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Intranasal treatment of cowpox virus respiratory infections in mice with cidofovir

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Abstract

Orthopoxvirus infections in mice have been effectively treated with cidofovir, a clinically approved drug given by intravenous infusion to treat cytomegalovirus infections. In a bioterrorist scenario it would be technically difficult to give this drug to a large number of exposed individuals. New treatment approaches are being sought, which include giving cidofovir by alternative routes or designing oral prodrugs of cidofovir. In this report, intranasal cidofovir was investigated as a treatment of pulmonary cowpox virus infections in BALB/c mice. Ninety to 100% of animals given a single intranasal drug treatment (10, 20 or 40 mg/kg) 24 h after virus challenge survived the infection, whereas all placebo-treated mice died. Doses of 2.5 and 5 mg/kg resulted in 60 and 80% survival, respectively. Single treatments of 20 and 40 mg/kg could be given up to 3 days after virus inoculation and still be 80–90% protective. A single 40 mg/kg treatment of infected mice given 1 or 2 days after infection also resulted in statistically significant decreases in virus titer in lungs and nose/sinus compared to the placebo group. Drug efficacy was found to be contingent upon treatment volume. A 10 mg/kg intranasal dose given 24 h after virus challenge was 100 and 50% effective in volumes of 40 and 20 μl, respectively. The same dose in 5 and 10 μl volumes caused no decrease in mortality. The results of these studies establish the utility of cidofovir treatment of poxvirus infections in mice by intranasal route. The data suggest the possibility that aerosol delivery of cidofovir to human lungs may be a viable alternative to intravenous dosing.

Keywords: Antiviral; Cidofovir; Cowpox virus; Intranasal treatment

1. Introduction

The concerns surrounding the use of smallpox or monkeypox viruses as bioterrorist weapons (Breman and Henderson, 1998; Orent, 1998) have prompted the need to investigate new therapies for the treatment of these infections. A limited number of antiviral treatments for orthopoxvirus infections have been studied. Ribavirin was effective against vaccinia virus infections in mice (De Clercq et al., 1976), and in combination with vaccinia immune globulin against a vaccinia infec-

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tion in an immunosuppressed human (Kesson et al., 1997). Ribavirin treatments delayed the mean day to death but did not prevent mortality in an intranasal cowpox model in mice (Smee and Huggins, 1998). Cidofovir has shown a high degree of efficacy against vaccinia virus infections in mice (Neyts and De Clercq, 1993) and in the cowpox virus model (Bray et al., 2000). Single (one time only) subcutaneous cidofovir treatments given at various times pre- or post-virus challenge were protective to cowpox virus-infected mice. Cidofovir has also been reported to be effective in treating molluscum contagiosum infections in immunosuppressed humans (Meadows et al., 1997). Overall, the potency of cidofovir far exceeds that of ribavirin against orthopoxvirus infections.

In a bioterrorist scenario the number of exposed individuals is expected to be large. The problem with using cidofovir under these conditions is that the drug must be given intravenously. Oral bioavailability is less than 5%, and subcutaneous injections of cidofovir cause localized tissue damage (Wachsman et al., 1996). Therefore, it is important to investigate other means whereby the drug may be administered. Certainly an orally active prodrug form of cidofovir would be ideal, but no such compound has been developed.

The approach used in this report was to study the treatment of the respiratory infection caused by cowpox virus with intranasally (i.n.) administered cidofovir. In this model the mice develop a severe upper and lower respiratory tract infection that in many ways mimics life-threatening smallpox infection. Cowpox virus infections in mice initiated by i.n. or aerosolized virus challenge have recently been characterized (Bray et al., 2000; Martinez et al., 2000). Visible illness (ruffled fur, slowed activity, and weight loss) are evident by day 5. Mice develop diffuse, bilateral viral pneumonitis, with lesions most commonly located in small bronchioles. Lesions are also evident in airways, trachea, nasal passages, and sinuses. Lung weights increase and may exceed three times normal weight by the time of death. The majority of the recovered virus is in the respiratory tract (lungs, nasal passages, and sinuses), with 1000-fold less virus found in the liver and spleen (Bray et al., 2000). Virus is rarely detected in the blood. In this report we use the cowpox i.n. infection model in mice to evaluate the efficacy of i.n. cidofovir treatment, and discuss the potential of such treatment to combat orthopoxvirus infections in humans.

2. Materials and methods

2.1. Antiviral compound

1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl] cytosine (cidofovir) (Hitchcock et al., 1996) was obtained from Norbert Bischofberger of Gilead Sciences (Foster City, CA). It was dissolved in sterile saline for i.n. treatments of mice.

2.2. Virus and cells

Cowpox virus (Brighton strain) was obtained from John Huggins, US Army Medical Research Institute of Infectious Diseases (Ft Detrick, Frederick, MD) as a twice plaque-purified isolate. The virus originated from the Centers for Disease Control and Prevention (Atlanta, GA). The virus was propagated in African green monkey kidney (Vero) cells, obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in Eagle's medium containing 10% fetal bovine serum. The serum concentration was reduced to 2% for titrations and for virus propagation.

2.3. Antiviral experiments in animals

Female BALB/c mice weighing about 15 g each were obtained from B&K Universal (Fremont, CA). The animals were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) followed by i.n. administration of 5×10^5 plaque forming units (PFU) of cowpox virus per animal in a 50 μl volume. Intranasal treatments with cidofovir and placebo (saline) were performed in mice similarly anesthetized with ketamine. The standard treatment regimen was administration of drug or placebo in a 50 μl volume 24 h after virus challenge, with modifications as noted in Tables 2 and

3. For all studies, only a single i.n. treatment was given. Uninfected toxicity control mice (five per group) similarly treated i.n. with cidofovir or placebo were used to establish the safety of the treatments.

2.4. Titration of virus from animal tissues

At selected times the lungs from infected mice were removed. The nose, nasal passages, and sinuses (hereafter referred to as nose/sinus tissue) were collected as a unit by removing the animal's face from inside the upper jaw to just below the eyes. The necropsied tissues were stored frozen at −80°C in 1 ml of cell culture medium. Later the tissues were thawed and homogenized using sterilized mortars and pestles, then were sonicated 30 s each. Homogenates of three mice from each treatment group were titrated separately for virus by plaque assay in Vero cells. Twelve-well plates of cells were infected with 10-fold virus dilutions in 0.1 ml increments. After virus adsorption for 1 h (rocking plates every 5 min during that time), the cells were fed maintenance medium containing 2% serum. At 2 days the cells were fixed, stained (using 0.1% crystal violet in 50% ethanol/3% buffered formalin), and plaques counted.

Table 1
Effect of i.n. treatment with cidofovir on a cowpox virus respiratory infection in mice

Cidofovir[a] (mg/kg)	Survivors/total	MDD ± SD[b]
40	10/10**	>21
20	9/10**	9.0 ± 0.0
10	10/10**	>21
5	8/10**	13.0 ± 2.8*
2.5	6/10*	10.5 ± 0.6*
0	0/10	8.4 ± 0.7

[a] Treatments were given one time only 24 h after virus challenge.

[b] Mean day to death ± standard deviation of mice that died prior to day 21.

* $P<0.05$; ** $P<0.001$; compared to the saline-treated control group.

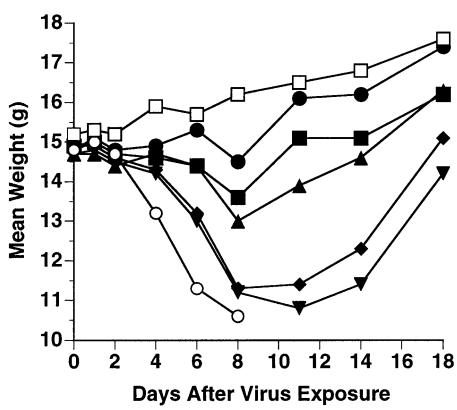

Fig. 1. Mean weights of mice during the course a cowpox virus respiratory infection. Animals were treated i.n. one time only at 24 h post-virus exposure with cidofovir or placebo. Symbols: ○, placebo; ●, cidofovir, 40 mg/kg; ■, cidofovir, 20 mg/kg; ▲, cidofovir, 10 mg/kg; ◆, cidofovir, 5 mg/kg; ▼, cidofovir, 2.5 mg/kg; □, untreated, uninfected.

2.5. Statistical evaluations

Statistical interpretations of increases in numbers of survivors were determined by the Fisher exact test. The Mann–Whitney *U*-test statistically analyzed increases in mean day to death and tissue virus titer reduction. Significant differences in mean animal weights on infection day 8 were determined using the Student's *t*-test. All statistical evaluations were two-tailed, and compared cidofovir-treated groups to the respective placebo control group.

3. Results

3.1. Dose–response effect of intranasal cidofovir

Mice were infected with cowpox virus and treated the next day with cidofovir at five different concentrations (Table 1). All of the placebo-treated animals died of the infection between 7 and 9 days after virus challenge. Intranasal treatment with cidofovir was highly protective, especially at doses of 10, 20 and 40 mg/kg. Significant numbers of animals survived in groups treated with 2.5 and 5 mg/kg. The mean days to death of mice that died were longer than that of the placebo group. Weight loss during the course of the infection, which is a characteristic feature of the disease (Bray et al., 2000), is shown in Fig. 1.

Table 2
Effect of varying the i.n. cidofovir treatment time on a cowpox virus respiratory infection in mice

Cidofovir[a] (mg/kg)	Time of treatment[b]	Survivors/total	MDD ± SD[c]
40	1	10/10***	>21
20	1	10/10***	>21
40	2	10/10***	>21
20	2	9/10***	12.0 ± 0.0
40	3	8/10***	13.5 ± 2.1
20	3	9/10***	9.0 ± 0.0
40	4	6/10*	13.0 ± 2.5**
20	4	0/10	11.7 ± 2.1***
40	5	0/10	10.7 ± 0.8***
20	5	0/10	12.3 ± 3.3**
Placebo	1	0/10	8.5 ± 0.7

[a] Treatments were given one time only on the day indicated.
[b] Day post-virus exposure.
[c] Mean day to death ± standard deviation of mice that died prior to day 21.
* $P<0.05$; ** $P<0.01$; *** $P<0.001$; compared to the saline-treated control group.

It is evident from this figure that even though the majority of mice treated with 2.5 and 5 mg/kg survived the infection, they became very ill (as indicated by severe weight loss) and were near death at the peak of the infection (day 8). Weight losses during the course of the infection in the 10, 20 and 40 mg/kg groups were much less severe, indicating milder disease. On day 8 of the infection, the mean weights of the 10, 20, and 40 mg/kg groups were significantly different ($P<$ 0.01) than the placebo controls.

Uninfected mice treated a single time with cidofovir at doses of 40 and 20 mg/kg were compared to the placebo group for changes in weight. Mean weights of groups initially declined as a result of ketamine anesthesia plus i.n. treatment. Mean weight between days 0 and 1 were -0.4, -0.4, and -0.3 g for the 40, 20, and 0 mg/kg groups, respectively. The weight differences between days 0 and 5 were 0, $+0.2$, and $+0.3$ g, respectively. Overall, the i.n. delivered drug appeared to be well tolerated.

3.2. Effect of time of initiation of treatment on outcome

Bray et al. (2000) showed that subcutaneous treatments with cidofovir could be given at varying times before and after virus inoculation to provide survival benefit. Our studies were designed to determine whether similar results could be obtained using i.n. treatments given after virus challenge. Mice were treated one time with either 40 or 20 mg of cidofovir/kg at 1, 2, 3, 4 or 5 days after virus inoculation (Table 2). A high rate of survival was evident in groups treated on days 1–3. The 40 mg/kg dose provided some protection to the group treated on day 4. All other treatments did not prevent mortality, but did extend the mean day to death significantly. Groups of animals treated with placebo and 40 mg/kg were sacrificed on a daily basis to determine virus concentrations in the lungs and nose/sinus (Fig. 2). Lung and nose/sinus virus titers were reduced in mice treated on days 1–3 relative to the placebo group. After that time of treatment initiation, the tissue virus titers had already peaked. Mean virus titers were calculated for days 2–8 of the infection. The 40 mg/kg dose given on day 1 reduced lung and nose/sinus virus titers by 150- and >10 000-fold, respectively. The dose given on day 2 reduced titers by 15- and 50-fold, respectively. These results were statistically significant ($P<0.01$). Treatments caused a greater suppression of virus titer in the nose/sinus than they did in the lungs.

3.3. Effect of treatment volume on drug efficacy

It was reasoned that treatment volume is a factor in determining how much drug penetrated into the lungs, which could directly impact on treatment efficacy. A large drug volume should deliver more drug to the lungs than a smaller volume. To determine the impact of drug volume on treatment efficacy, infected mice were treated i.n. with 10 mg/kg in varying treatment volumes (Table 3). The 5 μl dose was a highly concentrated solution that essentially only penetrated to the nose/sinus region. The larger volumes were expected to reach deeper into the mice. All of the animals receiving the 40 μl dose survived the infection. Half of the mice treated with the 20 μl dose survived. The 10 and 5 μl doses were not effective in preventing mortality, although at 10 μl/mouse the animals as a group lived significantly longer than placebo-treated mice. These results clearly demonstrate that a large treatment volume (relative to the size of the animal) was needed for maximum efficacy.

4. Discussion

The results of these studies demonstrate that i.n. cidofovir treatment of cowpox virus infections in mice are effective in preventing mortality, reducing virus concentrations in the lungs and nose/sinus tissues, and reducing morbidity in surviving animals (as evidenced by improved weight gain). Concentrations of drug as low as 2.5 mg/kg given as a single i.n. instillation provided significant survival benefit to the animals. Treatments with

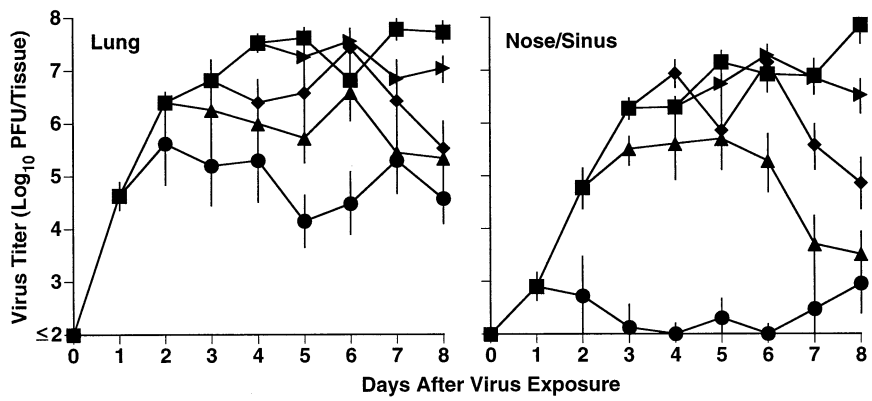

Fig. 2. Effects on lung and nose/sinus virus titers in mice treated i.n. with 40 mg/kg of cidofovir at varying times after i.n. cowpox virus challenge. Symbols: ■, placebo treatment on day 1; ●, cidofovir treatment on day 1; ▲, cidofovir treatment on day 2; ◆, cidofovir treatment on day 3; , cidofovir treatment on day 4.

Table 3
Effect of varying the i.n. cidofovir treatment volume on a cowpox virus respiratory infection in mice

Cidofovir[a] (mg/kg)	Treatment volume (μl)	Survivors/total	MDD ± SD[b]
10	40	10/10***	>21
10	20	5/10*	11.8 ± 1.5***
10	10	0/10	10.3 ± 1.5**
10	5	0/10	9.5 ± 0.5
0	40	0/10	8.9 ± 0.6

[a] Treatments were given one time only 24 h after virus challenge.
[b] Mean day to death ± standard deviation of mice that died prior to day 21.
* $P<0.05$; ** $P<0.01$; *** $P<0.001$; compared to the saline-treated control group.

doses of 20 and 40 mg/kg were even beneficial when given three days after virus challenge.

Recently Bray et al. (2000) reported the treatment of intranasal and aerosol cowpox virus infections in mice by subcutaneous administration of cidofovir. Single treatments of 100 mg/kg increased survival by $>70\%$ when given on various days from 8 days before to 3 days after virus challenge. Effective doses prevented weight loss and suppressed lung virus titers as we have shown. They found multiple treatments (every 3 days) to be more effective than a single dose. Extrapolating from their results, we would expect intranasal cidofovir treatments to be more effective when given multiple times. Such studies are planned for the future.

Single i.n. treatments of cidofovir did not cause overt toxicity to the animals. The drug is known to cause renal toxicity in humans when administered intravenously, and is given with probenecid to reduce this toxicity (Naesens et al., 1997). Intranasal delivery of cidofovir, which provides a more localized effect than by parenteral administration (albeit the drug must be cleared through the kidneys in order to exit the body), may possibly be a safer method of delivery. Higher localized concentrations of drug in the respiratory tract may be achieved than by parenteral administration.

The efficacy of cidofovir in the treatment of cowpox virus respiratory infection was greatly influenced by treatment volume. Complete protection was only achieved when a 10 mg/kg dose was administered in a 40 μl volume, with the 20 μl volume being only 50% protective. High volumes would tend to distribute more drug to the lungs. In a related study, intranasal instillation of a radioactive nucleoside to anesthetized mice in a 50 μl volume was shown to distribute compound to the lungs, nose/sinus region, mouth, and stomach (Smee et al., 1990). But only 12% of the recovered radioactivity was found in the lungs. These results combined with the mortality data in Table 3 suggest that administration of low treatment volumes would afford minimal delivery of cidofovir to the lungs. Lower volumes should produce a localized antiviral effect in the nose/sinus region; this amount of drug would then presumably be cleared through the intestinal tract. Previous research has shown that cidofovir has very low oral bioavailability (Wachsman et al., 1996), thus, drug passing through the gut would provide no benefit to the host.

The survival results obtained when cidofovir was given in small volumes cast doubt on the feasibility of treating poxvirus infections in humans by this route. It is not possible to instill large doses of liquid into the human respiratory tract without undue risk. The 40 μl volume required for maximum protection of mice is quite large relative to the size of the animal. This equates to a dose of 2666 μl/kg of animal (for a 15 g mouse). If a 70 kg human received 1 ml of drug through an inhaler or spray device, this would equate to a volume of only 14 μl/kg. An effective human mg/kg dose cannot be achieved merely by increasing the drug concentration because of solubility limitations. Frequent applications of the compound would be a way to increase the total dose, however.

Zanamivir is effective in the treatment of influenza virus infections in humans when administered by an oral inhalation device (Monto et al., 1999). It is probable that a lower dose of zanamivir is required to treat influenza than for cidofovir to treat an orthopoxvirus infection. This is because zanamivir is about 30-fold more potent than cidofovir against the respective virus in cell culture (zanamivir inhibits influenza virus in cell culture at 0.1 μM (Woods et al., 1993) compared to cidofovir activity against cowpox virus at 3 μM (D.F. Smee, unpublished).

Aerosol delivery of cidofovir given over a period of time may be a viable alternative route of cidofovir administration. In a bioterrorist scenario where many exposed individuals would need to be treated, small particle aerosol administration using aerosol delivery devices available in many hospitals may be feasible. Because the drug is very effective when given even a single time, coupled with the fact that treatment can be delayed for a few days after virus exposure, this would allow time for a number of patients to be treated in this manner.

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