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Antiviral Research 66 (2005) 1–7
Antiviral Research
www.elsevier.com/locate/antiviral

Review

The efficacy of cidofovir treatment of mice infected with ectromelia (mousepox) virus encoding interleukin-4

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Received 16 February 2004; accepted 8 December 2004

Abstract

Improved vaccines and therapies for virulent poxvirus infection are required, particularly in the light of recent threats of bioterrorism. Cidofovir (HPMPC) is an acyclic nucleoside analog with proven efficacy against poxviruses. Here, we evaluated HPMPC in mice given a recombinant ectromelia virus (ECTV) encoding interleukin-4 (ECTV-IL-4) that is highly immune suppressive. Mousepox-sensitive BALB/c mice given HPMPC for five consecutive days after infection were protected against the lethal effects of a control ECTV recombinant, although they suffered a chronic form of mousepox disease. High doses of the drug resulted in a milder localized disease. In contrast, HPMPC failed to protect mousepox-resistant C57BL/6 mice against ECTV-IL-4, although its lethal effects were delayed by five daily doses of 20 mg/kg or a single dose of 100 mg/kg. Higher daily doses further delayed mortality, although the majority of animals eventually succumbed to infection. It appears that HPMPC inhibited ECTV-IL-4 replication without clearance, with the virus having a lethal effect when the drug was removed. Resistance of ECTV-IL-4 to HPMPC treatment may relate to the virus's ability to inhibit antiviral cell-mediated immunity. Interestingly, ECTV-IL-4-mediated immune suppression was not accompanied by a reduction in systemic IFN-γ expression, suggestive of an alternative or highly localized suppressive mechanism.

Keywords: Cidofovir; Mousepox infection; Interleukin-4; Immune suppression

Contents

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doi:10.1016/j.antiviral.2004.12.003

1. Introduction

In 1980 smallpox was officially declared globally eradicated by the World Health Organisation (WHO). Routine vaccinations against smallpox were discontinued by 1979, and consequently the majority of the world population is now either immunologically naïve or may have diminished immunity (Lane et al., 2001). The recent threat of bioterrorism has raised concerns that severe poxvirus infections, such as those due to variola (VARV, smallpox), monkeypox virus or other virulent agent, will once again pose a threat to public health. Currently effective smallpox vaccination practices, which use live vaccinia virus (VACV), can give rise to life-threatening complications such as disseminated and progressive infections, particularly in immune deficient individuals (Bray, 2003; Bray and Wright, 2003). The current high prevalence of immune deficiency in the population, mainly due to HIV infection, together with the risk of complications of VACV inoculation in healthy individuals, have raised questions concerning the reintroduction of this form of vaccination. Alternative immunization strategies and the development of effective, safe antiviral therapies for the treatment of poxvirus infections are now of major scientific interest. Indeed, in 1999 a WHO advisory committee on variola stressed the importance of drug development for the treatment of human smallpox (or other poxvirus) infections should they re-emerge (Neyts and De Clercq, 2003).

The development of safe and effective antiviral drugs is now a major focus of poxvirus research. Cidofovir [(*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC)], an acyclic nucleoside analog known to target DNA polymerase and prevent viral transcription and replication, is currently licensed as an antiviral agent (Vistide®) for the treatment of cytomegalovirus (CMV) retinitis in AIDS patients (De Clercq, 2003). To date, HPMPC has not been used to treat orthopoxvirus infections in humans; however, extensive testing has been conducted in laboratory animals, with the drug shown to be protective against a variety of poxviruses. In 1993 the successful use of HPMPC was reported in preventing VACV infection in immunosuppressed [severe combined immune deficiency (SCID)] mice (Neyts and De Clercq, 1993). In addition, the antiviral drug has been identified as an inhibitory agent against camelpox virus, and monkeypox virus, as well as intranasal cowpox virus (CPXV) and vaccinia virus infection of mice making it of particular interest for the possible treatment of smallpox (Bray et al., 2000; Smee et al., 2001, 2002; De Clercq et al., 1987; De Clercq, 2001; Nettleton et al., 2000). Recently, orally active ether lipid analogs of HPMPC were shown to be highly effective in blocking viral replication in ectromelia virus (ECTV)-challenged A/NCR mice (Buller et al., 2004).

ECTV, a close relative of VARV and VACV, is an orthopoxvirus and a natural pathogen of laboratory mice causing mousepox. The mousepox disease model has been widely used to study pathogenesis of generalised viral infections, and genetic resistance to infection (Fenner, 1949; Karupiah et al., 1996; Blanden and Gardner, 1976; Müllbacher et al., 1999). Virulence of ECTV infection is determined by host genetic background. In mousepox-sensitive (e.g., BALB/c) mice, the disease presents as an acute systemic infection with high viral titers in the visceral organs and high mortality. "Mousepox-resistant" mice (e.g., C57BL/6 strain) generally have subclinical infections with low levels of viral replication in the visceral organs and development of non-fatal lesions. The genetic resistance and recovery of mice from ECTV infection can be attributed to innate and adaptive cell-mediated immune responses, including natural killer (NK) cells, alpha interferon (IFN-α), IFN-β, IFN-γ, activated macrophages, and inducible nitric oxide production (Jacoby et al., 1989; Karupiah et al., 1998, 1993a,b; Ramshaw et al., 1997). Early activation of a strong virus-specific cytotoxic T-lymphocyte (CTL) response and the production of Type-1 cytokines interleukin-2 (IL-2), IL-12 and IFN-γ in mousepox-resistant mice are key factors in recovery. (Blanden, 1970a,b; Karupiah et al., 1996; O'Neill and Brenan, 1987; Chaudhri et al., 2004).

As a natural pathogen in mice, ECTV is an appropriate vector for vaccine development. Indeed, we recently engineered a thymidine kinase positive (TK^+) recombinant ectromelia virus-expressing mouse IL-4 (ECTV-IL-4) as a potential immunocontraceptive vaccine for use in mouse plagues (Jackson et al., 2001). In the course of our studies, we found the recombinant ECTV-IL-4 to be highly virulent, suppressing NK and CTL cytolytic activity as well as IFN-γ expression by splenic $CD8^+$ T lymphocytes. In normally mousepox-resistant C57BL/6 mice, ECTV-IL-4 infection resulted in acute mousepox infection with a high mortality rate. This model appears ideal for testing the efficacy of HPMPC in the setting of a highly virulent poxvirus infection. Previously, footpad inoculation of the control virus ECTV (TK^+) in both mousepox-resistant and—sensitive strains of mice caused acute disease symptoms similar to those normally seen in mice infected with the Moscow strain of ECTV (Fenner and Buller, 1997; Jackson et al., 2001). We have therefore examined the efficacy of treating mice infected with ECTV (TK^+) or ECTV-IL-4 (TK^+) with a range of HPMPC doses and evaluated the immune response of treated mice. We report here that HPMPC treatment was protective against both disease

symptoms and mortality that is associated with ECTV infection in mousepox-sensitive BALB/c mice. Although development of disease in C57BL/6 mice infected with the more virulent ECTV-IL-4 was delayed in response to high doses of HPMPC, once drug treatment was removed animals developed acute mousepox infection with high mortality.

2. Materials and methods

2.1. Virus

Construction of recombinant ectromelia viruses ECTV-602-TK$^+$ (ECTV-TK$^+$) and ECTV-602-IL-4-TK$^+$ (ECTV-IL-4) was described previously (Jackson et al., 2001)

2.2. Animals, inoculations and drug treatment

Animal studies were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Specific-pathogen-free 6- to 8-week-old female mice were obtained from the Australian National University Animal Services Division for use in these studies. Cidofovir (HPMPC; Gilead, Foster City, CA, USA) was diluted in sterile phosphate-buffered saline (PBS) to make a 10 mg/ml stock and was given by intraperitoneal injection of doses ranging from 1 to 100 mg/kg/day in sterile PBS, either as a single dose or daily for 5 days. Survival data are shown as the mean days ± standard deviation (S.D.) with *P*-values determined using Student's *t*-test (significance being $P \leq 0.05$).

2.3. Evaluation of HPMPC dosage on viral infection and immune response

The influence of HPMPC treatment on the immune response to ECTV infection was studied in ECTV resistant C57BL/6 mice. Mice were infected by inoculation into the right hind leg footpad with 10^3 PFU of ECTV-TK$^+$ or ECTV-IL-4. 24 h post viral inoculation mice in each group ($n = 5$) were given either 20 mg/kg or 100 mg/kg of HPMPC, or PBS placebo (control groups), once daily for 5 days via intraperitoneal injection for evaluation of the immune response. Mice were tail-bled and euthanised at 4 days after cessation of drug treatment. Data are shown as mean viral titers ± S.D. *P*-values were determined using Student's *t*-test (significance being $P \leq 0.05$).

2.4. Mouse Th1/Th2 cytokine cytometric bead array (CBA) assay

Blood was collected by tail-bleeding at day 6 p.i. from recombinant ECTV-infected C57BL6 mice (both HPMPC treated and untreated). Concentrations of IL-2, IL-4, IL-5, IFN-γ, and tumor necrosis factor-α (TNF)-α in sera were analysed using the mouse Th1/Th2 cytokine CBA assay according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA). Statistics were obtained using the BD CBA Software (BD Biosciences). Data are shown as the mean ± S.D. The minimum sensitivity of the assay was ≤20 pg/ml and the maximum quantifiable level ≤5000 pg/ml.

2.5. Virus titration

C57BL/6 mice infected with recombinant ECTV (HPMPC-treated or untreated) were sacrificed at day 6 or day 11 p.i. Footpads (site of viral inoculation) and spleens were removed and placed into pre-weighed Eppendorf Safe-Lock tubes (Eppendorf, Hamburg, Germany), the tissue weight was determined and 1 mL of 4 °C PBS, 1 mM HEPES (Gibco), 1% FCS (Gibco) was added to each tube. Tissues were homongenized then frozen and thawed three times with vigorous vortexing to release virus. Serial dilutions (six 10-fold dilutions) in Gelatin Saline, 1 mM HEPES (Gibco) were set-up and 0.5 ml of each virus dilution was placed on BSC-1 cells in 6-well plates (Linbro) for 1 h at 37 °C. Virus was removed and wells were overlaid with 2 ml of MEM containing 1% (w/v) low-melting-point agarose and were grown at 35 °C for 72 h. Plaques produced by viruses expressing β-galactosidase were visualized by overlaying the infected cell monolayers with a further 2 ml of MEM-1% agarose containing X-Gal (5′-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 300 μg/ml).

3. Results

3.1. Infection of mice with recombinant ECTV results in acute mousepox

Footpad inoculation of "mouse-pox resistant" mice (C57BL/6) with 10^3 PFU ECTV-TK$^+$ caused necrosis and sloughing of the infected limb with full recovery in all mice. In contrast, infection of mousepox sensitive mice (BALB/c) with 10^3 PFU ECTV-TK$^+$ caused acute mousepox with 100 % lethality, with a mean survival time of 10 ± 1.0 day.

Similar to our previous experiments, footpad inoculation of C57BL/6 mice with 10^3 ECTV-IL-4 caused acute mousepox infection with 100% lethality and a mean survival time of 8.5 ± 1.0 days. The acute mousepox symptoms seen in ECTV-TK$^+$- infected BALB/c mice and ECTV-IL-4-infected C57BL/6 mice comprised footpad inflammation of inoculated feet by day 6 p.i. (post-infection), lethargy, eye inflammation, ruffled fur and a hunched posture. In addition, pale enlarged spleens and livers containing numerous discrete white necrotic lesions were found post-mortem.

3.2. HPMPC treatment is effective against infection with ECTV-TK$^+$ but not ECTV-IL-4

To study the effects of HPMPC dosage on the outcome of ECTV infection, BALB/c mice were infected by inoculation

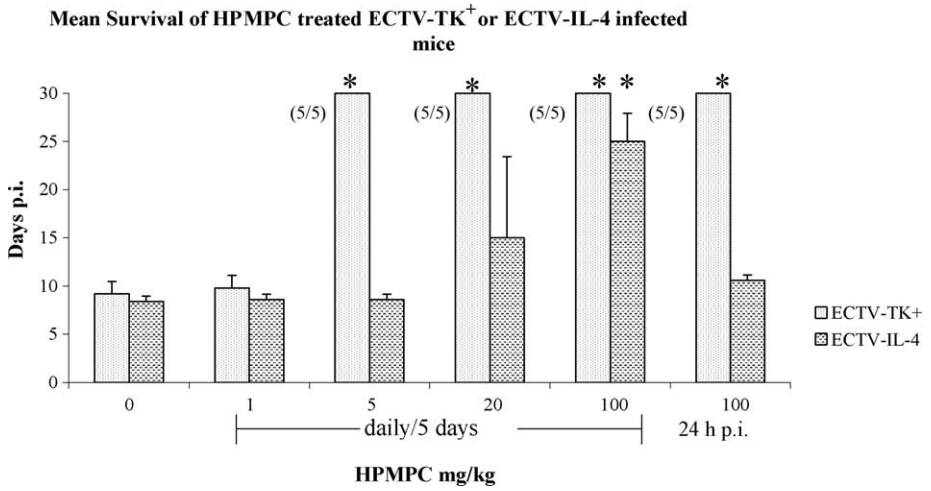

Fig. 1. BALB/c mice infected with 1×10^3 PFU of ECTV-TK$^+$ and C57BL/6 mice infected with 1×10^3 PFU ECTV-IL-4 via footpad inoculation were treated with intraperitoneal injections of HPMPC (excluding control mice). Each treatment regime commenced 24 h post-viral inoculation as described in Section 2.2. Mice were observed for signs of disease and survival was recorded for the duration of the experiment (30 days p.i.). 5/5 indicates 100% survival of mice given ECTV-TK$^+$. Group means ± S.D. are shown, $^*P \leq 0.05$ compared to untreated mice or those given 1 mg/kg/day.

into the right hind footpad with 10^3 PFU of ECTV-TK$^+$ control virus, while the C57BL/6 mice were given 10^3 PFU of ECTV-IL-4. After 24 h both strains of mice were weighed, and given doses of HPMPC ranging from 1 to 100 mg/kg/day over 5 days via intraperitoneal injection, while one group of mice of each strain was given a single dose of 100 mg/kg HPMPC at 24 h after viral inoculation.

HPMPC treatment, particularly at higher doses of the range examined, was clearly protective against symptoms of mousepox infection of mice with ECTV-TK$^+$ (Fig. 1). BALB/c mice given 1 mg/kg/day of the drug succumbed to the infection, albeit with disease symptoms becoming more chronic, and including development of lesions over the body, face, and tail, edema of all limbs and nose, eye inflammation, ruffled fur, and hunched posture. Average survival time was 12.0 ± 4.0 days. Mice given 5 mg/kg/day of HPMPC exhibited signs of mild, chronic mousepox with minor eye inflammation and footpad swelling but had a 100% recovery. Those given higher doses of HPMPC had mild footpad swelling and 100% recovery from infection. HPMPC treatment (20 mg/kg/day) of resistant C57BL/6 mice infected with ECTV-TK$^+$ completely abrogated all overt disease symptoms (data not shown).

In contrast to its activity against ECTV-TK$^+$, HPMPC was poorly effective against infection with ECTV-IL-4, except at a dosage of 100 mg/kg/day which significantly delayed time of death compared to untreated controls (Fig. 1). C57BL/6 mice receiving 1 mg/kg/day or 5 mg/kg/day developed acute mousepox infection with 100% lethality and a mean survival time of 8.5 ± 1.0 day. Mice treated with 20 mg/kg/day or a single dose of 100 mg/kg showed a delay in developing disease symptoms, having mild footpad inflammation by day 3 p.i. More severe footpad swelling developed by day 10 p.i, along with eye inflammation, ruffled fur and lethargy; 80% of these mice died with a mean survival time of 11.5 ± 1.5 days. Animals given 100 mg/kg/day of the drug appeared initially to respond to treatment, with only very mild footpad inflammation being observed until day 21 p.i. However, disease symptoms, footpad inflammation, eye inflammation, ruffled fur, and lethargy increased thereafter with 80% (4/5) succumbing to infection by day 27 p.i. The remaining mouse survived the course of the experiment.

3.3. HPMPC treatment is associated with reduced viral load but does not prevent systemic spread of ECTV-IL-4

C57BL/6 mice infected with either ECTV-TK$^+$or ECTV-IL-4 had reduced viral loads in footpad and splenic tissues following HPMPC treatment compared to infected mice not given the drug ($P \leq 0.05$, Table 1). Virus was found both locally and systemically in untreated ECTV-TK$^+$-infected mice, with mean titers of $\log_{10}$ 6.6 PFU/g of footpad tissue and $\log_{10}$ 4.2 PFU/g of splenic tissue. HPMPC treatment markedly reduced viral levels in footpad tissue ($P \leq 0.05$, $\log_{10}$ 2.0 PFU/g) and virus was not detected in splenic tissue by day 6 p.i. Virus was apparently cleared from footpad tissues in 80% of the mice by day 11 p.i. with the exception of a single mouse having apparently localized the virus (detected at 1×10^4 PFU/g). Untreated ECTV-IL-4-infected mice had high viral titers both locally (mean titer of $\log_{10}$ 7.6 PFU/g) and systemically ($\log_{10}$ 7.0 PFU/g). HPMPC treatment reduced these virus levels ($P \leq 0.05$); however, unlike ECTV-TK$^+$, systemic spread of ECTV-IL-4 was prevalent in mice treated with the drug at 20 mg/kg, with a mean splenic viral titer of $\log_{10}$ 2.2 PFU/g by day 6 p.i. and virus loads in both footpad and splenic tissue returning to similar high levels as those found in the untreated controls by day 11 (Table 1). ECTV-IL-4-infected mice treated with a larger dose of HPMPC (100 mg/kg) had high levels of virus in footpad tissue ($\log_{10}$ 3.7 PFU/g) but with no evidence for systemic

Table 1
Viral load of HPMPC-treated and untreated C57BL/6 mice infected with either ECTV-TK$^+$ or ECTV-IL-4

	Virus			
	ECTV-TK$^+$		ECTV-IL-4	
	Day p.i. ($n = 5$/group)			
	6 p.i.	†11 p.i.	6 p.i.	†11 p.i.
Footpad PFU/g $\log_{10}$				
Untreated control	6.6 ± 4.9	NT	7.6 ± 5.9	NT
HPMPC 20 mg/day	$2.0 \pm 1.3^*$	$<10^{2\,\#}$	$3.8 \pm 2.6^*$	7.6 ± 6.2
HPMPC 100 mg/day	$<10^{2\,*}$	NT	$3.7 \pm 2.8^*$	NT
Spleen PFU/g $\log_{10}$				
Untreated control	4.2 ± 2.9	NT	7.0 ± 5.9	NT
HPMPC 20 mg/day	$<10^{2*}$	$<10^2$	$2.2 \pm 1.2^*$	7.5 ± 6.0
HPMPC 100 mg/day	$<10^{2*}$	NT	$<10^{2*}$	NT

C57BL/6 mice infected with 1×10^3 PFU of either ECTV-TK$^+$ or ECTV-IL-4 were treated daily with 20 mg/kg or 100 mg/kg of HPMPC for five consecutive days commencing at 24 h p.i. Virus titers were assayed on day 6 p.i. and day 11 p.i. and are shown as group means ± S.D. (PFU/g $\log_{10}$). $<10^2$ indicates virus was not detected within assay sensitivity limits. $^\#$Indicates 4 logs of virus were detected in a single mouse in this group and with no evidence of systemic spread. NT: not tested. †Day 11 p.i. data are from a repeat experiment. Statistical comparisons between untreated and HPMPC-treated groups (20 mg/kg/day and 100 mg/kg/day) showed significant differences ($^*P \leq 0.05$) at day 6 p.i. for both ECTV-TK$^+$- and ECTV-IL-4-infected mice. Comparisons between uninfected and infected mice on day 11 p.i. were not performed as the mice given placebo did not survive the duration of the experiment.

spread at day 6 p.i. Thus, HPMPC treatment resulted in reduced viral loads but did not appear to completely abrogate virus replication thereby allowing for systemic spread of the virulent IL-4 recombinant from the site of inoculation.

3.4. High levels of IFN-γ expression in mice infected with recombinant ECTV-IL-4

To investigate the nature of the immune suppression induced by ECTV-IL-4, cytokine expression profiles in the serum of HPMPC-treated or untreated C57BL/6 mice infected with ECTV-TK$^+$or ECTV-IL-4 were measured using a cytometric bead assay (Table 2). ECTV-TK$^+$-infected mice had increased levels of serum IFN-γ that could be detected as early as day 3 p.i. (data not shown) and were found at high mean levels of 978.5 pg/ml by day 6 p.i. Interestingly, high levels of IFN-γ were also detected in the sera of ECTV-IL-4-infected mice (1012.9 pg/ml) along with very high levels of IL-4 (>5000 pg/ml). Our additional experiments using an IFN-γ ELISPOT assay have confirmed this finding of high levels of IFN-γ expression in both ECTV-TK$^+$ and ECTV-IL-4-infected animals (data not shown). IFN-γ was not detected in ECTV-TK$^+$-infected mice treated with HPMPC at 20 mg/kg per day for five consecutive days, consistent with the observed inhibition of virus replication at this time. In contrast, ECTV-IL-4 infected mice continued to express both IFN-γ and IL-4 at day 6 p.i. after treatment with HPMPC. Serum levels of the Th1 cytokines IL-2 and TNF-α, or the Th2 cytokine IL-5, were not increased either in HPMPC-treated or in untreated mice infected with either ECTV-TK$^+$ or ECTV-IL-4.

4. Discussion

In this study we evaluated the efficacy of the antiviral phosphonylmethylether nucleotide analog HPMPC (cidofovir) in virulent models of mousepox virus infection in two strains of mice. There is currently widespread interest in HPMPC efficacy against virulent poxvirus infection in the light of recent

Table 2
Cytokine expression in sera HPMPC-treated and untreated C57BL/6 mice infected with either ECTV-TK$^+$ or ECTV-IL-4

Sera day 6 p.i.	Virus					
	ECTV-TK$^+$ ($n = 5$)			ECTV-IL-4 ($n = 5$)		
	Untreated control	HPMPC 20 mg/day	HPMPC 100 mg/day	Untreated control	HPMPC 20 mg/day	HPMPC 100 mg/day
Cytokines (pg/ml)						
TNF-α	22.0 ± 4.4	≤20	≤20	≤20	≤20	≤20
IFN-γ	978.5 ± 699.9	≤20	≤20	1012.9 ± 575.8	50.8 ± 24.5	62.8 ± 26.4
IL-4	≤20	≤20	≤20	>5000	33.9 ± 6.3	21.4 ± 0.8
IL-5	≤20	≤20	≤20	≤20	≤20	≤20
IL-2	≤20	≤20	≤20	≤20	≤20	≤20

C57BL/6 mice infected with 1×10^3 PFU of either ECTV-TK$^+$ or ECTV-IL-4 were treated daily with 20 mg/kg or 100 mg/kg of HPMPC for five consecutive days commencing at 24 h p.i. Control mice received PBS placebo injections. Data were analysed using BD Th1/Th2 Mouse Cytokine software, group means ± S.D. are shown. The minimum sensitivity of the assay was ≤20 pg/ml and the maximum quantifiable level was ≤5000 pg/ml.

threats of bioterrorism. Firstly, we used mousepox-sensitive BALB/c mice and found that doses of 5 mg/kg/day of the drug given for five consecutive days after infection with a control ECTV-TK$^+$ were fully protective against the normally lethal effects of this virus, although these animals suffered a chronic form of mousepox disease. A far milder form of disease, limited to localized footpad inflammation, was observed in mice given higher doses of the drug. Secondly, we used the highly virulent ECTV-IL-4 construct in mousepox-resistant C57BL/6 mice. Mice of this strain are normally refractory to the lethal effects of ECTV infection but succumb to infection with ECTV-IL-4 (Jackson et al., 2001). In contrast to its efficacy in BALB/c mice given ECTV-TK$^+$, HPMPC failed to protect C57BL/6 mice against infection with ECTV-IL-4, although daily doses of 20 mg/kg/day for five consecutive days after infection or a single dose of 100 mg/kg delayed mortality for 3 days. Daily doses at 100 mg/kg further delayed the lethal effects of this virus, although the majority of animals succumbed between 14 and 26 days after infection. It appears, therefore, that HPMPC may have inhibited ECTV-IL-4 replication without mediating clearance and the virus that emerged with lethal effects when the drug was removed.

Strong cell-mediated immune responses are normally required for resolution of ECTV infection (Jacoby et al., 1989; Karupiah et al., 1998, 1993a,b; Ramshaw et al., 1997; Chaudhri et al., 2004). In our study, low doses of HPMPC inhibited viral replication in mouse-pox sensitive BALB/c mice, facilitating ECTV clearance in these animals that normally exhibit poor antiviral cell mediated immune responses. In contrast, mousepox resistant mice infected with recombinant ECTV-IL-4 responded temporarily to relatively high doses of the drug with delayed mortality; however, once treatment ceased they succumbed to infection. HPMPC did not completely abrogate viral replication, nor are ECTV-IL-4 infected mice able to mount effective antiviral immune responses (Jackson et al., 2001). Mechanisms underlying the ability of the ECTV-IL-4 recombinant to evade immune clearance remain to be clarified; however, our earlier studies have highlighted the immune suppressive nature of this construct. ECTV-IL-4 suppressed NK and CTL cytolytic activity and IFN-γ production by splenic CD8$^+$ T cells (Jackson et al., 2001), while vaccinia virus-encoded IL-4 is associated with down regulation of antiviral CTL responses linked to reduced proliferation of CD8$^+$ T cells in IL-4 transgenic mice (Rolph and Ramshaw, 2003). The ability of IL-4 to inhibit perforin-mediated cytotoxicity (Aung and Graham, 2000) may also be important in this model, since this process appears to play a key role in recovery from ECTV infection (Müllbacher et al., 1999).

To further investigate the inability of mice to clear ECTV-IL-4, we assayed cytokine expression profiles in sera of infected mice. Interferon gamma (IFN-γ), in particular, is a cytokine known to inhibit virus replication and spread via direct antiviral or indirect immunoregulatory activities and normally plays a key role in the immune clearance of ECTV infection (Jacoby et al., 1989; Karupiah et al., 1993a; Ramshaw et al., 1997). Indeed, ECTV can subvert the antiviral immune response by inhibition of IFN-γ through secretion of virus-encoded soluble receptors (Smith and Alcami, 2002). The very low levels of IFN-γ found in control ECTV-TK$^+$ infected mice on day 4 p.i. in our study are probably reflective of the observed inhibition of virus replication in HPMPC-treated mice. Unexpectedly, however, high serum levels of IFN-γ were found in conjunction with very high levels of IL-4 in the mice infected with ECTV-IL-4. We had anticipated that virus-encoded IL-4 would drive the immune response towards a Th2 cytokine profile, while our previous study had shown a suppression of IFN-γ expression by CD8$^+$ T cells following ECTV-IL-4-infection (Jackson et al., 2001). Continued production of IFN-γ in ECTV-IL-4-infected mice in the present study may be due to CD4$^+$ T cells or NK cells and studies are underway to clarify this issue. In any case, the high serum IFN-γ levels did not facilitate clearance of the IL-4 construct, with 80% mortality observed even in mice receiving high doses of HPMPC. As noted above, HPMPC reduced ECTV-IL-4 loads when given at high levels but did not prevent systemic spread from the footpad site of inoculation.

We chose to investigate the efficacy of HPMPC (Vistide®) as a therapeutic agent against ECTV-TK$^+$ and ECTV-IL-4 infection as it is currently licensed for treatment of CMV retinitis in AIDS patients and has also proven to be an effective antiviral drug against other poxviruses in vitro. While effective against infection with control ECTV recombinants, it did not contain the more virulent ECTV-IL-4 except at high doses and only during the period of treatment. While these higher levels of HPMPC were effective in delaying viral symptoms, doses of this magnitude may not be appropriate in clinical situations, due to the risk of adverse side effects. The current recommended dosage of HPMPC for the treatment of CMV retinitis is 5 mg/kg of body weight given as an intravenous infusion once every two weeks, and possible adverse reactions to the drug include nephrotoxicity, neutropenia, decrease of intraocular pressure, and metabolic acidosis (Gilead Sciences, Inc., 2000). Cidofovir in its cyclic form (cHPMPC) and the alkyloxyalkyl 1-*O*-hexadecyloxypropyl (HDP) ester (HDP-HPMPC) have greater bioavailability and enhanced potency than the drug tested in this study (Bradbury, 2002). Recently, alternative alkoxyalkanol esters of HPMPC have been shown to be highly effective in the treatment of poxvirus infection, in particular octadecyloxyethyl (ODE)-CDV, a derivative of HPMPC, completely inhibited replication of ECTV in a normally lethal aerosol challenge model in A/NCR (Buller et al., 2004; Quenelle et al., 2004). The increased bioavailability of this type of compounds may provide the best current hope for effective control of virulent poxvirus infections in combination with improved rational vaccination strategies.

Acknowledgements

We gratefully acknowledge Mr. Geoff Osbourne and Ms. Sabine Gruninger for their assistance with FACS Analysis

of the cytokine cytometric bead array assay, and the Animal Services Division of the Australian National University for the supply and care of mice. This work was supported by NIH Grant R21-AI053290 (A.J.R. and I.A.R.).

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