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The Effect of (S)-1-(3-hydroxy-2-phosphonyl-methoxypropyl)cytosine (HPMPC) on bovine herpesvirus-1 (BHV-1) infection and reactivation in cattle

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Summary

A reproducible pattern of respiratory disease was produced in calves inoculated intranasally with a pathogenic strain of bovine herpesvirus-1 (BHV-1). A latent infection was established which could be reactivated by means of corticosteroid administration. Groups of calves were given a single dose of 20 mg/kg of (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC) either the day before or the day following virus inoculation. The drug markedly reduced clinical signs and virus replication; the therapeutic dose appeared to be more effective than the dose given one day before virus inoculation. The establishment of latency was not prevented and a single dose of HPMPC, the day before a course of dexamethasone (6 weeks after the acute infection), did not prevent virus shedding.

Bovine herpesvirus; HPMPC; BHV-1; Calf

Introduction

Cattle are widely infected with several distinct members of the herpesviridae (Kahrs, 1981). Bovine herpesvirus-1 (BHV-1) is considered to be the most important of these infections accounting for significant economic losses in both dairy and beef cattle (Wiseman et al., 1984). The infection is commonly associated with an upper respiratory tract disease known as infectious bovine

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rhinotracheitis (Straub, 1990). BHV-1 infection, however, also leads to other clinical conditions including conjunctivitis, mastitis, and encephalomyelitis (Kahrs, 1981). BHV-1 is a typical alphaherpesvirus and establishes latency in ganglionic neurons during the acute infection (Narita et al., 1978). Subsequent latent infections may reactivate with virus shedding and transmission to susceptible hosts. Virus reactivation occurs naturally following a variety of stressful stimuli (Snowdon, 1965) and readily can be demonstrated experimentally following the administration of corticosteroids (Narita et al., 1978; Davies and Duncan, 1974).

Mortality following primary infection by BHV-1 is generally low but high rates of morbidity are common in crowded, stressful environments such as feedlots; mixed infections and superinfection with opportunist microbes are common (Kahrs, 1981). Vaccines are available (Straub, 1990) but are of questionable efficacy and their use is complicated by the establishment and reactivation of latent virus. Use of chemotherapy to treat bovine herpesvirus infections has been investigated with the use of various compounds (Babiuk et al., 1983; Mohanty et al., 1980) but results from these tests have shown poor antiviral efficacy.

The phosphonyl derivatives of purines and pyrimidines are a family of 'nucleotide analogues' which have been shown to have a wide spectrum of activity against both DNA and certain RNA viruses (De Clercq, 1991; De Clercq and Holy, 1991; De Clercq et al., 1986; Gordon et al., 1991; Li et al., 1990; Soike et al., 1991). (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine (HPMPC) has already been shown to be active against several different herpesviruses in tissue culture and in animal models (De Clercq and Holy, 1991; Maudgal and De Clerq, 1991; De Clercq et al., 1987; Gibson et al., 1992). Of particular note is the effectiveness of a single administration of the drug. For example, a single dose of HPMPC given as late as 4 days after virus inoculation was protective in HSV-infected mice (De Clercq and Holy, 1991). The importance of the present study is that the efficacy of HPMPC was evaluated in the natural host infected under experimental conditions. These results appear to confirm the promising data already generated for HPMPC therapy using infection models in rodents.

Materials and Methods

Antiviral compound

HPMPC was obtained from Bristol-Myers Squibb, Wallingford, USA. The drug, supplied as a dry powder, was dissolved in dimethylsulfoxide and diluted in phosphate buffered saline (PBS) for injection. The solution was filter-sterilised before use.

Calves

Eleven Friesian bull calves, 12–16 weeks old, were obtained from a certified

BHV-1-free herd. All calves were castrated 4 days after purchase and were then kept under observation for 6 weeks before use. All animals were confirmed to be BHV-1 antibody free on the basis of the ELISA and virus neutralisation tests (see below).

Tissue culture

Madin Darby bovine kidney (MDBK) cells were grown in Eagle's minimal essential medium (EMEM) supplemented with antibiotics, fungizone and 10% fetal calf serum (FCS). Serum concentration was reduced to 2% for growth of virus and for antiviral tests. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Virus

BHV-1 wild type (w/t) strain 6660 was that described by Brown and Field (1990). This strain is of the 'Cooper' type based on the DNA restriction pattern (Owen and Field, 1988). Virus working stocks were grown in MDBK cells infected at a low multiplicity.

Plaque reduction assay

The sensitivity of BHV-1 to the compound was tested in vitro by means of a plaque reduction assay in MDBK cell monolayers using methods similar to those described previously (Field and Reading, 1987). The ED_{50} of HPMPC was found to be approximately 10 μg/ml (range 7–13 μg/ml). This compares with an ED_{50} for the same strain of 0.01 μg/ml for BVDU (Mittal and Field, 1989).

Serum ELISA test

Sera were tested for whole BHV-1 immunoglobulin by an indirect ELISA based on the protocol described by Voller et al. (1982), and modified by Brown and Field (1990).

Calf biochemistry

Before administering HPMPC to infected calves, preliminary tests were performed on a single uninfected calf inoculated subcutaneously (s.c.) once with a dose of 20 mg/kg, HPMPC (the dose proposed for the antiviral investigation). Urine and blood samples were collected at regular intervals from 4 days prior to 8 days after drug administration. Samples were analysed for hepatic and renal function by monitoring serum levels of alkaline phosphatase, aspartate amino transferase, gamma-GT, sorbitol dehydrogenase, bile salts, creatinine and urea. Levels of alkaline phosphatase, aspartate amino transferase and gamma-GT were measured in urine.

Virus inoculation of calves and administration of HPMPC

Ten calves aged 4–6 months were inoculated with an aerosol of BHV-1 by introducing 1 ml of virus suspension (2.5×10^7 plaque-forming units (PFU/ml))

into each nostril using a nebuliser (Sigma). Calves were divided initially into 3 separately penned groups. Group A (n = 3) was treated with a single prophylactic dose of HPMPC at 20 mg/kg of calf body weight one day before virus inoculation (day −1 p.i.); group B (n = 3) was treated with the same dose of HPMPC the day after virus inoculation (day +1 p.i.); group C (n = 4) was untreated during the acute phase of the experiment. An eleventh calf (which had been used for preliminary biochemistry) was mock-infected with medium to provide an uninfected control and this calf was not given further drug.

Clinical signs and virological specimens

Rectal temperatures and general clinical signs were monitored daily from day −2 to day +10 p.i and on days 13, 14, 17 and 21 p.i. Ocular and nasal swabs were also taken according to this schedule; these were tested quantitatively for the presence of infectious virus by plaque titration in MDBK cells. Blood samples were taken weekly over this period for serology. Clinical signs observed in the calves ranged from general malaise and serous nasal discharge to respiratory distress and mucopurulent nasal discharge. Clinical signs were graded from 1 to 3 according to severity (Table 1).

Virus reactivation

On day 42 p.i. all animals were treated i.v. with 0.5 mg dexamethasone/kg/day for 3 days. Animals in group C were sub-divided into two groups of 2 calves (designated C1 and C2); C1 was given a single subcutaneous dose of HPMPC at 20 mg/kg on day 41 p.i. (i.e. 1 day before commencing the course of dexamethasone). Clinical, virological and serological indications of BHV-1 reactivation were monitored, as for infection, from day 35 to day 59 p.i.

Results

Lack of toxic effects of HPMPC

No obvious clinical signs of drug toxicity were seen in any calves treated with the compound under test. Clinical biochemistry revealed no significant evidence of acute organ damage or malfunction after a single s.c. dose of 20 mg/kg/calf.

Clinical signs following BHV-1 inoculation

Animals that were inoculated with BHV-1 but received no treatment throughout the acute phase infection suffered obvious clinical signs of a BHV-1 infection (Table 1); these signs were consistent with previous observations (Brown et al., 1990; Gilliam et al., 1992), whereas the uninoculated control calf remained normal at this time. Clinical signs were first apparent on day 2 p.i. with a peak at day 5 p.i; at two weeks p.i., calves were considered clinically normal. Both groups of calves that were treated with a single dose of HPMPC on the day before (group A) or the day after inoculation (group B) showed

TABLE 1

Scoring system for clinical signs

Score 1	Score 2	Score 3
Serous ocular discharge	Mucoid ocular discharge	Mucopurulent nasal discharge
Serous nasal discharge	Mucopurulent ocular discharge	Enlarged lymphnodes (bilateral)
Soft faeces	Mucoid nasal discharge	Respiratory distress cough
	Diarrhoea	Nasal ulcer
	Swollen eyes	
	Lethargic	
	Enlarged lymphnode (monolateral)	

Each clinical sign indicated was given a score according to the above scheme.

milder clinical signs compared with the untreated animals. Minimal signs only were observed for both groups over a 15-day period from day 2 p.i. (Fig. 1). The uninoculated calf remained clinically and virologically normal throughout the experimental period (data not shown).

Rectal temperatures

Calves that were given no HPMPC developed raised temperatures consistent with the respiratory signs described above. Pyrexia occurred over a 5-day period peaking around day 4 p.i. In contrast, the temperatures of animals in both groups that received HPMPC remained within the normal range (Fig. 2). The uninfected calf showed no rise in rectal temperature during the experimental period (data not shown).

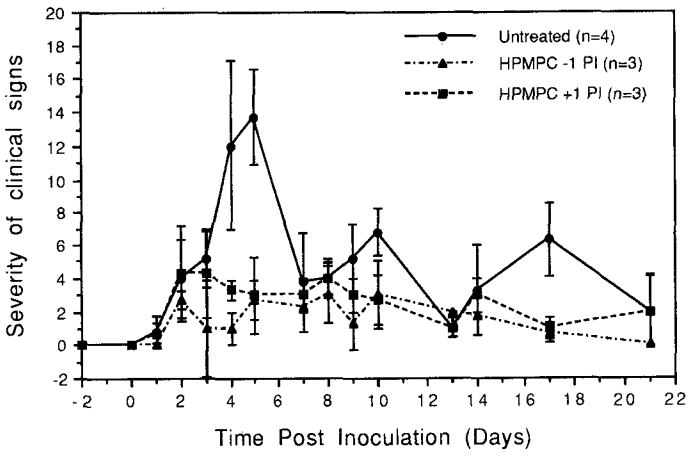

Fig. 1. Clinical signs of BHV-1-inoculated calves with or without HPMPC. Ten calves were inoculated i/n with 5×10^7 PFU of BHV-1. Animals were monitored daily for clinical signs using an arbitrary scale scored according to the scheme shown in Table 1. Four calves remained untreated throughout the period of observation; three were given a single dose (20 mg/kg) of HPMPC one day before virus inoculation (−1 p.i.) and 3 were given an identical dose of HPMPC the day after virus inoculation (+1 p.i.). The points are arithmetic means for each group with standard deviation shown by the bars.

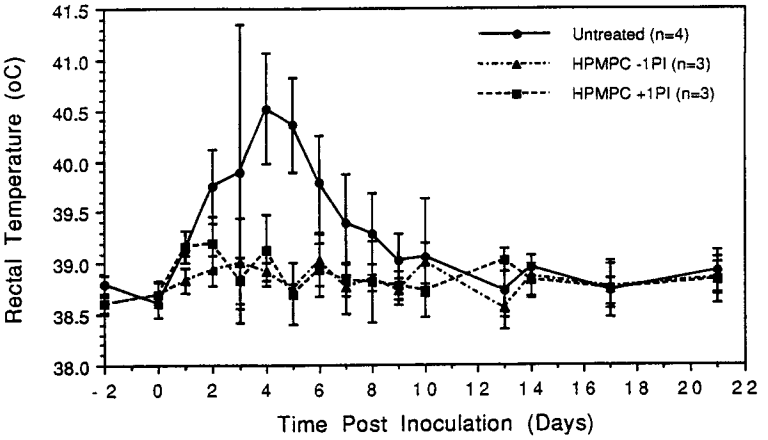

Fig. 2. Rectal temperatures of BHV-1-inoculated calves with or without HPMPC. Legend, as for Fig. 1. except that rectal temperature was measured.

Ocular virus

Ocular swabs titrated for BHV-1 by plaque assay indicated positive virus secretion in all groups of animals during the acute phase infection period. Virus was recovered from the ocular secretions of all 4 untreated animals at high titre between days 3 and 10 p.i. with peak titres ranging between 4 and 6 $\log_{10}$ PFU/sample on days 3 and 4 p.i. (Fig. 3a).

Ocular virus was detected in 2 of 3 calves treated with drug the day before virus inoculation and in one case (calf 5) the period of secretion was both delayed and extended up to day 17 p.i although peak virus titres were similar to those of the untreated animals (Fig. 3b).

Virus was detected in ocular swabs from only one of the three animals treated on the day after virus inoculation (Fig. 3c). Virus was detected in this animal (calf 8) intermittently from day 5 p.i. with a peak virus titre of approximately 4 $\log_{10}$ PFU/sample 7 days p.i.; this being 3 days later than the peak ocular virus titre in untreated controls (Fig. 3a).

Nasal virus

All 3 groups of calves were found to secrete nasal virus during the acute infection. Virus was recovered from all 4 untreated animals from day 2 or 3 p.i., reached peak titres between days 4–6 p.i. and was cleared in all cases by day 13 (Fig. 4a).

Nasal virus was isolated from all 3 calves treated with HPMPC on the day before virus inoculation (c.f. ocular virus shedding) but the time of peak titre was delayed for two animals (calves 5 and 6) in this case by 3–4 days (Fig. 4b). The total amount of virus detected during the acute infection was less in the case of the treated animals.

For animals treated on the day after virus inoculation, virus was undetectable in the nasal secretions of one animal (calf 9) and was detected

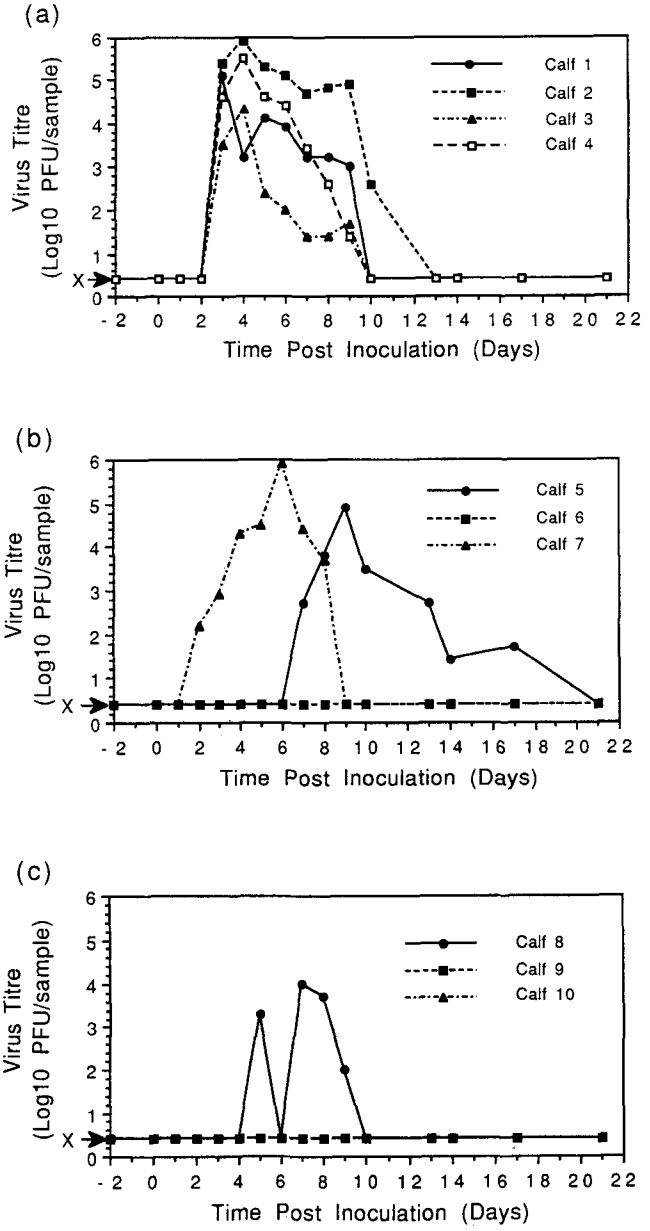

Fig. 3. Ocular virus titres of BHV-1 inoculated calves with or without HPMPC. Ocular swabs obtained from individual calves during the acute phase infection were tested for the presence of infectious virus by plaque assay. The titres were plotted for each calf in the groups (a) untreated; (b) single dose of HPMPC (20 mg/kg) administered day −1 p.i.; (c) single dose of HPMPC (20 mg/kg) administered day +1 p.i. 'X' indicates the limit of test sensitivity. The areas under the curve were used to measure the total virus recovered and reductions compared using the *t*-test (two tailed). The reduction in virus in (c) was significant ($P < 0.05$).

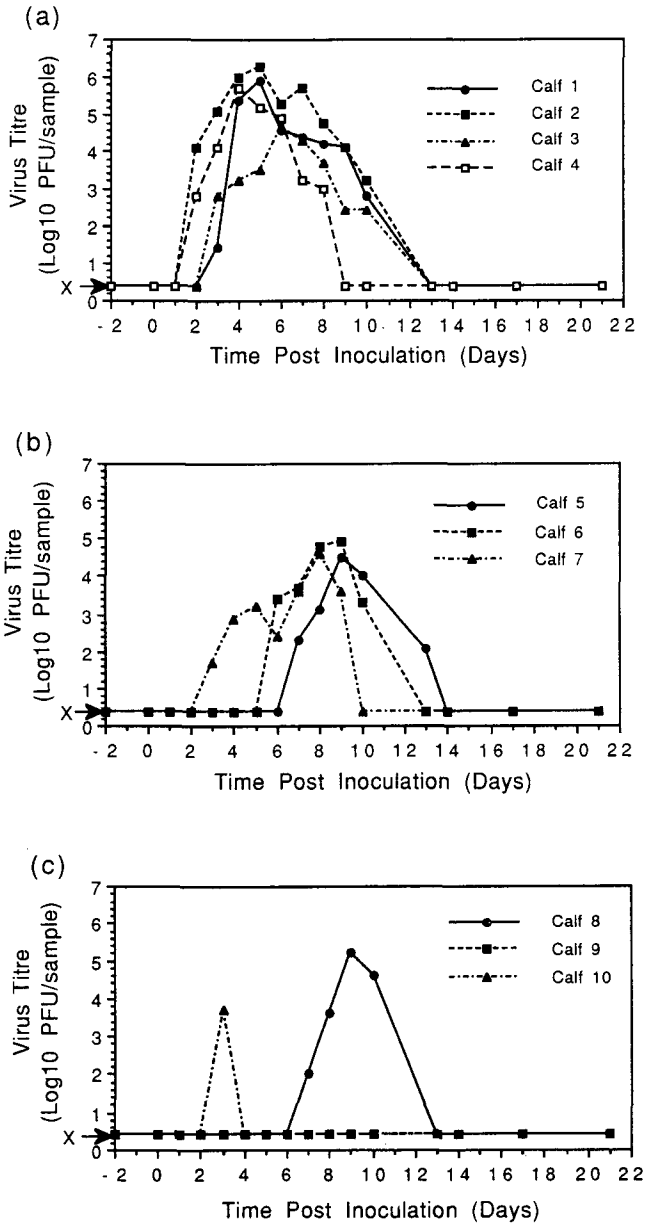

Fig. 4. Nasal virus titres of BHV-1 inoculated calves with or without HPMPC. Nasal swabs obtained from individual calves during the acute phase infection were tested infectious virus as for Fig. 3. The areas under the curve were measured and compared using the *t*-test (two tailed). Total virus in (c) was found to be significantly reduced ($P < 0.01$).

on one day only for a second animal (calf 10) (Fig. 4c). The remaining animal in this group (calf 8) secreted virus for a 4-day period, but this was markedly delayed compared with the untreated animals. The reduction in the total amount of virus shed by the treated group of animals was highly significant compared with the untreated group (Fig. 4).

Virus reactivation

No virus was detected in the ocular swab samples taken from any of the animals following dexamethasone administration. Virus was, however, isolated from nasal swab samples from animals in all four groups during the period after the reactivation stimulus.

The two calves from group C1 which were untreated at the time of primary inoculation, and which remained untreated during the attempted reactivation phase, were both found to secrete nasal virus for a 3- or 4-day period starting on the 5th and 6th day after the dexamethasone treatment began (Fig. 5a). One of the two calves in group C2 (which were also untreated during the acute phase, but were given a prophylactic dose of HPMPC one day before the start of dexamethasone) also showed evidence of virus reactivation although virus was recovered later, on days 8 and 9, after commencing the dexamethasone treatment (Fig. 5b).

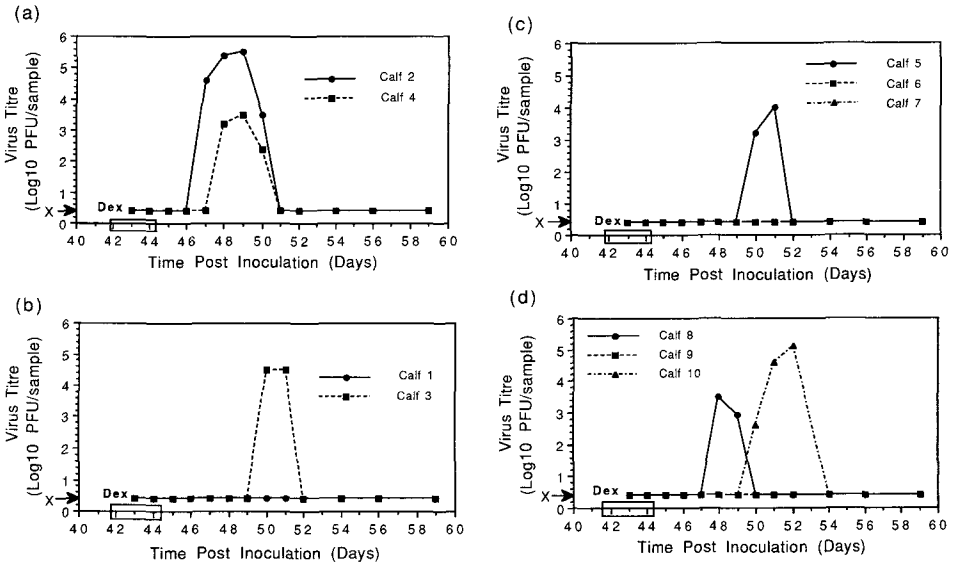

Fig. 5. Nasal virus titres of BHV-1 inoculated calves following reactivation with or without HPMPC. Nasal swabs obtained from individual calves following reactivation stimulus were tested for the presence of infectious virus by plaque assay. The titres were plotted for each calf in the groups (a) untreated; (b) single dose of HPMPC (20 mg/kg) administered day before dexamethasone (−1 dex); (c) single dose HPMPC day −1 p.i.; (d) single dose HPMPC day +1 p.i. The rectangle on the *X*-axis indicates the period during which dexamethasone was administered to reactivate latent virus. 'X' indicates the limit of test sensitivity.

Virus was also recovered from 3/6 calves which had been treated only during the acute phase of the experiment (Fig. 5c and d). One animal (calf 9) from which neither ocular nor nasal virus was recovered after primary virus inoculation again did not yield virus following the reactivation stimulus, however, the serum ELISA antibody titres suggested that an infection was established in this calf.

Antibody response

Serum samples were monitored for seroconversion to BHV-1 antigen using an ELISA technique. Peak antibody titres were reached by 14 days p.i. for the 4 animals that were inoculated but untreated during the acute phase of infection (data not shown).

The antibody responses in the three animals that were treated with HPMPC the day before virus inoculation were not significantly different from untreated controls, although the titres were marginally lower and peaked between days 14 and 21 p.i. The calves treated after virus inoculation also seroconverted, including the calf (no. 9) from which no virus was isolated during the study. The ELISA results from serum antibody were confirmed by a test for BHV-1 neutralising antibody. The control, uninfected calf did not seroconvert by either test during the experimental period.

Discussion

An experimental infection model was established using a pathogenic strain of BHV-1 and seronegative calves. Intranasal inoculation of virus resulted in a reproducible pattern of clinical signs and virus shedding, moreover latency could be established and BHV-1 reliably reactivated using a corticosteroid.

The model was used to test the effects of a single dose of the nucleotide analogue, HPMPC given either before or after virus inoculation. Prophylaxis or therapy produced a marked reduction in clinical signs; the most objective measurement was rectal temperature that rose uniformly in the untreated, infected animals but remained within normal limits in calves given HPMPC.

Ocular and nasal virus secretion was reduced in the groups which received drug and the compound appeared to be more effective when given the day after virus inoculation. However, since there was a delay of up to two or three days before virus could be isolated from the respiratory specimens in untreated animals, the antiviral effects of dosing before inoculation may have waned before the peak phase of virus replication. In treated calves, a pattern emerged of delayed virus shedding with peak titres at 6–10 days p.i. compared to 3–6 days p.i. in untreated controls. The experimental design was constrained by the limited availability of compound which did not allow repeated administration, thus the pattern of virus replication may reflect a waining of antiviral effect with time, suggesting that further doses could be beneficial. No measurements of tissue concentration of compound or its metabolites were undertaken in this

study, clearly such information will help to elucidate the potential for improved therapeutic effects.

It was of interest that the establishment of latency was not prevented in HPMPC-treated groups. In a previous experiment all animals in a group of 3 calves, infected with the present strain of BHV-1, showed virus shedding following dexamethasone (Gilliam et al., 1992) and in the present study reactivated virus was recovered from 2 of 2 animals which were untreated prior to steroid administration.

No obvious toxicity was observed during the experiment and none of the biochemical tests on serum and urine specimens indicated significant alteration from normal. The compound HPMPC has been shown to be efficacious against several herpesviruses in cell culture and in various animal models (De Clercq and Holy, 1991; De Clercq et al., 1987; Maudgal and De Clercq, 1991). To our knowledge this is the first report of its effects against BHV-1 in the natural host. We are also reporting elsewhere the potent effects of the same compound against equine herpesvirus-1 (EHV-1) demonstrated in a murine model and in experimentally infected foals (Gibson et al., 1992). These results are important because they demonstrate beneficial effects of a single dose administration of HPMPC in the natural hosts for two herpesviruses with veterinary importance. There remain considerable practical difficulties in the development of chemotherapy for veterinary applications (Rollinson, 1992), however, the present results should encourage further research into the feasibility of antiviral chemotherapy in large animals.

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