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Susceptibility of Bovid Herpesvirus 1 to Antiviral Drugs: In Vitro Versus In Vivo Efficacy of (*E*)-5-(2-Bromovinyl)-2′-Deoxyuridine

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The relative efficacies of a variety of antiviral drugs against bovid herpesvirus 1 was investigated. (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine and trifluorothymidine were found to be inhibitory at doses of 0.01 μg/ml in in vitro yield reduction and plaque reduction assays. In contrast, acylovir was inactive even at concentrations as high as 1,000 μg/ml. Other drugs, including phosphonoformic acid, 9-β-D-arabinofuranosyladenine, 5-iodo-2-deoxyuridine, and 1-β-D-arabinofuranosylcytosine were active at concentrations previously shown to inhibit herpes simplex virus. Oral administration of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine to calves infected with bovid herpesvirus 1 had no effect on the level of virus shedding, clinical signs, or susceptibility to secondary bacterial infection with *Pasteurella haemolytica*. The reason for this lack of in vivo activity was that sufficient levels of the drug in blood were not achieved by oral administration.

A wide variety of drugs have been tested for their antiviral effects on herpesviruses, particularly herpes simplex virus (2, 3, 7, 8, 13, 17–19, 22, 23, 25, 26). Although these drugs have been shown to be most effective in limiting virus replication in vitro, they have also been shown to be at least partially effective in vivo under both experimental and natural conditions (9, 10, 13, 14, 17, 20, 31). These observations have stimulated the continued search for better drugs which could be used as a means of controlling herpesvirus infections or preventing recrudescent disease in humans. In addition to infecting humans, herpesviruses cause disease in many animal species (6, 15, 21). Unfortunately, little information is available on the in vitro and in vivo efficacies of these drugs in controlling the replication of animal herpesviruses (1, 32).

Our laboratory has been particularly interested in bovid herpesvirus 1 (BHV-1), an economically important pathogen of cattle. The bovine system serves as an excellent model for understanding virus-host cell interactions as well as the immune response of the host to the virus (4, 5, 27–29). In addition, this virus has proven to be very useful for studying synergistic interactions between viruses and bacteria. Thus, infection of the respiratory tract with BHV-1 does not kill the animal but appears to alter the resistance of the animals to *Pasteurella haemolytica*, possibly by replicating in the alveolar macrophages (11, 12) or by altering influx and activity of polymorphonuclear leukocytes.

Cattle exposed to aerosols of only *P. haemolytica* remain healthy, whereas those exposed to BHV-1 only develop tracheitis and transient interstitial pneumonia. However, those given BHV-1 followed 4 days later by *P. haemolytica* develop acute, often fatal, fibrinous bronchopneumonia (15). Also, vaccination with BHV-1 will prevent the subsequent development of *Pasteurella pneumonia* by limiting the degree of virus replication and, presumably reducing the degree of immunosuppression caused by the virus. Considering the active role of the virus in predisposing animals to superinfection with *P. haemolytica*, we felt that, if we could reduce the degree of virus replication by antiviral drugs, then the bacterial clearance mechanisms would prevent the establishment of bacteria in the lung.

The present report analyzes the effects of commonly used anti-herpesvirus drugs on the replication of BHV-1 in vitro. One of the most active drugs (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVdU), was then tested for its efficiency in vivo in our BHV-1–*P. haemolytica* model.

MATERIALS AND METHODS

Drugs. 5-Iodo-2′-deoxyuridine, 1-β-D-arabinofuranosylcytosine, 9-β-D-arabinofuranosyladenine, phosphonoformic acid, and trifluorothymidine (F_3dT) were obtained from Sigma Chemical Co., St. Louis, Mo.

(*E*)-5-(2-Bromovinyl)-2'-deoxyuridine (BVdU) was synthesized essentially as described previously (16). Acyclovir (ACV) was obtained as a gift from Burroughs Wellcome Co., Research Triangle, N.C. For in vivo studies, all drugs were dissolved at the required concentration in Eagle minimal essential medium and sterilized by filtration just before use. For in vivo studies, BVdU was dissolved in water.

Cell cultures. Georgia bovine kidney (GBK) cells were cultured as described previously (5). Confluent monolayers were prepared by seeding approximately 5×10^4 cells into each well of microtiter tissue culture plates (no. 3040; Falcon Plastics, Oxnard, Calif.), or at 2.5×10^5 cells per well onto a 24-well plate (no. 16-24-TC; Linbro). The cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere. The monolayers were confluent within 24 h and were used for virus infection at that time.

Virus and antibody. BHV-1 strain P8-2 or 108 was prepared as described previously (5). Antibodies to BHV-1 were prepared by immunizing young heifers twice intramuscularly with 10^9 PFU of BHV-1. At the end of the immunization period, animals were bled and their sera were collected, heat inactivated at 60°C for 30 min, and stored frozen until use.

Yield reduction assays. Confluent cultures of GBK cells were infected with 100 to 1,000 PFU of BHV-1. The virus was allowed to adsorb for 1 h, the monolayer was washed twice to remove unadsorbed virus before the addition of fresh minimal essential medium supplemented with 5% fetal bovine serum and containing the appropriate concentration of each specific drug. After 48 h, the cultures were harvested and the quantity of virus was determined by a plaque assay (5).

Plaque reduction assays. Confluent GBK cell cultures in microtiter plates were infected with 20 to 50 PFU of BHV-1 per well as described previously (5). After adsorption for 1 h, monolayers were washed once with Hanks balanced salt solution and overlaid with minimal essential medium plus 1% fetal bovine serum containing four neutralizing units of BHV-1 antibody plus various concentrations of each drug. Plaques were allowed to develop for 48 h before fixation, staining, and enumeration (5). In each experiment, toxicity controls (containing test compound and medium only), virus controls (containing virus alone), and cell controls (containing cells alone) were run simultaneously.

Infectious-center assays. GBK cells cultured in 24-well culture plates were infected with 100 to 1,000 PFU of BHV-1 per well. After a 1-h adsorption period, monolayers were washed to removed unadsorbed virus and overlaid with minimal essential medium containing anti-BHV-1 serum and various concentrations of BVdU. Forty-eight hours later, cells were removed by trypsinization and diluted in minimal essential medium plus anti-BHV-1. Diluted cells were added to confluent GBK cells in 24-well culture plates. The cells were allowed to settle for 2 days, after which time the monolayers were fixed and stained and the viral plaques enumerated (5).

In vivo experiments. Six- to eight-month-old, specific pathogen-free (BHV-1 and *P. haemolytica*) Hereford calves were infected intranasally with 10^8 PFU of BHV-1 108, administered as an aerosol generated with a Devilbis 65 nebulizer (15). Animals were divided into four groups. Two groups were given 5 mg of BVdU per kg of body weight daily, resuspended in 300 ml of distilled water by gavage for 5 consecutive days starting on the day of virus challenge. Four days after BHV-1 challenge, one group of calves given drug and one group not given drug were challenged with an aerosol of *P. haemolytica* (10^7 CFU) with a Devilbis 65 nebulizer as described above. Nasal swabs were taken daily and titrated for the presence of BHV-1 as described previously (24). Serum samples were taken daily to determine the levels of BVdU. Briefly, heat-inactivated serum was added to 50 PFU of BHV-1 in microtiter plates in a plaque reduction assay as described above. The numbers and sizes of plaques were assessed 48 h later.

Postmortem analysis. The calves were necropsied as soon as they died. The entire respiratory tract was removed and, after gross examination and assessment of lung involvement, samples of lung tissue were taken from the cranial, middle, and caudal lobes for viral, bacterial, and histological assessment. The presence of virus was determined by culturing in GBK cells as described previously. Bacteria were isolated by culturing on blood agar plates at 37°C. Histological examination was made in 5-μm-thick hematoxylin-eosin-stained paraffin sections.

RESULTS

In vitro effects of antiviral drugs on BHV-1 replication. In an attempt to determine which anti-herpesvirus drug was most effective against BHV-1, we evaluated the effect of various drugs on the replication and yield of BHV-1 in vitro. 5-Iodo-2'-deoxyuridine, BVdU, and F_3dT were the most potent (Fig. 1). These drugs reduced virus yields by 99% at a concentration of 1 μg/ml or less. In contrast, ACV, which is very effective against some other herpesviruses, was totally ineffective against BHV-1. Other drugs, such as phosphonoformic acid and 9-β-D-arabinofuranosyladenine, were effective at concentrations higher than those previously shown to prevent herpes simplex virus replication (Fig. 1 and Table 1). The efficacies of BVdU and F_3dT were shown even more dramatically in a plaque reduction assay in which these drugs totally inhibited plaque development at concentrations of >1 μg/ml. Thus, at a concentrations of 0.01 μg, the number of visible plaques was less than 50% of those seen in the control cultures (Table 1). Plaques that did develop were much smaller, suggesting that, even though the virus replicated at these concentrations, the number of infectious particles released and, consequently, the number of contiguous infected cells were greatly reduced. This hypothesis was further substantiated by the observed reduction in the number of infectious centers produced by the drug-treated cultures (Table 2). Thus, upon infection of cultures with 100 PFU of BHV-1, there was only a slight increase in the number of infected cells (100 versus 600) after 48 h in the presence of as little as 0.1 μg of BVdU per ml. At lower

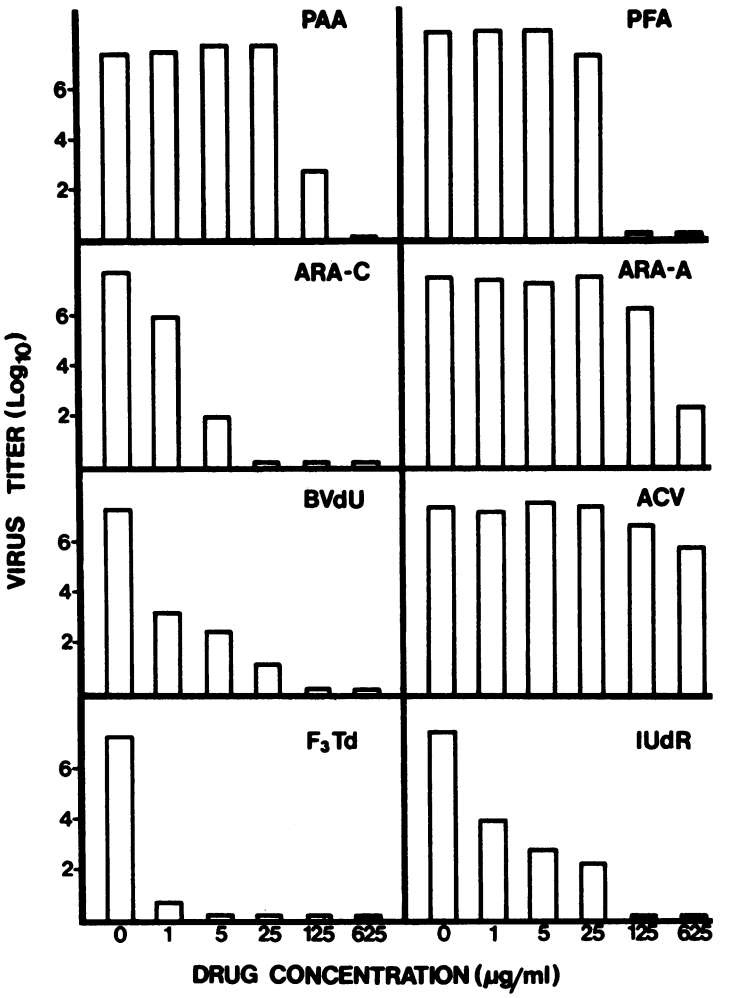

FIG. 1. Reduction of BHV-1 replication by commonly used anti-herpesvirus drugs. Individual drugs were added immediately after infection. Virus yields were determined 48 h later by a plaque assay. PAA, phosphonoacetic acid; PFA, phosphonoformic acid; ARA-C, 1-β-D-arabinofuranosylcytosine; ARA-A, 9-β-D-arabinofuranosyladenine; IUdR, 5-iodo-2'-deoxyuridine.

concentrations of BVdU or F_3dT, there was a gradual increase in the level of virus spread.

In vivo effect of BVdU. Since BVdU was extremely effective against BHV-1 and also much less toxic than F_3dT, as evidenced by its therapeutic index (Table 1), we felt that this drug may prove to be the most useful one of those tested in inhibiting BHV-1-induced pneumonic pasteurellosis. Thus, an in vivo trial was initiated. Unfortunately, the drug reduced neither the quantity of virus present in the nasal passages nor the temperature elevation as a result of virus replication in animals given BHV-1 virus alone (Fig. 2).

All animals infected with BHV-1 plus *P. hemolytica* died with typical fibrinous pneumonia regardless of whether they were given BVdU. Furthermore, there was no difference in the time of death or the pulmonary lesions between drug-treated and untreated animals; the mean day of death for drug-treated animals was the same as that for untreated animals. Lesions in both groups consisted of fibronecrotic pharyngitis, laryngitis, and tracheitis. Various proportions of the pulmonary parenchyma were consolidated. In some cases, the entire lobe was involved, whereas others had as little as 10% involvement. The consolidated areas were reddish-black in color, hard in consistency, and often raised above the surrounding normal lung. The interlobular septa of the involved areas and those peripheral to these areas were distended, with pale, straw-colored edema. In some animals, there was marked subpleural emphysema and

TABLE 1. BHV-1 plaque reduction by commonly used anti-herpesvirus drugs

Drug[a]	MIC (μg/ml)[b]	MTC (μg/ml)[c]	TI[d]
PFA	30	500	17
Ara-C	0.5	5	10
F_3dT	0.01	0.5	50
IdU	5	10	2
ACV	>1,000	500	
Ara-A	200	100	0.5
Ara-AMP	750	1,000	1.3
BVdU	0.01	50	5,000

[a] PFA, Phosphonoformic acid; Ara-C, 1-β-D-arabinofuranosylcytosine; IdU, 5-iodo-2'-deoxyuridine; Ara-A, 9-β-D-arabinofuranosyladenine; Ara-AMP, 9-β-D-arabinofuranosyl-AMP.

[b] MIC, Minimal inhibitory concentration of drug inhibiting virus plaque formation by >50%.

[c] MTC, Minimal toxic concentration of drug inducing visible microscopic alterations in confluent GBK cell monolayers.

[d] TI, Toxicity index; calculated by dividing the lowest concentration inducing evidence of toxicity by the lowest concentration inhibiting virus plaque production.

bullous emphysema dissecting the septa of the caudal lobe of the lung. There was little evidence of fibrin on the visceral or parietal pleura. The histological lesions confirmed the gross findings at necropsy and included lobules obscured by hemorrhage, collection of alveolar macrophages resembling oat-cell macrophages, bronchiolitis, alveolar edema, subpleural fibrin, and distended interlobular septa present in both control and BVdU-treated animals. There were no discernible differences between the histological lesions of the treated animals and those of the untreated animals. Furthermore, large numbers of virus and bacteria were isolated from the lungs of both groups.

In an attempt to determine the reason why the drug, which was so effective in vitro, had no effect in vivo, two approaches were used. First, it was hypothesized that the virus became resistant to the drug, as often occurs for herpesviruses grown in the presence of antiviral drugs (30). To test this possibility, the virus shed from nasal passages of animals was tested for its susceptibility to BVdU in vitro. In no case could we detect increased resistance to the drug between the original challenge virus and recovered virus (data not shown). Second, we assayed the serum for the presence of BVdU by a virus inhibition bioassay. We were unable to detect any drug in the sera of BVdU-treated animals, even though our bioassay could demonstrate inhibition of virus in the infectious center assays or plaque reduction assays at BVdU concentrations of 0.01 μg/ml.

DISCUSSION

Most of the chemicals that have been previously shown to be capable of inhibiting herpes simplex virus replication (7) were shown in the present study to have similar activity against BHV-1. One obvious exception was ACV, which has recently gained popularity as a drug for treating herpesvirus infections in humans (14, 22, 31). In the case of BHV-1, ACV was totally ineffective even at 1,000 μg/ml, at which both the number and size of plaques was unaltered. We do not feel that this resistance to ACV was due to accidentally choosing an isolate that had an altered viral thymidine kinase which could not convert the drug to the monophosphate or because of an altered DNA polymerase (30), since a similar resistance pattern was observed with a large number of BHV-1 isolates (data not shown). Thus, we feel that BHV-1 is inherently resistant to ACV. Our present results support those of our previous studies demonstrating that ACV could not be phosphorylated by virus-induced thymidine kinase (32). Other drugs which act at the level of the viral DNA polymerase or thymidine kinase are equally effective against BHV-1 and herpes simplex virus, suggesting that although the BHV-1 virus-specified enzymes have some similarities to herpes simplex virus enzymes, there are also some important differences.

When the susceptibility of BHV-1 to BVdU was compared with that reported for herpes simplex virus types 1 and 2, it was found that the susceptibility pattern of BHV-1 more closely resembled that of HSV-1 (7, 8). Thus, the minimal inhibitory concentration was about 0.01 μg/ml for both BHV-1 and HSV-1. In contrast, the minimal inhibitory concentration for HSV-2 is 10 to 100 times as high (7, 8).

TABLE 2. Effect of F_3dT and BVdU on infectious-center production by BHV-1

Drug	No. of infectious centers produced by drug concn (μg/ml) of[a]:			
	0	0.01	0.05	0.1
F_3dT	$6 \times 10^4 \pm 1 \times 10^4$	$5 \times 10^4 \pm 0.7 \times 10^4$	$8.5 \times 10^3 \pm 0.5 \times 10^3$	$4.6 \times 10^2 \pm 0.5 \times 10^2$
BVdU	$6 \times 10^4 \pm 1 \times 10^4$	$5.5 \times 10^4 \pm 0.6 \times 10^4$	$3.4 \times 10^3 \pm 0.6 \times 10^3$	$6.2 \times 10^2 \pm 0.7 \times 10^2$

[a] Numbers represent mean ± standard deviation infectious centers produced per culture infected with 100 PFU of BHV-1 48 h earlier.

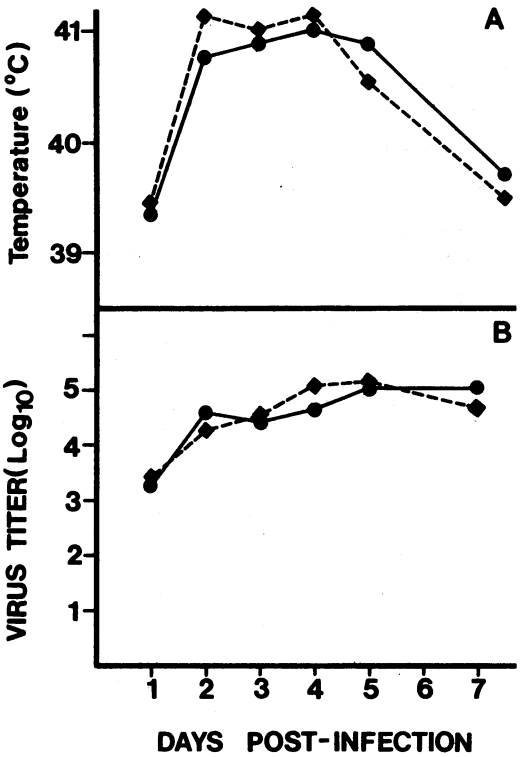

FIG. 2. Temperature and BHV-1 virus shedding in control or BVdU-treated calves. (A) Temperature responses of BVdU-treated (●——●) or untreated (■------■) BHV-1-infected animals; (B) BHV-1 virus shedding in nasal secretion of BVdU-treated (●——●) or untreated (■------■) BHV-1-infected animals.

Since BVdU was so effective against BHV-1 and previous studies have shown that oral administration of BVdU is effective in preventing herpes simplex virus type 1 replication in animals (9, 10, 20), we felt that this drug might provide an attractive means of controlling BHV-1-induced pasteurellosis. This seemed especially attractive since most cases of pasteurellosis occur shortly after entry of cattle into feedlots, when exposure to stress and viral agents predispose the animals to this complex disease. Thus, it appeared feasible to administer the drug during this short time period to reduce losses due to the synergistic interaction of virus and bacteria. Unfortunately, the drug did not have any effect on virus replication in vivo or viral-bacterial synergistic interactions under the present circumstances. We surmise that the main reason for this lack of activity was that the drug did not enter the bloodstream. Since the bioavailability of any drug given orally or parenterally is the limiting factor in its efficacy, it appears that the oral administration of BVdU is not the route of choice in cattle. A possible reason that the drug has been effective when given orally in other animals is that other studies involved monogastric animals. The rumen is known to have a totally different flora than the stomach of monogastric animals, and therefore many nutrients and drugs are broken down in the rumen which would pass unchanged through the stomach of monogastric animals. This is possibly what happened with BVdU, since it was not present in the bloodstream. However, the drug might be very effective in vivo when administered intramuscularly, intravenously, or by aerosols directly into the lung. The reason that this approach was not used in this study is that the aqueous solubility of BVdU is <5 mg/ml. Thus, to receive a dose of 5 mg/kg per day, an average animal would require a daily injection or aerosolization of approximately 300 to 500 ml. This appeared to be impractical at the time of this study; however, it may be possible to develop other delivery systems for intramuscular use or by packaging BVdU in a capsule that would be released only in the intestines. Such studies are presently under way.

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