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Effects of cidofovir treatment on cytokine induction in murine models of cowpox and vaccinia virus infection

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Abstract

Cytokine profiles during cowpox and vaccinia (WR strain) virus infections were characterized in intranasal (i.n.) and intraperitoneal (i.p.) models in BALB/c mice. The time-course of induction and effects of cidofovir treatment on interferon (IFN)-γ, IFN-γ inducible protein (IP)-10, interleukin (IL)-6, and monocyte chemoattractant protein (MCP)-1 were determined. The four mouse infection models have distinct patterns of cytokine induction. Cowpox virus i.p. and vaccinia virus i.n. infections showed increased induction throughout the time studied. Cowpox virus i.n. infection resulted in delayed induction of IFN-γ and IP-10. Cytokine levels were fairly constant during vaccinia virus i.p. infections. Cidofovir treatment (100 mg/kg/day i.p. for 2 days) significantly suppressed certain cytokine (IFN- γ, IL-6, IL-10, IL-11, IP-10, LIF, MCP-1, MCP-3, MCP-5, MIP-1γ, and TIMP-1) levels to near normal relative to uninfected animals, as well as prevented mortality and reduced virus titers significantly. Characterization of cytokine responses has implications for understanding the immune responses and pathogeneses of viral infections in these mouse models.

Keywords: Orthopoxvirus; Cowpox; Vaccinia; Cidofovir; Antiviral; Cytokines

1. Introduction

Smallpox is a deadly scourge that has invoked terror through the ages. With routine vaccinations ceasing in the 1970s many of the world's population are left unprotected from a potential bioterrorist attack employing this pathogen. Although studies show that individuals vaccinated still maintain some protection from the disease many years after vaccination (Crotty et al., 2003; Hammarlund et al., 2003), the potential for terrorists to use genetically modified smallpox would render these individuals prone to infection. Furthermore, the World Health Organization (WHO) is establishing a global vaccine reserve for use in a potential deliberate release of smallpox. Together these facts are indications that the potential use of smallpox as a weapon of bioterrorism cannot be ignored. Additionally, monkeypox virus has emerged as a potential threat, causing mortality among humans in central Africa (Learned et al., 2005). Understanding viral pathogenesis and finding antiviral agents against poxviruses are important to modern day public health.

At this time, the best animal models for studying smallpox are monkeypox and variola virus infections in nonhuman primates (Hooper et al., 2004; Jahrling et al., 2004; Stittelaar et al., 2005). Unfortunately these nonhuman primate models cannot be widely utilized due to animal costs and biosecurity issues. Thus, other laboratories are limited to studying other orthopoxvirus infection models, such as cowpox and vaccinia virus infections in mice (reviewed in Smee and Sidwell, 2003).

In order to understand the pathogenesis of poxviruses and effects of antiviral compounds thereon, it is requisite that researchers utilize in vivo models of infection. Modulation of cytokines demonstrates their integral role in the antiviral immune response. For example, infection with a recombinant vaccinia virus encoding IL-2 enhanced survival of immunodeficient mice and reduced morbidity (Flexner et al., 1987; Ramshaw et al., 1987). C57BL/6 mice which are normally resistant to ectromelia virus were rendered susceptible when deficient in IFN-γ and IFN-α/β or when infected with a recombinant ectromelia virus expressing IL-4 (Jackson et al., 2001; Ramshaw et al., 1997). Furthermore, cytokines can be administered to assist the host in overcoming viral infections. Liu et al. (2004) demonstrated that IFN-α and IFN-γ successfully reduced virus titers 1000- to 10,000-fold and decreased mortality in a vac-

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cinia virus intranasal (i.n.) infection. The fact that some viral immunomodulators target cytokine function further illustrates their important role in viral infections.

An examination of the effects of vaccinia and cowpox viruses on the systemic cytokine profile in mouse models has not been well established. One study reported that IFN-γ, TNF-α and certain chemokines in the bronchoalveolar lavage (BAL) fluid of mice infected i.n. with vaccinia (WR) peaked at days 7 and 10, then decreased by day 15 after infection (Reading and Smith, 2003a). Rock et al. (2004) documented systemic cytokine profiles in human vaccines. However, vaccination does not represent a severe infection and does not usually require antiviral compounds to control. Other studies have analyzed the effects of specific factors on certain cytokine levels in lungs and serum of mice (Reading and Smith, 2003b; Rees et al., 2005).

The use of antiviral compounds on orthopoxviruses has been widely studied (Smee and Sidwell, 2003). (*S*)-1-(3-Hydroxy-2-phosphonylmethoxypropyl)cytosine (cidofovir or HPMPC), is one of the most potent antiviral agents against orthopoxviruses. It successfully aided in survival of mice infected with lethal doses of cowpox and vaccinia viruses (Bray et al., 2000; Smee et al., 2001a,b, 2004). In the present report we characterized cytokine induction during i.n. and intraperitoneal (i.p.) infections of mice with cowpox and vaccinia (WR strain) viruses, and studied the effects of cidofovir treatment on these infections.

2. Materials and methods

2.1. Antiviral compound, viruses and cells

Cidofovir was obtained from Mick Hitchcock of Gilead Sciences (Foster City, CA). Doses of drug were prepared in sterile saline for treatment of mice. Sterile saline served as the placebo control. Vaccinia virus (WR strain) was purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Cowpox virus (Brighton strain) was obtained from John Huggins at the US Army Medical Research Institute of Infectious Diseases (USAMRIID, Ft. Detrick, Frederick, MD). Joseph Esposito of the Centers for Disease Control and Prevention (Atlanta, GA) originally provided this virus to USAMRIID. High titer virus pools were prepared in African green monkey kidney (MA-104) cells purchased from BioWhittaker (Walkersville, MD). The cowpox virus was passed through African green monkey kidney (Vero 76) cells (from ATCC) and a high titer pool of a syncytium-forming plaque purified isolate was obtained (Smee et al., 2002). For vaccinia virus, the virus was then passed once through immunosuppressed hairless mice and a high titer pool obtained from skin lesions (Smee et al., 2004). The virus was collected once from mice to get a large pool. It is not a mouse adapted strain as this usually takes multiple passages, and the virus is not more virulent than the original. Another line of African green monkey kidney (Vero) cells, purchased from ATCC, was used for plaque assays. These cells were cultured in Eagle's medium (MEM) containing 5% fetal bovine serum. For plaque assays, MEM containing 2% fetal bovine serum was used.

2.2. Mouse infections and serum collection

Female BALB/c mice (13–15 g) were obtained from Charles River Laboratories (Wilmington, MA). They were quarantined for approximately 48–72 h after arrival prior to experiment initiation. Mice were infected with approximately 3×10^5 PFU/mouse i.n. or $(3–5) \times 10^6$ PFU/mouse i.p. with cowpox or vaccinia virus. Intraperitoneal infections required much greater virus challenge doses to induce equivalent mortality. Virus stocks were sonicated 20–30 s before dilution in MEM media. Virus doses were given in 50 μl volume i.n or 0.1 ml volume i.p. The 50 μl volume i.n. virus dose is routinely used and is intended to develop an infection in the lung. During i.n. infections mice were anaesthetized using ketamine (100 mg/kg i.p.) diluted in sterile saline. For antiviral studies, mice were treated i.p. with 100 mg/kg cidofovir in 0.1 ml volumes once a day on days 1 and 2 after virus exposure. Previous work has demonstrated that this dose of drug protects mice against poxvirus infections (Bray et al., 2000; Smee et al., 2001b). Efficacy of the drug increases when given in multiple doses (Smee et al., 2001a).

Serum was collected by anaesthetizing the animals using Avertin diluted to 5% in sterile water and given 0.15 ml i.p. After the mice had succumbed to the anesthesia, the brachial artery was severed, and in some cases the heart was also severed. Blood was collected from the site of incision. The blood was allowed to clot and then centrifuged at approximately $4800 \times g$ for 7 min. The serum was stored at −80 °C until analyzed for cytokines.

2.3. Cytokine profiles in infected mice

Groups of at least six mice were infected with the viruses for the four models studied. Pooled serum was used to obtain the cytokine profiles in the following manner. Serum from the i.n. infections were initially analyzed using the TranSignal Mouse Cytokine Antibody Array 1.0 (Panomics, Redwood City, CA). For this assay, serum was collected on days 5 and 6 for vaccinia and cowpox virus infections, respectively. Mice from these experiments received placebo treatment. Later in the study, pooled serum samples were shipped to Charles River Laboratories (Wilmington, MA) for analysis via rules-based medicine mouse multi-analyte profile (MAP) testing. For the MAP analyses, the serum samples were collected on day 6 for the i.n. models, which also received placebo treatment, and day 4 for the i.p. models, which did not have any treatment. Days of serum collection differed in the i.n. and i.p. models based upon time to death for the particular infection.

2.4. Determination of specific cytokines

Specific cytokine concentrations for the time-course and antiviral treatment studies were determined using commercially available ELISA kits. IL-6 and MCP-1 concentrations were quantified using ELISA kits from Pierce Endogen (Rockford,

IL). The BD Biosciences (San Diego, CA) mouse IFN-γ kit and the R&D Systems (Minneapolis, MN) IP-10 ELISA kit (Minneapolis, MN) were used for the detection of IFN-γ and IP-10, respectively. The sensitivities of the assays were <7 pg/ml for IL-6, <4 pg/ml for MCP-1, 14 pg/ml for IFN-γ, and 2.2 pg/ml for IP-10. All samples were assayed in duplicate. If undiluted samples were below the standard curve the values were reported as the appropriate sensitivity.

A time-course of cytokine induction was determined for all four models of infection. Serum was collected from mice on days 1, 3, and 5 post-infection for all models except for the cowpox virus i.n. model, during which serum was collected on days 1, 3, 5, and 7 p.i. This schedule of collection was based upon time to death for each model. Concentrations of IFN-γ, IL-6, IP-10, and MCP-1 were also measured during an antiviral study. For each model of infection one group of mice received cidofovir treatments while the other group received placebo. Serum collection occurred on day 5 p.i. for all models except for the cowpox virus i.n. model in which serum was collected on day 7 p.i. Pooled serum from each group was also sent to Charles River Laboratories for MAP testing.

2.5. Cidofovir treatment studies

Groups of 10–12 infected mice were treated with cidofovir or placebo. These mice were held for 21 days to observe mortality. Other mice were sacrificed on day 5 (i.p. infections) or day 6 (i.n. infections) after virus exposure, at which time the brains, spleens, livers, lungs, snouts, and serum were collected. If the mice died prior to the day of sacrifice, tissues were collected post-mortem to obtain enough samples (five to seven per group) for analysis. Tissues were homogenized in cell culture medium and stored at −80 °C. The snouts were homogenized with a mortar and pestle or a mechanical blender while the other tissues were homogenized by hand in stomaching bags. Virus titers were determined by plaque assay (Smee et al., 2001a). Samples were centrifuged to obtain supernatant fluid which was serially diluted with medium then titrated in confluent Vero cell monolayers in 12-well plates. The plates were rocked every 5 min for 45 min after addition of the dilutions to ensure a high degree of virus adsorption. After this time, MEM with 2% fetal bovine serum was added to each well. The cells were incubated for 72 h. Following incubation the plaques were visualized with 0.2% crystal violet stain and counted. Data were converted to plaque forming units (PFU) per gram of tissue.

2.6. Statistical methods

Statistical analyses were made between cidofovir and placebo treated groups. Cytokine levels were compared using the Student's *t*-test with Welch correction. Virus titers and mean day of death were analyzed using the Mann–Whitney *U*-test. The Fisher's exact test was used to determine differences in survivor ratios. All *P*-values reported are two-tailed analyses. Calculations were performed using GraphPad InStat version 3.0 for Macintosh, GraphPad Software (San Diego, CA).

3. Results

3.1. Cytokine profiles in infected mice

Cytokine induction was studied in i.n. and i.p. poxvirus infections of untreated mice to determine extent of induction relative to uninfected animals. The results from the Panomics assays demonstrated differences in cytokine induction for the cowpox and vaccinia virus i.n. models (Table 1). The data indicated strong granulocyte-colony stimulating factor (G-CSF), IL-6, and IL-10 induction during cowpox virus i.n. infections. Vaccinia virus i.n. infection stimulated marked induction of G-CSF with slight induction of IFN-γ and four other cytokines. MAP testing analyzed 59 cytokines quantitatively from pooled serum samples, the results of which from uninfected and infected mice will be presented later in conjunction with cidofovir studies. Based upon the results of the Panomics and MAP assays, four cytokines were selected for continued study: IFN-γ, IL-6, IP-10, and MCP-1. These specific cytokines were selected to avoid similarity in cytokine function.

3.2. Time-course of cytokine induction

Serum was collected on days 1, 3, and 5 after virus exposure for all the models except cowpox virus i.n. infection in which serum was also collected on day 7 after virus exposure. This schedule was used to determine the time that maximum cytokine induction occurred after virus exposure and before death. In most of the models, cytokine concentrations increased throughout the time studied (Fig. 1). However, this was not true for cowpox virus i.n. and vaccinia virus i.p. infection models. During the cowpox virus i.n. infection, IFN-γ and IP-10 had a delayed induction. In the vaccinia virus i.p. infections, IP-10 and MCP-1 were already

Table 1
Cytokines induced in cowpox virus i.n. and vaccinia virus i.n. infected mice[a] as determined by Panomics assay

Cytokine[b]	Cowpox virus i.n.	Vaccinia virus i.n.
G-CSF	+++	++
MIG	−	+
IFN-γ	−	+
IP-10	−	+
RANTES	+	+
IL-6	+++	+
IL-10	+++	−

−, not detected; +, weak; ++, moderate; +++, strong signal.

[a] Pooled serum from five mice per group was collected on day 6 after virus exposure for cowpox virus and day 5 after virus exposure for vaccinia virus infected mice. Mice received placebo treatment i.p. once a day for 2 days after virus exposure.

[b] No induction of the following cytokines occurred: M-CSF, GM-CSF, MIP-1α, TNF-α, VEGF, IL-2, IL-4, IL-5, IL-12, and IL-13. Definitions: G-CSF, granulocyte-colony stimulating factor; M-CSF, macrophage-colony stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; MIG, monokine induced by interferon-γ; MIP, macrophage inflammatory protein; IFN, interferon; TNF, tumor necrosis factor; IP, interferon-γ inducible protein; RANTES, regulated upon activation, normally T-cell expressed and secreted; VEGF, vascular endothelial cell growth factor; IL, interleukin.

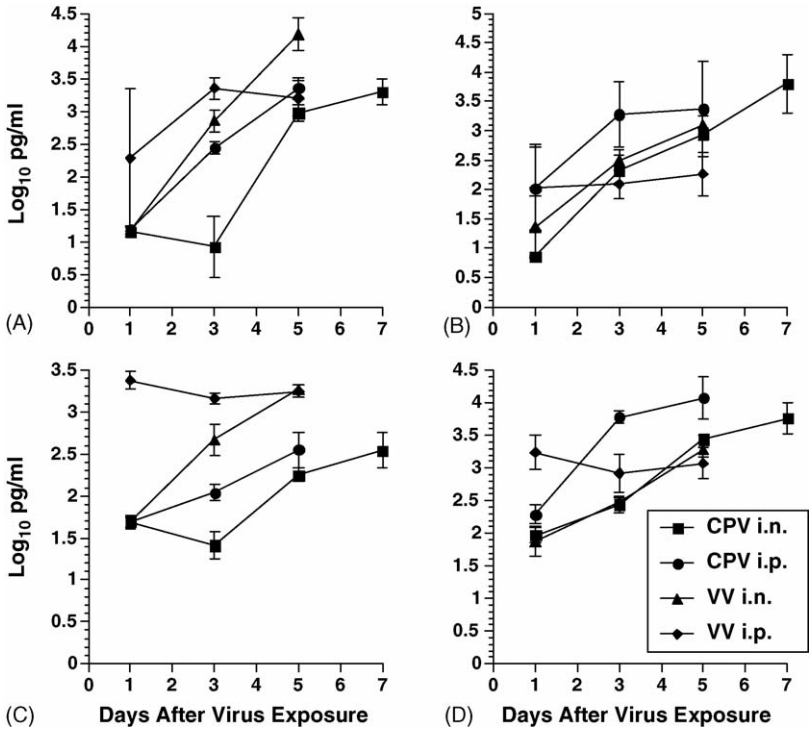

Fig. 1. Time-course of IFN-γ (A), IL-6 (B), IP-10 (C), and MCP-1 (D) induction in cowpox virus and vaccinia virus infections. Data points represent mean ± S.D. (four to seven mice per group [fewer mice were available at the last time-point due to death]). The concentrations for uninfected mice were as follows: IFN-γ (six mice) 1.15 $\log_{10}$ pg/ml; IL-6 (six mice) 1.24 ± 0.55 $\log_{10}$ pg/ml; IP-10 (five mice) 1.56 ± 0.13 $\log_{10}$ pg/ml; MCP-1 (five mice) was 2.15 ± 0.23 $\log_{10}$ pg/ml. CPV = cowpox virus infection, VV = vaccinia virus infection.

elevated by day 1 p.i. and cytokine levels remained fairly constant throughout the time studied.

3.3. Cidofovir effects on survival, mean body weight, and lung virus titer

During the infections where cytokine profiles were presented, other mice were held for death to assess different infection parameters. Antiviral treatments were given to determine the effects of cidofovir on these parameters. Cidofovir treatment significantly decreased the mortality in the cowpox i.n., cowpox i.p., and vaccinia i.n. models. Cidofovir caused less of a preventive effect in the vaccinia i.p model, which was conducted twice (Table 2). The results of the second vaccinia i.p. experiment were not statistically significant due to two survivors in the placebo group. The vaccinia i.p. infection was conducted twice to help clarify an issue regarding mean day of death differences between cidofovir-treated and placebo groups. In these two experiments

Table 2
Effects of cidofovir on survival, mean day of death, and tissue virus titers of infected mice in four infection models

Virus infection and route	Treatment[a]	Survivors/total	MDD[b]	Tissue virus titer ($\log_{10}$ PFU/g ± S.D.)[c]				
				Brain	Lung	Liver	Spleen	Snout
Cowpox i.n.	Cidofovir	10/10***	>21***	<2.0	7.5 ± 0.1**	<2.0	2.2 ± 0.6	7.6 ± 0.5
Cowpox i.n.	Placebo	0/10	7.7 ± 0.5	2.2 ± 0.4	8.1 ± 0.1	2.2 ± 0.5	2.9 ± 0.7	7.0 ± 0.6
Cowpox i.p.	Cidofovir	10/10***	>21***	<2.0	3.6 ± 0.5**	2.5 ± 0.8**	2.3 ± 0.7**	5.2 ± 0.8
Cowpox i.p.	Placebo	0/12	5.3 ± 0.5	2.4 ± 0.7	7.3 ± 0.6	7.9 ± 0.3	7.8 ± 0.5	6.0 ± 0.9
Vaccinia i.n.	Cidofovir	9/10**	8.0	3.1 ± 0.8*	7.1 ± 0.7*	2.4 ± 0.7*	2.4 ± 0.8*	7.1 ± 0.5*
Vaccinia i.n.	Placebo	1/10	6.4 ± 0.5	5.5 ± 0.2	8.2 ± 0.2	5.0 ± 0.9	7.1 ± 0.5	8.1 ± 0.3
Vaccinia i.p.	Cidofovir	6/10*	3.8 ± 1.7	<2.0	<2.0**	<2.0**	<2.0**	2.8 ± 1.3**
Vaccinia i.p.	Placebo	0/10	5.9 ± 3.5	2.6 ± 0.8	6.0 ± 0.4	5.8 ± 0.3	5.6 ± 0.9	6.5 ± 0.9
Vaccinia i.p.[d]	Cidofovir	6/10	2.5 ± 1.0	<2.0**	2.1 ± 0.4**	2.2 ± 0.4**	<2.0**	3.6 ± 1.1**
Vaccinia i.p.[d]	Placebo	2/10	4.1 ± 1.2	3.3 ± 0.4	5.7 ± 0.8	5.6 ± 0.7	5.0 ± 0.6	7.1 ± 1.0

*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

[a] 100 mg/kg cidofovir or placebo given i.p. once a day for 2 days starting 24 h after virus exposure.

[b] Mean day of death ± S.D. of mice that died prior to day 21 of the infection.

[c] Determined on day 5 for i.p. virus challenges or on day 6 for i.n. virus exposures. Five to six mice per group were used, except for the vaccinia i.p. repeated study where seven mice per group were used.

[d] Repeated experiment.

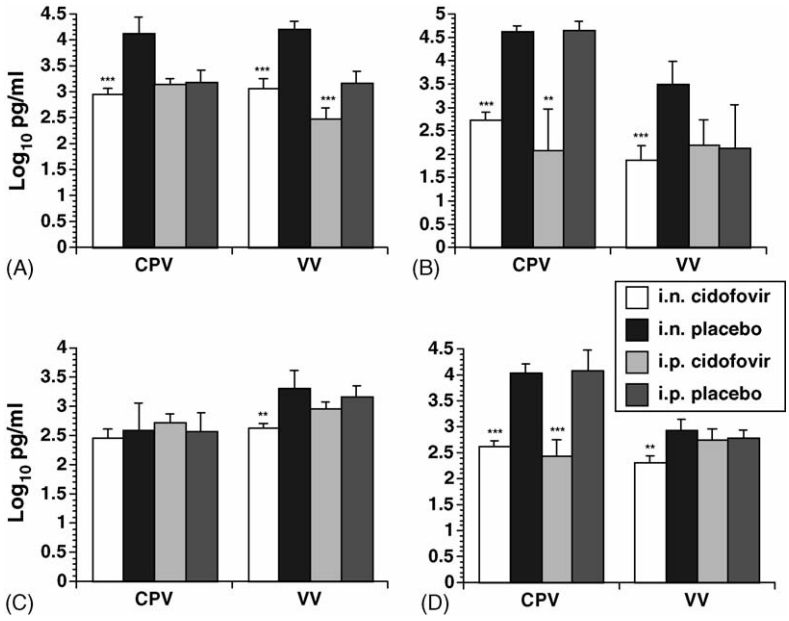

Fig. 2. Effects of cidofovir treatment on IFN-γ (A), IL-6 (B), IP-10 (C), and MCP-1 (D) induction in cowpox virus and vaccinia virus infections. Mice were treated once a day i.p. for 2 days with cidofovir (100 mg/kg) or placebo starting 24 h after virus exposure. Serum was collected on day 5 after virus exposure, except for cowpox virus i.n. infection collections on day 7. There were three to six mice per group (influenced by survival). $^{**}P < 0.01$, $^{***}P < 0.001$. CPV = cowpox virus infection, VV = vaccinia virus infection.

the mice that died in the cidofovir groups died earlier than in the respective placebo control groups. What appeared to be happening in the vaccinia i.p. model was a few early deaths in both the cidofovir-treated and placebo groups. With the high initial virus challenge required to initiate these infection to ensure high mortality combined with cidofovir treatments starting 1 day after virus challenge, the drug was not able to prevent death in some of the animals.

The efficacy of cidofovir was demonstrated by the reduction of virus titers in cidofovir-treated mice in each model studied (Table 2). The cowpox virus i.n. infection resulted in high titers in the lungs and snouts. Antiviral treatment of these mice significantly decreased virus titers in the lungs. On the other hand, during vaccinia virus i.n. infection high titers were obtained in all the tissues examined, which is consistent with our previous results (Smee et al., 2001a). Virus titers during the vaccinia i.n. were also reduced significantly by cidofovir. Despite the effects of cidofovir treatment, virus titers in the lungs and snouts still remained high in the cowpox and vaccinia i.n. infected animals. Mice infected with cowpox virus i.p. or vaccinia virus i.p. developed high titers in all tissues examined except the brains. Cidofovir treatment significantly decreased viral load in the spleens, livers, and lungs of the cowpox virus i.p. mice and in the spleens, livers, lungs, and snouts of the mice infected i.p. with vaccinia virus.

3.4. Cidofovir effects on cytokines induced during infection

As part of the antiviral analyses, treatments with cidofovir were conducted during infections with cowpox and vaccinia viruses to determine drug effects on cytokine production. Based on the above time-course results, serum was collected on day 5 p.i., except for the cowpox virus i.n. model in which it was collected on day 7 p.i. Although some cytokines were elevated earlier during infection, these days were chosen to ensure enough time for cidofovir to provide a beneficial effect and for maximum cytokine induction. Moreover, by taking most of the samples on the same day it allowed for the same sample to be used in multiple assays thus reducing cost.

Antiviral treatment resulted in significant reductions of cytokine concentrations in most models as determined by ELISA assays (Fig. 2). Cidofovir treatment significantly reduced IFN-γ, IL-6, and MCP-1 in the cowpox virus i.n. model; IL-6 and MCP-1 in the cowpox virus i.p. model; IFN-γ, IL-6, IP-10, and MCP-1 in the vaccinia virus i.n. model; and IFN-γ in the vaccinia virus i.p. model. However, drug treatment did not significantly affect IP-10 in the cowpox virus i.n. model; IFN-γ and IP-10 in the cowpox virus i.p. model; or IL-6, IP-10, and MCP-1 in the vaccinia virus i.p. model.

When taking into account variation and use of pooled serum samples, the MAP analysis shows similar effects of cidofovir as the ELISA kits for the respective assays (Tables 3 and 4). The MAP data indicate that other cytokines were also markedly elevated during the infections in placebo groups, namely IL-10, IL-11, LIF, MCP-3, MCP-5, MIP-1γ, and TIMP-1. These serum factors were greatly reduced by cidofovir treatment. Comparing the cytokine profiles from cidofovir-treated mice to those of uninfected animals (Tables 3 and 4) indicated a substantial return to normal values in the drug treated group.

Although it may appear that there are discrepancies between the Panomics assay and MAP assays (Tables 1, 3 and 4), these differences may be attributed to differing levels of sensitivity. The Panomics assay had levels of sensitivity in the 1000–2000 pg/ml range. The Panomics assay also did not reflect

Table 3
Analytes decreased by cidofovir treatment[a] in cowpox virus infections as determined by MAP analysis of pooled serum

Analyte[b]	Units	Cowpox virus i.n. infection		Cowpox virus i.p. infection		Average uninfected[c]
		Cidofovir	Placebo	Cidofovir	Placebo	
IFN-γ	pg/ml	26	639	80	203	<20
IL-6	pg/ml	80	7260	73	17400	<14
IL-10	pg/ml	147	7670	943	36600	183
IL-11	pg/ml	35	896	100	880	<30
IP-10	pg/ml	129	1580	272	420	156
LIF	pg/ml	258	1380	265	3240	86
MCP-1	pg/ml	774	3460	2310	3260	159
MCP-3	pg/ml	350	2970	1190	6380	247
MCP-5	pg/ml	337	>6260	832	>6260	145
MIP-1γ	ng/ml	34	>694	45	>694	11
TIMP-1	ng/ml	4.5	42	6.6	58	1.6

[a] Mice treated i.p. with cidofovir (100 mg/kg) or placebo once a day for 2 days starting 24 h post-infection. Serum was collected from four to five mice per group on day 7 after virus exposure for i.n. infected mice and day 5 after virus exposure for i.p. infected mice. Values are from pooled serum samples.
[b] Definitions: IFN, interferon; IL, interleukin; LIF, leukemia inhibitory factor; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; TIMP, tissue inhibitor or metalloproteinase.
[c] Mean of two independent assays.

Table 4
Analytes decreased by cidofovir treatment in vaccinia virus infections[a] determined by MAP analysis of pooled serum

Analyte[b]	Units	Vaccinia virus i.n. infection		Vaccinia virus i.p. infection		Average uninfected[c]
		Cidofovir	Placebo	Cidofovir	Placebo	
IFN-γ	pg/ml	102	1690	52	144	<20
IL-6	pg/ml	<14	464	51	73	<14
IL-10	pg/ml	538	1080	347	893	183
IL-11	pg/ml	21	522	93	124	<30
IP-10	pg/ml	286	1830	331	1140	156
LIF	pg/ml	304	528	211	215	86
MCP-1	pg/ml	108	1820	916	1170	159
MCP-3	pg/ml	290	2970	1810	2380	247
MCP-5	pg/ml	217	1140	730	523	145
MIP-1γ	ng/ml	15	38	29	24	11
TIMP-1	ng/ml	3.5	20	52	50	1.6

[a] Mice treated i.p. with cidofovir (100 mg/kg) or placebo once a day for 2 days starting 24 h p.i. Serum was collected from three to five mice per group (influenced by survival) on day 5 after virus exposure. Values are from pooled serum samples.
[b] Definitions are same as in Table 3.
[c] Mean of two independent assays.

fold changes as the MAP assay did but merely indicated cytokines with elevated concentrations above a certain level. Considering the sensitivity of the Panomics assay, the concentrations measured in the MAP data coincide with most of the levels of induction seen in the Panomics assay. The only data point that does not correspond well between the two assays is the IL-6 concentration for vaccinia virus i.n. placebo mice. The concentration according to the MAP analysis (464 pg/ml) should not have shown up well on the Panomics assay though a slight induction was recorded. This apparent discrepancy may be due to qualitative nature of the Panomics assay or perhaps the fact that the pooled serum may have contained an extreme value in one sample.

4. Discussion

Cidofovir is an effective antiviral compound for cowpox virus and vaccinia virus infections initiated by different inoculation routes. The drug significantly increased the number of survivors in all models. Cowpox virus and vaccinia virus i.n. infections resulted in different tissues being affected. Cowpox virus i.n. infection resulted in high titers mainly in the lung and snout while vaccinia virus i.n. infection resulted in high titers throughout the body. These differences in viral distribution in cowpox virus and vaccinia virus i.n. infections have been previously documented (Smee et al., 2001a). The fact that cidofovir did not reduce titers in the lungs and snouts as dramatically as in other tissues of vaccinia virus i.n. infected animals at the time-point studied have also been reported (Smee et al., 2001a,b). The presence of high titers in the lung and snout even after antiviral treatment indicates that the infection had not entirely been resolved. As the number of survivors indicates, the cowpox virus and most of the vaccinia virus i.n. infected animals were able to overcome infection later in the time period studied. Since the lungs and snouts were the site of first infection in the i.n. infected animals and as they developed the high titers, it is rea-

sonable that they would show viral clearance last of the tissues examined. The i.p. infections resulted in high titers throughout the body excluding the brain. Cidofovir treatment reduced virus titers in all models studied demonstrating its antiviral properties. The ability of cidofovir to reduce mortality and virus titers in the cowpox virus and vaccinia virus i.n. models has been previously demonstrated (Bray et al., 2000; Quenelle et al., 2003; Smee et al., 2001a,b). To our knowledge, the effects of cidofovir on i.p. infections in immunocompetent mice with these viruses have not been previously reported. However, Quenelle et al. (2003) documented the effects of cidofovir on cowpox virus and vaccinia virus i.p. infected severe combined immunodeficient mice. These researchers found that cidofovir did not prevent mortality in i.p. cowpox virus or vaccinia virus infections in severe combined immunodeficient mice, though treatments did delay the mean day of death and reduced or delayed virus titers.

The data demonstrate distinct differences in the pathogenesis of infection in the four mouse models studied. Based on viral distribution, the cowpox virus i.n. model was mainly a respiratory infection while the other models were systemic infections. The cowpox virus i.n. model also had a unique pattern of cytokine induction. IL-6 and MCP-1 appeared to be the first components of the cytokine response followed by IFN-γ and IP-10. The delayed induction of the two cytokines in the cowpox virus i.n. model correlated well with the later mean day of death, indicating that this infection took a longer time to establish. The placebo cowpox virus i.n. infected animals had very high levels of IFN-γ, IL-6, and MCP-1. Antiviral treatment resulted in a significant reduction of these concentrations, although IFN-γ concentrations were still highly elevated. IL-6 levels also were elevated after treatment. As some cytokines remained elevated after treatment, it may be that they provided some antiviral functions after 7 days of infection. The need for these cytokines could be explained by the fact that the cidofovir-treated groups infected with cowpox virus i.n. show some viral clearance in the lungs but not the snouts at day 6 p.i. During the cowpox virus i.p. infection, the concentrations of all cytokines continued to increase throughout infection. IFN-γ, IL-6, and MCP-1 concentrations were stimulated to a great extent. However, cidofovir treatment only reduced IL-6 and MCP-1 levels. Once again IP-10 and IFN-γ concentrations remained elevated. Almost all infected organs except the snouts showed viral clearance by day 6 indicating that the animals had not completely recovered from infection at this time.

Infection with vaccinia virus by the i.n. route markedly stimulated IFN-γ, IL-6, and IP-10. Induction of these cytokines was observed by day 3 and continued to increase by day 5. Cidofovir treatment significantly suppressed induction of all four cytokines in this model, with IFN-γ and IP-10 remaining high on day 5 p.i. after treatment. At this time-point the mice were still recovering from the infection because the lungs and snouts of the cidofovir-treated groups still had elevated titers. In the vaccinia virus i.p. model the immune system responded rapidly, and IP-10 and MCP-1 were elevated by day 1 p.i. Interestingly, the concentrations of the cytokines appeared to remain fairly constant throughout the infection. The early induction of cytokines in this model correlates well with the rapidity and severity of this infection model. During this infection model, highly elevated concentrations of IFN-γ and IP-10 were reached, although antiviral treatment only significantly reduced IFN-γ levels.

It is clear that not all of the cytokines were affected by cidofovir treatment. It is assumed that cidofovir reduces cytokine concentrations as a function of inhibiting viral replication. As indicated by the reported virus titers, most of the animals still had elevated titers in some tissue at the time studied. Apparently the infection had not been completely resolved at this point. Presumably, the presence of certain cytokines after cidofovir treatment depends on the need for their continued antiviral functions. It can be surmised that the cytokines that were reduced by treatment were no longer required.

MAP testing revealed other cytokines that were markedly induced during infection, namely IL-10, IL-11, LIF, MCP-3, MCP-5, MIP-1γ, and TIMP-1. Overall, cowpox virus infections induced greater cytokine induction than did vaccinia virus infections. Greater inductions of IL-6, IL-10, LIF, and MCP-3 were seen with i.p. cowpox virus compared to i.n. cowpox virus infections. In contrast, i.n. vaccinia virus infections induced more cytokines than i.p. vaccinia virus infections.

To employ these cytokines as measures of infection, each model must be considered separately. For a particular model, a particular cytokine would need to be selected based on strong induction during infection and significant reduction during antiviral treatment. Based upon the MAP analysis, for the cowpox virus i.n. and i.p. infections, excellent candidates would be IL-6, IL-10, MCP (1, 3, or 5), and MIP-1γ. IFN-γ, IL-6, IP-10, and MCP (1, 3, or 5) could be used for the vaccinia virus i.n. infection analysis. TIMP-1 was the factor most induced by i.p. vaccinia virus infection. Other cytokines that could be followed during this infection include IFN-γ, IP-10 and MCP (1 and 3). Realizing that the i.n. infection models are the most commonly used, IL-6 may be the best single factor to analyze in these studies.

T helper (Th) 1 and Th2 polarization has been shown to be important in the antiviral immune response. Resistance or susceptibility to ectromelia virus is correlated with Th1 or Th2 responses, respectively. C57BL/6J mice, which are resistant to ectromelia virus, strongly induce Th1 cytokines IL-2, TNF, and IFN-γ compared to susceptible BALB/c mice in response to footpad inoculation with ectromelia virus infections (Chaudhri et al., 2004). Intravenous (i.v.) infection with recombinant vaccinia virus expressing IL-4, a Th2 cytokine, delayed viral clearance as well as reduced cytotoxic T lymphocyte precursor cells, cytotoxic T lymphocyte lysis, and expression of Th1 cytokines IL-12, IL-2, and IFN-γ mRNA (Sharma et al., 1996). Likewise, footpad infection with a recombinant ectromelia virus encoding IL-4 abrogated the natural resistance C57BL/6 mice have to this virus (Jackson et al., 2001). Cidofovir treatment of these mice did not protect them from infection but only delayed the mean day to death (Robbins et al., 2005). Furthermore, i.p. infection with vaccinia virus (WR strain) in C57BL/6 mice lacking IL-12, which stimulate a Th1 response, resulted in higher virus titers than controls while on the other hand i.p. infection in IL-10 negative mice and IL-4 negative mice showed reduced virus titers (van den Broek et al., 2000). These findings indicate that

Th2 responses hinder recovery from poxvirus infections. In the present study, there was no clear Th1 or Th2 profile. Both Th1 and Th2 cytokines were elevated above background levels in the models.

Other studies also found that these cytokines were strongly induced by poxvirus infection which correlates with the findings of this study. IFN-γ concentrations were increased in serum of humans vaccinated with vaccinia virus by approximately 200% (Rock et al., 2004). When spleen cells immune to vaccinia virus were re-stimulated with the virus they showed a rapid induction of IFN-γ and IL-6 (Carpenter et al., 1994). Additionally, vaccinia virus (WR strain) infection of human fibroblasts stimulated IL-6 mRNA as early as 4 h p.i. with protein levels increasing by 48 h p.i. to about 2000 pg/ml (Rokita et al., 1998). Amichay et al. (1996) noted IP-10 mRNA induction in BALB/c mice during vaccinia virus (WR strain) i.p. infections. In another study, mice infected with vaccinia virus (WR strain) by i.p. route demonstrated high levels of IP-10 mRNA on days 3 and 6 p.i. in the uteri, ovaries, spleen and liver (Mahalingam and Karupiah, 2000). In i.v. variola infections of monkeys, elevated concentrations of IFN-γ, IL-6, and MCP-1 were observed (Jahrling et al., 2004). Reading and Smith (2003a) noted peak levels of IFN-γ of about 2000 pg/ml and strong induction of MCP-1 in BAL fluid of mice infected i.n. with vaccinia virus (WR strain).

The response of these cytokines to poxvirus infections is not surprising given their reported antiviral functions. Although the present study did not ascertain the function of these cytokines in the four models of infection, a brief look at their antiviral roles may help explain why these cytokines were induced. The discussion will be limited to the four cytokines studied in detail in this report, namely IFN-γ, IL-6, IP-10, and MCP-1.

IFN-γ has a well established role in the antiviral immune response. It induces the antiviral state, induces nitric oxide synthase, activates natural killer (NK) cell activity, and increases MHC antigen expression (Biron and Sen, 2001; De Maeyer and De Maeyer-Guignard, 1998). Additionally, IFN-γ is also important in the polarization of the T helper response. Th1 cells produce IFN-γ which inhibits Th2 differentiation (De Maeyer and De Maeyer-Guignard, 1998).

The cytokine IL-6 has many different functions that are important in the antiviral response. It is involved in antibody production, differentiation of cytotoxic T lymphocytes, the inflammatory response, the fever response, the mucosal immune response, and the acute-phase response (Biron and Sen, 2001; Hirano, 1998).

Research has also established a role for IP-10 in the resolution of viral infections. Using recombinant vaccinia virus expressing IP-10, Mahalingam et al. (1999) demonstrated that athymic nude mice were able overcome i.v. infection at doses of 10^4 or 10^5 PFU with no recorded morbidity; however, higher doses of the recombinant vaccinia virus, 10^6 or 10^7 PFU, only delayed the mean day of death in these mice. IP-10 may play a role in polarizing the Th response as it attracts Th1, but not Th2 cells (Bonecchi et al., 1998; Sallusto et al., 1998; Siveke and Hamann, 1998). The antiviral effects of IP-10 are also due to NK cells. During the study by Mahalingam et al. (1999), it was demonstrated that the enhanced antiviral response to recombinant vaccinia virus encoding IP-10 was dependent on NK cells as depletion of these effector cells resulted in significantly higher titers in mice infected i.v. with recombinant vaccinia virus IP-10 but did not affect control virus replication. Thus, the antiviral role of IP-10 is well established.

MCP-1 is a CC chemokine that is chemotactic for monocytes, basophils, T cells, and NK cells (Proost et al., 1998). Through these effects MCP-1 can be important in the resolution of viral infections. The influence MCP-1 has on the Th response is debatable. Some evidence suggests a role in Th2 polarization for MCP-1. For example, MCP-1 deficient mice could only produce low levels of Th2 cytokines (Gu et al., 2000) and MCP-1 indirectly promoted Th2 differentiation from naïve T cells (Karpus et al., 1997). However, other research contradicts this evidence as MCP-1 is chemotactic for both Th1 and Th2 cells depending on concentration (Bonecchi et al., 1998; Siveke and Hamann, 1998). The fact that vaccinia virus and cowpox virus encode a chemokine binding protein that binds CC chemokines but not CXC or C chemokines also indicates the potential importance of this chemokine in poxvirus infections as this immunomodulator binds MCP-1 with high affinity (Alcamí et al., 1998).

Although this study shows distinct differences in the immune responses during cowpox virus and vaccinia virus infection models there is still much to be learned about these infections. With further research the immune responses and effects of cidofovir in poxvirus infections can be further established. As antiviral research continues there is the hope that the threat of smallpox will be eliminated due to the development of highly effective treatment measures.

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