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Differential pathogenesis of cowpox virus intranasal infections in mice induced by low and high inoculum volumes and effects of cidofovir treatment

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Abstract

The causes of death from intranasal cowpox virus infections in mice remain unclear. Hypotheses include severe pneumonitis, hepatitis, and/or hyperproduction of cytokines and chemokines. This work explores these hypotheses by studying the influence of low and high volume virus inoculums on viral pathogenesis. BALB/c mice were infected intranasally with a syncytium-forming variant of cowpox virus in 5- μ l or 50- μ l volumes containing the same infectious virus challenge dose. The 50- μ l infection produced a more rapidly lethal disease associated with severe pneumonitis, high lung and nasal virus titers, and increases in cytokine and chemokine levels in lungs and nasal tissue, while liver infection was minimal. The 5- μ l inoculum infection was also lethal, but the infection was primarily confined to the upper respiratory tract, and included elevated nasal cytokine and chemokine levels. The pro-inflammatory cytokine, interleukin-6, was particularly high in both infections. Treatment of the infections with cidofovir (100 mg/kg/day for 2 days starting 24 h after virus exposure) led to survival and suppression of tissue virus titers. Treatment reduced pneumonitis in the 50- μ l infection, and lessened cytokine hyperproduction in both infections. We conclude that 5- μ l volume inoculum of cowpox virus causes a lethal upper respiratory tract infection, while the 50- μ l inoculum targets both upper and lower respiratory tracts, with excessive release of systemic pro-inflammatory factors occurring. Cidofovir effectively treated both infections and slowed viral replication sufficiently to subdue the exaggerated release of pro-inflammatory mediators.

Keywords

orthopoxvirus; cowpox; antiviral; cidofovir; cytokines; chemokines

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1. Introduction

Intranasal (i.n.) infection of mice with cowpox (Brighton strain) virus has been used as a small animal model to study treatments with antiviral agents [1,2]. Mice develop severe pneumonitis by this route of infection [3], and the virus disseminates to many organs and tissues [4]. Questions have been raised about the pathogenic factors that contribute to death of the animals, with three hypotheses proposed. The first hypothesis is that death results from severe pneumonitis. Results from the mouse model appear to support this hypothesis [1–3]. However, other investigators believe hepatitis is the cause of death. The rationale for this hypothesis is that although cidofovir protects mice from lethal cowpox virus infection, the surviving mice have only moderate decreases in lung virus titer, yet exhibit much more profound declines in liver virus titer [5,6]. A third proposed factor that may lead to death is the overproduction of inflammatory mediators (commonly referred to as a “cytokine storm”) during such infections. Recent work by our group demonstrated highly elevated levels of a number of cytokines and chemokines during i.n. cowpox and vaccinia virus infections in mice [7]. In that report, i.n. and intraperitoneal (i.p.) infections with cowpox and vaccinia viruses were compared; the i.n. infection leading to lethal pneumonitis and the i.p. infection producing fatal hepatitis with an absence of pneumonitis. Thus, data supporting all three of the hypotheses exist.

This research explored whether the contributions of severe pneumonitis, hepatitis, or cytokine hyperproduction are necessary components of lethal cowpox virus infections in mice. The work was facilitated by the discovery that low (5- μ l) volume i.n. infections with a syncytium-forming cowpox virus strain (the strain of virus was first described by Smee et al. [8]) confines the infection primarily to the upper respiratory tract, yet is sufficient to kill mice. Various features of the pathogenesis of the low, compared to high, virus inoculum models using the same dose of infecting virus are characterized, and the effects of cidofovir treatment on the infections are presented.

2. Materials and methods

2.1. Animals

Female 13–15 g specific pathogen-free BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA, USA). They were quarantined 48 h prior to use and maintained on commercial rodent chow and tap water in the AAALAC-accredited Laboratory Animal Research Center at Utah State University.

2.2. Virus

A clone of wild-type cowpox virus (Brighton strain) that produces syncytia in cell culture [8] was isolated at the U.S. Army Medical Research Institute of Infectious Diseases (Ft. Detrick, Frederick, MD, USA). This virus is designated as a syncytium-forming (SF) cowpox virus. It was selected for these studies because this strain of virus was more virulent in titration experiments than the more typical form of cowpox virus causing rounded cell cytopathology when given by i.n. route to mice in both small (5 μ l) and large (50 μ l) volume inoculations (D.F. Smee, unpublished data). Neither of these virus strains was passaged in mice to enhance virulence, but they are by nature lethal by i.n. infection route when given in sufficient infectious doses. The SF virus was propagated in African green monkey kidney (MA-104) cells for use in these experiments.

2.3. Antiviral compound

Cidofovir was obtained from Mick Hitchcock of Gilead Sciences (Foster City, CA, USA). It was dissolved in sterile saline for treatment of the mice. Sterile saline was used as the placebo control.

2.4. Animal experiment design

Intranasal infections were performed under ketamine anesthesia (100 mg/kg by i.p. injection). Mice were infected i.n. with approximately 5×10^5 plaque-forming units of virus per animal in either a 5 or 50 μ l volume. Ten mice per group were held until death. Tissue virus titer determinations were made using 5 additional mice per group at each time of sacrifice. For the cidofovir studies, mice were treated i.p. with either the drug (100 mg/kg/day) or placebo control once a day for 2 d starting 24 h after virus exposure. The short-term drug treatment regimen that was used was based upon successful therapy of other poxvirus infections [7,9]. Virus titer determinations were made from lung, snout, liver, spleen and brain tissues taken on various days of the infection. The lungs were removed and assigned a consolidation score ranging from 0 (normal appearance) to 4 (entire lung exhibiting an abnormal plum color), being categorically graded in 0.5-unit increments. The lungs and other tissues (after weighing) were frozen at -80° C. For virus titer determinations, thawed soft tissues were homogenized in 1 ml of cell culture medium using a stomacher. Snouts and associated sinus tissue were ground in 1 ml medium using sterilized mortars and pestles. After homogenization the samples were centrifuged at $600 \times$ gravity for 5 min to pellet debris. Samples were serially diluted in 10-fold increments and plaque titrated in African green monkey kidney (Vero) cells in 12-well microplates. Cell monolayers were stained at three days with 0.2% crystal violet in 10% buffered formalin, and virus plaques were counted with the aid of a light box. Plaque numbers were converted to plaque forming units (PFU) per gram of tissue.

2.5. Histopathological examinations

Tissues from sacrificed mice were immediately placed in 10% buffered formalin. The samples were thin sectioned, stained with hematoxylin-eosin, and examined microscopically at the Utah Veterinary Diagnostic Laboratory (Logan, UT, USA). A written description of the observed pathogenesis for each sample was recorded.

2.6. Cytokine and chemokine profiles in infected mice

Mice (6 animals per group) were sacrificed for cytokine and chemokine determinations 7 days after infection in the 50- μ l infection or 9 days after infection in the 5- μ l group. These times coincided with days near death of the respective placebo group. It was previously shown that cytokines and chemokines are maximal near death of infected mice [7]. Serum was first obtained from the sacrificed mice. Lungs and snouts (with accompanying sinus tissue) were collected and each sample was homogenized in 1 ml of cell culture medium as described above. The samples were frozen at -80° C, then were thawed and analyzed using a chemiluminescent ELISA-based assay (Quansys Biosciences Q-Plex™ Array, BioLegend, San Diego, CA) according to the manufacturer's instructions. Clarified tissue samples and serum were assayed for the following: GM-CSF, IFN- γ , IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, MCP-1, MIP-1 α , RANTES, and TNF- α . Definitions of abbreviations are: GM-CSF - granulocyte/macrophage colony stimulating factor; IFN - interferon; IL -interleukin; MCP - monocyte chemoattractant protein; RANTES - regulated on activation, normal T expressed and secreted; TNF - tumor necrosis factor. Some of the cytokines and chemokines were not appreciably elevated at the sampling times during the infection, thus, the data for those are not reported.

2.7. Statistical analysis

The two-tailed Fisher exact test was used to assess numbers of survivors. The two-tailed Mann-Whitney U-test was used to evaluate mean day of death, mean lung consolidation score, mean lung weight, mean tissue virus titer, and body weight (area under the curve) parameters. The Mann-Whitney test is a non-parametric test that does rank-sum analysis between sets of data, rather than comparing means and standard deviations. The two-tailed Student's t-test was used

for cytokine and chemokine analyses. Statistical comparisons were made between respective cidofovir-treated and placebo groups, except where indicated.

3. Results

3.1. Comparative pathogenesis of 5- and 50- μ l volume cowpox virus infections

Infection of mice with cowpox virus by i.n. route resulted in 90–100% mortality with mean days of death of approximately 10 and 8 days for the 5 and 50 μ l volume infections (each having the same virus infectious dose), respectively. Lung consolidation scores and lung weights were determined at various times after infection (Figure 1). The 50- μ l volume infection induced severe pneumonitis, as depicted by high lung consolidation scores and lung weights, particularly on day 8 post-infection. Minimal lung consolidation scores and lung weight increases were noted in the group infected with the 5 μ l volume of virus.

Cowpox virus titers in five tissues during the infections are shown in Figure 2. The 5- μ l infection resulted in high virus titers in nasal tissue. Considerably less (over 1000-fold) virus titers were detected in lungs, spleen, liver, and brain. The 50- μ l infection resulted in high viral loads in both lungs and in the nasal tissue, with lung virus titer being up to 10-fold greater than virus titer in the snout. Less virus was produced in the spleen, liver, and brain in the 50- μ l infection. Notably, barely detectable amounts of liver virus were seen during the course of the low volume infection, with only minimal levels present on day 8 of the higher volume infection, in contrast to the liver involvement reported with infections with other strains of cowpox and vaccinia viruses [4].

3.2. Histopathological analysis of infected tissues from mice infected with 5 and 50 μ l volumes of cowpox virus

Lungs and nasal tissues of infected animals were examined histopathologically. A description of the pulmonary pathology of placebo-treated mice that were near death from the 50- μ l volume infection (day 7) is as follows: Edema fluid and interspersed fibrin strand filled 70–80% of air spaces, and free erythrocytes filled some alveolar spaces. Many medium and small diameter bronchioles were lined by piled layers of swollen epithelial cells that occasionally occluded airway lumens. Large, clear, indiscrete cytoplasmic vacuoles were in the cytoplasm of bronchiolar epithelial cells. Most bronchiolar epithelial cells had variably-sized, but generally large, circular, brightly eosinophilic intracytoplasmic inclusion bodies. Alveolar macrophages and lymphocytes were scattered within bordering alveolar air spaces. These findings are consistent with severe pneumonitis reported previously [3]. A description of pathology of infected nasal tissues from these same mice is as follows: The lumen of the nasal cavity contained bands of fibrin and mucus mixed with large numbers of neutrophils, shards of organic foreign material and multiple small colonies of coccoid bacteria. Fibrinomucoid and suppurative rhinitis was moderate.

Lungs of placebo-treated mice that were near death from the 5- μ l volume infection (day 9) were normal in appearance. A description of pathology of the nasal tissues in the same infection is as follows: Multiple piled layers of proliferative epithelial cells lined the nasal cavity. Lining cells had multiple, indiscrete, clear vacuoles, and many contained eosinophilic, round, intracytoplasmic inclusion bodies. Sloughed epithelial cells, neutrophils, and moderate amounts of fibrin, mucus, organic foreign material and coccoid bacteria were within the lumen. The mucosuppurative rhinitis was severe.

3.3. Treatment of 5- and 50- μ l volume cowpox virus infections with cidofovir

Mice were infected with cowpox virus and then treated once a day for 2 days with cidofovir at 100 mg/kg/day (Table 1). The drug prevented death in all mice for both the 5- μ l and 50- μ l

volume infections. Lung consolidation scores, lung weights, and lung virus titers were low in the 5- μ l infection treated and placebo groups, and cidofovir treatment did not significantly reduce these parameters. Nasal virus titers were very high, and treatment with cidofovir significantly, although minimally, reduced virus production. In the 50- μ l infection groups, treatment with cidofovir caused statistically significant reductions in lung consolidation scores and lung weights. Lung and nasal virus titers were also high, and were not reduced by cidofovir treatment on the day of assay (day 8 after infection). Although this initially was a curious finding since the mice survived the infection and one would have anticipated seeing an antiviral effect, the results were clarified by further research (below).

Mean body weights during the infection are shown in Figure 3. Weight loss was more severe in the 50- μ l groups than in the 5- μ l groups ($P < 0.001$). Improvements in body weight were seen in the cidofovir-treated groups compared to respective placebos, but statistical significance ($P < 0.01$) was only evident in the 5- μ l infection. All of the infected mice lost weight compared to normal uninfected control animals.

Based upon the data shown in Table 1, it was evident that lung and nasal virus titers determined from tissues taken on day 8 of the infection were negligibly impacted by cidofovir treatment. This seemed anomalous since the mice survived the infection, suggesting that reductions in virus titer should have occurred. A follow-up study determined virus concentrations over the course of the acute phase of the infection to help clarify the matter (Figure 4). In the 50- μ l infection, lung virus titers were significantly reduced by cidofovir treatment on days 2, 4 and 6. Nasal virus titers were reduced by treatment on days 4 and 6. In the 5- μ l infection the amount of lung virus titer produced remained low at all times, and no significant reduction due to cidofovir treatment was evident. However, nasal virus titers were significantly reduced on days 2, 4, and 6 of the infection. By day 8 virus titers in both cidofovir-treated and placebo groups were similar for the 5- and 50- μ l infections, indicating virus rebound in the cidofovir group at that time during the infection. Virus rebound likely occurred as a result of the fact that treatment only went through day 2 of the infection.

The extent of serum, lung and nasal cytokine and chemokine production in placebos during the latter days of the infections are presented in Table 2. Mice infected with 5 and 50 μ l of virus had significantly higher levels of most of the factors reported in Table 2 in the lungs, serum, and nasal region compared to uninfected animals. Statistical comparisons were also made comparing factors induced during the 5- μ l infection versus the 50- μ l infection (Table 2). There were significantly lower levels of the majority of cytokines and chemokines in the lungs of mice infected with the 5- μ l volume compared to animals infected with the higher volume. Significantly higher levels of IL-6 were present in snouts of mice infected with the lower volume, however. The largest increases in cytokine production for both infections were IL-6 and IL-9 levels, with the exception of low IL-6 induction in lungs of mice infected with the 5- μ l volume.

The effects of cidofovir treatment in attenuating the cytokine response in mice, as a result of suppressing virus replication, are shown in Table 2. All cytokines in the serum were significantly reduced by drug treatment in 5- and 50- μ l infections, except for IL-9 in the 50- μ l infection. In the 5- μ l infection the following cytokines were reduced in lungs: IL-2, IL-9, and IL-10. IL-6 was significantly reduced in nasal tissue by cidofovir treatment. In the 50- μ l infection, IL-6, IL-9, and IL-10 were reduced in lungs by antiviral treatment, as was IL-6 in nasal tissue. In general, only moderate decreases in cytokine levels resulted from cidofovir treatment at the time of assay, since most of the factors were still well above the levels seen in uninfected mice. There were some exceptions where serum levels of IFN- γ , IL-6, and IL-10 were reduced to near background levels as a result of antiviral treatment in both infections.

4. Discussion

This report described a low volume (5- μ l) i.n. cowpox virus infection model in mice that induces a severe upper respiratory infection. The focus of the infection differs from the 50- μ l infection, which induces considerably more lung disease. This occurs because the larger inoculum volume carries more virus-containing medium into the lower respiratory tract to initiate the infection. The lethal 5- μ l infection was characterized by high virus titers in nasal tissue, with considerably less virus found in lungs, spleen, liver, and brain. There was minimal pulmonary consolidation or lung weight increases. Histopathologic examination of the mice indicated severe rhinitis without notable lung pathology. A high degree of cytokine production occurred in the snout during the 5- μ l infection, with IL-6 being elevated the most. IFN γ , IL-1 β , IL-2, IL-5, IL-9, and IL-10 were also highly elevated in the snouts of placebos relative to uninfected controls. TNF α was moderately elevated in the snout. The 50- μ l infection produced characteristic severe pneumonitis and moderate rhinitis. High virus titers were found in the lungs and in nasal tissue. Cytokine elevations were evident both in the lungs and in the snout, and were particularly high for IL-6, IL-9, and IL-10. TNF α was moderately elevated in the snout and in the serum. The serum contained higher levels of most of the factors in the 50- μ l-volume infection compared to serum from uninfected mice. The extent of elevation in cytokine and chemokine levels varied among samples (serum, lung, and snout) and was influenced by the virus inoculum volume.

The virus strain that was used differs from the more typical cowpox virus [1,4] because it disseminated less to organs and tissues away from the lungs and/or sinus region following either a 5- μ l or a 50- μ l inoculum volume challenge. Particularly, there was minimal liver infection observed. Otherwise, the type of infection in mice resulting from the 50- μ l i.n. challenge with the SF strain of cowpox virus is similar to the infection with the typical cowpox virus strain [1,5,6].

One objective of this research was to determine whether pneumonitis or hepatitis was a necessary component of lethal cowpox virus infection. The present data coupled with previously published work supports the premise that neither is essential, but either is sufficient to cause lethality [7]. In infected mice exhibiting pneumonitis (i.e., in the 50- μ l volume infection), there was more severe weight loss and an earlier time to death compared to the 5- μ l upper respiratory infection. The 50- μ l infection resulted in both lung and nasal tissues being infected, thus the overall virus burden was greater. This increase in virus burden would contribute to an earlier time to death, as would extensive pneumonitis. In lethal i.p. cowpox and vaccinia virus infections, mice had higher liver virus titers and lower lung virus titers compared to the i.n. infections [7], indicating hepatitis was more contributory to death than was the lung infection. Because the 5- μ l infection was confined to the upper respiratory tract and still produced lethality, this indicates that pneumonitis and hepatitis are not required components of lethal cowpox virus infections.

A common element in the 5- μ l and 50- μ l lethal cowpox virus i.n. infections was the hyperproduction of cytokines (cytokine storm response) in infected tissues and serum. The 5- μ l infection differentially induced more IFN γ , IL-1 β , IL-6, and IL-10 in the nasal tissue than in the lungs. The 50- μ l infection stimulated cytokine production in both lungs, and nasal passages. The larger inoculum volume deposits virus in both areas of the respiratory tract, whereas the 5- μ l infection confines the infection to the nasal area. Thus, hyperproduction of cytokines is associated with both types of infection. Previously we demonstrated cytokine and chemokine induction during cowpox virus infections [7], but not by studying the particular infection methodology (i.e., low volume) described here. In the earlier published experiments we analyzed pooled samples from mice for broad cytokine and chemokine screens. The present

method allowed for cost-effective evaluations of replicate samples and for statistical interpretation of the data.

The possible role of the hyperproduction of particular cytokines in disease progression or their contribution to disease severity (if any) is a complex issue. This present work does not attempt to correlate the immune factors that are hyper-produced to the observed pathogenesis. It does, however, shed light on the differences in cytokine and chemokine profiles that result from infections that cause distinct pathogenesis related to viral inoculum volume. We previously discussed the potential roles of the various induced cytokines and chemokines in poxvirus infections [7], with two cytokines highlighted here. One pro-inflammatory cytokine, IL-6, was markedly induced above all others in these cowpox virus infections. IL-6 is also induced to high levels in lethal influenza virus infections [10]. We feel that the hyper-induction of this cytokine is deleterious to the host. TNF- α may contribute to weight loss observed in the poxvirus model, since one of its known biological effects is to induce cachexia [11]. Cidofovir treatment attenuated some of the many cytokine and chemokine responses at the times assayed, and as was reported previously [7]. We believe this is due to suppression of virus production, particularly in the earlier course of the infection, rather than by an immunomodulatory mechanism. There is no published evidence suggesting that cidofovir has immunosuppressive properties. The main deleterious effect attributed to cidofovir is renal toxicity [12].

It is known that immunosuppression leads to prolonged orthopoxvirus infections, such as in SCID mice [13]. Other investigators have found that mice deficient in IFN- γ , IFN- γ receptor, TNF receptors, and IL-6 were highly susceptible to poxvirus infection, indicating the necessity of these factors for survival against vaccinia virus infections [14]. Clearly, the release of inflammatory mediators is critical to host defense against viral pathogens. However, the question of whether hyperproduction of these factors is deleterious to the host has not been adequately addressed. In severe RNA viral infections, there is evidence that the timely release of cytokines followed by a reduced level of production produces a more favorable outcome, whereas sustained high levels of certain cytokines can have a deleterious effect [15,16]. This may be addressed in future poxvirus studies involving partial immunosuppression.

Cidofovir was effective in preventing death and in attenuating other disease parameters in both the 5- μ l and 50- μ l infections. The drug treatment regimen used in the present investigations involved two consecutive days of treatment, as was done previously [7,9]. Other investigators have reported using lower daily doses of the compound, but by administering it for more consecutive days against poxvirus infections [5]. The shorter term of treatment mimics the one that will likely be given to humans, since the drug must be administered intravenously. The treatment regimen we employed allows for virus rebound to occur, as evidenced by the rise in virus titers over time (Figure 4). In the poxvirus infection models, an early suppression of virus titer appears to give the mice a better chance for survival. This may happen as a result of reducing the extent of weight loss, and by allowing time for the immune system to be activated yet not be overwhelmed by the consequences of the virus infection. With respect to these mouse models, both the 5- μ l and 50- μ l cowpox virus infections appear to be useful for studying treatments with antiviral agents.

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Figure 1.

Mean lung consolidation scores (A) and mean lung weights (B) $\pm$ SD (N=5) during syncytium-forming cowpox virus infections in mice following 5- μ l and 50- μ l volume i.n. virus exposures of the same infectious dose. Mice in the 50- μ l group (closed circles) were all dead after day 8, whereas animals in the 5- μ l group (open circles) all died by day 13.

Figure 2.

Tissue virus titers $\pm$ SD (N=5) during syncytium-forming cowpox virus infections in mice following 5- μ l and 50- μ l i.n. virus exposures of the same infectious dose. Mice in the 50- μ l group were all dead after day 8, whereas animals in the 5- μ l group died by day 13.

Figure 3.

Mean animal weight during cidofovir treatment of syncytium-forming cowpox virus infections in mice following 5- μ l and 50- μ l i.n. virus exposures of the same infectious dose. Cidofovir (100 mg/kg/day) was given i.p. once a day for 2 d starting 24 h after virus exposure.

Figure 4.

Lung and snout virus titers during cidofovir treatment of syncytium-forming cowpox virus infections in mice following 5-µl and 50-µl i.n. virus exposures of the same infectious dose. Cidofovir (100 mg/kg/day) was given i.p. once a day for 2 d starting 24 h after virus exposure. Error bars (SD, N=5) for the snout virus data are drawn only in one direction for clarity. * P<0.05, ** P<0.01.

Effect of treatment with cidofovir on survival in mice exposed to low (5-µl) and high (50-µl) i.n. inoculation volumes of the same infectious dose of a syncytium-forming cowpox virus.

Table 1

Treatment (mg/kg/ day) ^a	Volume of Virus Challenge	Survivors/ Total	Mean Lung Parameters ± SD (Day 8)		Nasal Virus Titer ^c ± SD
			MDD ± SD ^b	Score	Virus Titer ^c
Cidofovir (100)	5 µl	10/10 ^{***}	>21	0.2 ± 0.3	3.2 ± 0.3
Placebo	5 µl	1/20	9.8 ± 0.9	0.0 ± 0.0 ^{**}	3.0 ± 0.5
Cidofovir (100)	50 µl	10/10 ^{***}	>21	0.3 ± 0.3	7.9 ± 0.1
Placebo	50 µl	0/20	7.5 ± 0.5	4.0 ± 0.0	7.9 ± 0.1
					8.0 ± 0.3 [*]
					8.7 ± 0.2
					8.0 ± 0.3
					8.2 ± 0.3

^a Cidofovir and placebo were given i.p. once-daily for 2 days starting 24 h after virus exposure.

^b Mean day of death of mice that died prior to day 21 of the infection.

^c Log10 PFU/g.

* P<0.05,

** P<0.01,

*** P<0.001.

Table 2

Serum and tissue cytokine and chemokine levels and effects of cidofovir treatment on infections initiated by 5- μ l and 50- μ l i.n. inoculation volumes of the same infectious dose of a syncytium-forming cowpox virus.

Factor ^d	Uninfected	5- μ l Infection		50- μ l Infection	
		Cidofovir	Placebo	Cidofovir	Placebo
GM-CSF	serum	<0.1 $\pm$ 0.0	3.7 $\pm$ 2.4 ^{***}	1.0 $\pm$ 0.9 ^{***}	8.7 $\pm$ 8.5 ^{***} (>87)
	lungs	3.0 $\pm$ 2.5	9.8 $\pm$ 1.2 ^{***}	17 $\pm$ 1.6 ^{***}	22 $\pm$ 3.5 ^{***}
	snout	3.8 $\pm$ 2.2	12 $\pm$ 7.7	13 $\pm$ 8.1	11 $\pm$ 6.8 [*]
IFN- γ	serum	9.8 $\pm$ 8.5	<1 $\pm$ 0.0 [*]	<1 $\pm$ 0.0 [*]	65 $\pm$ 130
	lungs	95 $\pm$ 48	65 $\pm$ 18	645 $\pm$ 97 ^{***}	349 $\pm$ 109 ^{***}
	snout	42 $\pm$ 26	3,006 $\pm$ 1,042 ^{***}	2,679 $\pm$ 891 ^{***}	2,044 $\pm$ 825 ^{***} (49)
IL-1 β	serum	<1 $\pm$ 0.0	7.8 $\pm$ 2.2 ^{***}	10 $\pm$ 3.7 ^{***}	19 $\pm$ 5.0 ^{***} (>190)
	lungs	7.1 $\pm$ 8.4	34 $\pm$ 4.8	51 $\pm$ 13	68 $\pm$ 31 [*]
	snout	19.6 $\pm$ 8.0	1,233 $\pm$ 2,146 [*]	577 $\pm$ 356	1,092 $\pm$ 1,228 (56)
IL-2	serum	0.3 $\pm$ 0.5	1.0 $\pm$ 0.4 [*]	2.7 $\pm$ 1.4 ^{***}	6.9 $\pm$ 2.0 ^{***}
	lungs	0.1 $\pm$ 0.2	3.3 $\pm$ 0.4 ^{***}	15 $\pm$ 3.8	16 $\pm$ 3.7 ^{***} (16)
	snout	0.1 $\pm$ 0.2	4.4 $\pm$ 2.0	4.2 $\pm$ 1.6	3.9 $\pm$ 1.7 ^{***} (39)
IL-3	serum	<0.1 $\pm$ 0.0	<0.1 $\pm$ 0.0 [*]	0.5 $\pm$ 1.0 [*]	8.1 $\pm$ 11 ^{***} (>81)
	lungs	6.0 $\pm$ 4.0	5.4 $\pm$ 0.7 ^{***}	18 $\pm$ 4.5 ^{***}	43 $\pm$ 12 ^{***}
	snout	4.1 $\pm$ 1.4	19 $\pm$ 17 ^{***}	15 $\pm$ 8.8	16 $\pm$ 9.3 [*]
IL-5	serum	0.5 $\pm$ 0.8	1.9 $\pm$ 0.5 ^{***}	4.0 $\pm$ 1.1 [*]	15 $\pm$ 9.1 ^{***} (30)
	lungs	0.8 $\pm$ 1.3	7.8 $\pm$ 1.4 ^{***}	33 $\pm$ 9.9	37 $\pm$ 19 ^{***} (46)
	snout	2.3 $\pm$ 2.2	2.7 $\pm$ 4.7	1.5 $\pm$ 1.0 ^{***}	2.5 $\pm$ 2.8
IL-6	serum	39 $\pm$ 61	70 $\pm$ 54 [*]	254 $\pm$ 222 ^{***}	2,781 $\pm$ 1,583 ^{***} (71)
	lungs	2.5 $\pm$ 1.3	20 $\pm$ 27 ^{***}	12,667 $\pm$ 4,537 ^{***}	44,886 $\pm$ 5,208 ^{***} (17,954)
	snout	7.8 $\pm$ 4.1	18,723 $\pm$ 7,733 ^{***}	2,449 $\pm$ 3,558 ^{***}	36,770 $\pm$ 15,570 ^{***} (4,714)
IL-9	serum	<1 $\pm$ 0	201 $\pm$ 91 ^{***}	356 $\pm$ 336	664 $\pm$ 127 ^{***} (>664)
	lungs	<1 $\pm$ 0	550 $\pm$ 160 ^{***}	867 $\pm$ 218 ^{***}	1,280 $\pm$ 109 ^{***} (>1,280)
	snout	<1 $\pm$ 0	1,106 $\pm$ 271	907 $\pm$ 166	1,016 $\pm$ 260 ^{***} (>1,016)
IL-10	serum	17 $\pm$ 11	1.6 $\pm$ 2.7 ^{***}	18 $\pm$ 22 ^{***}	1833 $\pm$ 991 ^{***} (108)
	lungs	23 $\pm$ 3.0	14 $\pm$ 5.7 ^{***}	164 $\pm$ 78 ^{***}	1,726 $\pm$ 715 ^{***} (75)
	snout	15 $\pm$ 12	810 $\pm$ 562	444 $\pm$ 120	732 $\pm$ 469 ^{***} (49)
MCP-1	serum	314 $\pm$ 222	332 $\pm$ 57 ^{***}	589 $\pm$ 248	1,429 $\pm$ 401 ^{***}
	lungs	120 $\pm$ 56	437 $\pm$ 16	1,472 $\pm$ 205	1,709 $\pm$ 345 ^{***} (14)
	snout	243 $\pm$ 48	379 $\pm$ 361	488 $\pm$ 133	529 $\pm$ 197 ^{***}
TNF α	serum	2.7 $\pm$ 4.7	6.3 $\pm$ 6.0	6.4 $\pm$ 4.9 [*]	28 $\pm$ 17 ^{***} (10)
	lungs	8.4 $\pm$ 5.2	21 $\pm$ 12	30 $\pm$ 11 ^{***}	48 $\pm$ 17 ^{***}
	snout	19 $\pm$ 5	70 $\pm$ 86	95 $\pm$ 41 [*]	176 $\pm$ 75 ^{***}

^a Determined on day 7 of the 50- μ l volume infection and on day 9 for the 5- μ l volume infection, which was near the death of each group. Cidofovir was given once a day for 2 days starting 24 h after virus exposure. Levels are in pg/ml $\pm$ SD (N=6).

^b Values in parentheses are fold differences of placebos from untreated mice. Only placebos with fold increases ≥ 10 are shown.

* P<0.05,

**
P<0.01,

P<0.001, comparing effects of cidofovir treatment to the respective placebo control.

ϕ P<0.05,

$\phi\phi$ P<0.01,

$\phi\phi\phi$ P<0.001, comparing 5- μ l placebo to 50- μ l placebo.

ψ P<0.05,

$\psi\psi$ P<0.01,

$\psi\psi\psi$ P<0.001, comparing 5- μ l and 50- μ l placebo groups to uninfected animals.