1.1	5.9	1.5	3.1
EHV-1 (PR3)	0.1	0.9	0.3	1.2	0.6	0.4

[a] Data are differences in geometric mean virus titres in organs (log_{10}) with or without treatment with PCV. PCV was administered s.c. at 100 mg/kg/day from one day before virus inoculation until the end of the experiment.

in lungs than in turbinate bones (Table 2). No infectious virus was detected at day 7 in respiratory organs of mice infected either with w/t or PR3 mutant.

It was noted that mutant virus consistently achieved lower virus titres in both turbinate and lung tissues compared with w/t inoculated mice. EHV-1 was detected in the blood of mice by means of infectious-centre assay. Both mutant and w/t virus were found to cause viraemia in some mice (three of three, one of three and one of four mice tested on days 1, 3 and 5 for w/t-inoculated mice, and two of three and one of three tested on days 1 and 3 for mice inoculated with PR3 mutant). Treatment with PCV ablated viraemia and no virus-infected leucocytes were detected in the drug-treated animals. All of 11 virus isolates obtained from respiratory organs of mice inoculated with the PR3 mutant were tested and found to have ED_{50} values of approx. 60 μg/ml against PCV and retained the TK^- phenotype (data not shown).

Discussion

Antiviral chemotherapy against members of the herpesvirus family is now routinely practised in human medicine and considerable success has been proven with a variety of different drugs, most notably acyclovir. There is now much interest in alternative guanosine analogues, e.g., ganciclovir, for the treatment of retinitis caused by cytomegalovirus in patients with the acquired immunodeficiency syndrome (Jeffries, 1989). More recently the drug PCV has emerged for the therapy of herpes simplex in man (Boyd et al., 1988). Although the modes of action of these compounds are generally similar, each has subtle characteristics which may provide particular advantages in different clinical situations or species.

While good progress has been made in treating human virus infections, to date, there has been comparatively little published work concerning the use of antiviral chemotherapy in veterinary medicine although there are a number of important target diseases (Rollinson, 1987). EHV-1 is a common infection among equines with several damaging manifestations including abortion and

neurological disease while the infection has proved difficult to control by the available vaccines. Previously we have reported the efficacy of a phosphonyl derivative in a murine model for EHV-1 (Field and Awan, 1990). In the present paper, similar encouraging results were obtained using the nucleoside analogue, PCV.

The mechanism of action of PCV against HSV-1 has been well-described (Vere-Hodge and Perkins, 1989). It appears that the drug is phosphorylated by virus-encoded TK and this underlies its selectivity for HSV-infected cells. We have shown that the equine herpesvirus is also sensitive to PCV in tissue culture suggesting that the analogue is also a substrate for the EHV-1 TK. This hypothesis was further substantiated by the observation that a series of EHV-1 mutants selected for resistance to PCV were all defective in TK induction. Moreover, a mutant which had been selected for resistance to the phosphonyl derivative, HPMPA remained sensitive to PCV. This is consistent with the proposed mechanism action of HPMPA which is believed to act independently of virus-coded TK (Votruba et al., 1987).

The two drugs HPMPA and PCV were found to inhibit EHV-1-infected RK cell monolayers at ED_{50} concentrations of 0.1 and 1.6 μg/ml (corresponding to 0.3 and 6.0 μM), respectively. This compares with values of 2.0 and 0.6 μM, for acyclovir and ganciclovir respectively, reported by Rollinson and White (1983) for the Rac-H strain of EHV-1 tested in porcine (PK-15) cell monolayers. HPMPA and PCV were compared in mice using doses of 50 mg/kg and 100 mg/kg per day, respectively. Both drugs were effective in preventing the development of clinical signs and no mortality was observed among treated animals. It was notable that viraemia was not detected in the treated mice. This is an important result since viraemia plays a key role in the production of disease in the natural host, including abortion and neurological signs (Bryans 1969; Charlton et al., 1976).

Both compounds (PCV and HPMPA) produced marked reduction of virus growth in the respiratory tissues, as measured by the levels of infectious virus present. These results were substantiated by the histological findings. We draw particular attention to the apparent protection of the ciliated mucosal lining of the bronchioles which was relatively spared in the treated animals. If a similar effect can be obtained in the equine host this would be a very important aspect of chemotherapy since secondary infections tend to complicate recovery from EHV-1 owing to the compromised state of the respiratory tract following damage by the virus. However, we noted that the clearance of virus was less effective in the turbinate samples. We have previously encountered similar findings in the treatment of HSV in mice following i.n. inoculation (Goldthorpe et al., 1991). This may reflect the relative difficulty of maintaining active drug concentrations in upper respiratory secretions. It remains to be seen whether or not this will be an important constraint on chemotherapy in the natural host.

The PCV-resistant mutant PR3 was found to multiply slightly less well in the murine model. This is broadly consistent with the finding that drug-resistant mutants selected in vitro frequently have some reduction in pathogenicity

(Field, 1989). However, following chemotherapy with PCV the reduction in virus titre was consistently less in the case of the resistant mutant than for wild type and, as discussed above, this was most marked in the turbinate samples. Although the reduction in virus titre in the mutant virus-infected, treated mice was not significant, consistently less virus was isolated from all sites on all occasions from treated mice. This suggests that the drug tissue levels perhaps may have approached the concentration (approx. 60 μg/ml, i.e. the in vitro dose) required to inhibit the mutant although this is considered unlikely. Levels of 21 μg/ml in the blood have been reported in PCV-treated mice (Boyd et al., 1988).

In summary, these results encourage us to believe that several different compounds, including PCV and HPMPA are very active against EHV-1 in tissue culture and that these drugs were also effective in vivo in a murine model. We have now unequivocal evidence that neither drug prevents the establishment of a latent EHV-1 infection that can be reactivated by means of suitable stimuli (Field et al., 1992). However, the results for the treatment of acute disease in mice are sufficiently encouraging to suggest that trials in the natural host will now be appropriate.

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