Cidofovir Protects Mice against Lethal Aerosol or Intranasal Cowpox Virus Challenge

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The efficacy of cidofovir for treatment of cowpox virus infection in BALB/c mice was investigated in an effort to evaluate new therapies for virulent orthopoxvirus infections of the respiratory tract in a small animal model. Exposure to 2–5 \( \times 10^6 \) pfu of cowpox virus by aerosol or intranasally (i.n.) was lethal in 3- to 7-week-old animals. One inoculation of 100 mg/kg cidofovir on day 0, 2, or 4, with respect to aerosol infection, resulted in 90%–100% survival. Treatment on day 0 reduced peak pulmonary virus titers 10- to 100-fold, reduced the severity of viral pneumonitis, and prevented pulmonary hemorrhage. The same dose on day −6 to 2 protected 80%–100% of i.n. infected mice, whereas 1 inoculation on day −16 to −8 or day 3 to 6 was partially protective. Cidofovir delayed but did not prevent the death of i.n. infected mice with severe combined immunodeficiency. Treatment at the time of tail scarification with vaccinia virus did not block vaccination efficacy.

Orthopoxviruses pose major risks to human health. By the time smallpox was finally eradicated in the late 1970s, the virus had killed tens of millions of people and disfigured countless millions more [1]. The subsequent discontinuation of vaccination programs has rendered most humans vulnerable to smallpox infection, and it is now feared that variola virus, the agent of smallpox, might be used as an agent of biological warfare or terrorism [2]. Meanwhile, monkeypox, a disease transmitted to humans from wild animals, continues to occur in central Africa [3]. Human monkeypox infection resembles smallpox but is less lethal and has a lower secondary attack rate [4, 5].

There are, therefore, several public health and biological defense reasons to improve our ability to prevent or treat virulent orthopoxvirus infections. In either case, it is the human respiratory tract that must be defended. While endemic smallpox was still occurring, it was noted that infection typically spread only to persons in close contact with patients, probably by means of infectious droplets arising from oropharyngeal enanthema. However, under certain circumstances, variola was transmitted as a true airborne agent, with devastating consequences. A German hospital outbreak in 1966 demonstrated unequivocally that even brief exposure to low levels of airborne virus is sufficient to initiate infection [6]. Laboratory accidents provide further proof of the danger of aerosolized variola [1].

There are no approved medications for smallpox or monkeypox infection. However, some new drugs now in clinical use for treating severe herpesvirus infections also inhibit orthopoxviruses. In particular, cidofovir (1-[(S)-3-hydroxy-2-(phosphonomethoxy)-propyl]cytosine; HPMPC), a phosphonylethynyl derivative of cytotoxic, which is approved for the treatment of cytomegalovirus (CMV) infection in immunocompromised patients, also inhibits vaccinia virus (VV) replication [7]. Cidofovir has a broad spectrum of activity against DNA viruses. The drug is protective in murine models of VV, herpes simplex virus, and CMV infection [7–12]. In addition, we recently found that cidofovir is highly active in vitro against other orthopoxviruses: cowpox virus (CPV), camelpox, monkeypox, and 3 isolates of variola (present authors, unpublished data).

To test cidofovir and other drugs for effectiveness against virulent orthopoxvirus infections targeting the respiratory tract, we first needed to identify an appropriate murine disease model. Variola and monkeypox viruses do not cause disease in adult mice, making it necessary to use surrogate viruses. Previous antiviral drug evaluations in mice have used VV administered intravenously (i.v.) or intracerebrally [13–15]. A reduction in the number of VV tail lesions has been used as an index of the antiviral activity of a range of compounds, including ribavirin, cytosine arabinoside, methisazone, interferon, and interferon inducers [13–17]. A lethal model of VV infection in mice with severe combined immunodeficiency (SCID mice) has also been
used [9]. However, neither the iv nor the intracerebral route simulates the respiratory exposure that takes place in natural smallpox or monkeypox infection or that would occur if smallpox were used as a biologic weapon.

Intranasal (inl) infection with the Western Reserve strain of VV causes lethal encephalitis in mice, but the disease has little in common with severe orthopoxvirus infections of humans, which do not involve the central nervous system [18]. However, the Brighton strain of CPV, which produces localized skin lesions in humans, has been shown to be lethal for mice when inoculated by a variety of pathways and to cause fatal pneumonia when administered by the inl route [19–21]. In a pilot study, we confirmed that CPV is uniformly lethal for inl infected weanling BALB/c mice and found that a series of subcutaneous (sc) doses of cidofovir, or a single dose on the day of infection, protected mice from death (present authors, unpublished data).

To our knowledge, there are no published reports of aerosol CPV infection. We therefore expanded our evaluation to include infection of mice by means of a small-particle aerosol of CPV. We studied the effect of cidofovir treatment on the course of illness in mice lethally infected by the aerosol or inl route and measured the effect of drug treatment on the extent of viral replication and the progression of pathologic abnormalities. We examined the role of the immune response in the protective efficacy of cidofovir by challenging immunocompetent or SCID mice inl with CPV and treating them once or repeatedly with cidofovir. We also evaluated the interval required for VV vaccination to induce protection against CPV and determined whether drug treatment close to the time of vaccination interfered with subsequent protection against lethal challenge.

Materials and Methods

Viruses, cells, and antiviral drug. The Brighton strain of CPV and the Copenhagen strain of VV were provided by J. Esposito (Centers for Disease Control and Prevention, Atlanta). Vero and Vero clone E6 monkey kidney cells (VERO C1008, ATCC CRL 1586) were propagated in Eagle MEM with Earle’s salts (EMEM), nonessential amino acids, 10% fetal bovine serum, glutamine, penicillin, and streptomycin at 37°C in a 5% CO2 atmosphere. Cidofovir (Vistide) was provided by Norbert Bischofberger (Gilead Sciences, Foster City, CA). The compound was dissolved in PBS at concentrations appropriate for injection in a 0.1-mL volume.

Aerosol and inl CPV infection. Female weanling BALB/c mice and 5- to 6-week-old SCID mice were obtained from the National Cancer Institute (Frederick, MD), housed in filtertop microisolator cages, and given commercial mouse and ad libitum. Autoclaved cages, food, and water were used for immunodeficient animals. Both viruses were used under biosafety level 3 containment at the US Army Medical Research Institute of Infectious Diseases (Frederick, MD).

Mice were exposed to aerosolized CPV suspended in EMEM or to EMEM alone in a nose-only system contained within a class III biological safety cabinet. Aerosols were generated by a 3-jet Collison nebulizer (BGI, Waltham, MA) at a flow rate of 7.5 L/min and a predicted median particle diameter of 1.2 μm [22, 23]. The aerosol was mixed with secondary air for a total system flow rate of 12 L/min and was then divided. Half flowed to the mouse nose ports, where it was distributed evenly through metering orifices. The other half was sampled continuously, using an all-glass impinger to drive the aerosol into EMEM containing 2% fetal bovine serum and an antifoaming agent (Antifoam A Emulsion; Sigma, St. Louis). The amount of virus collected was determined by plaque assay, and the inhaled virus dose was calculated on the basis of exposure time and Guyton’s formula for minute volumes administered to rodents [24]. Susceptibility to aerosol infection was tested in 4-week-old (11–13 g) BALB/c mice, which were exposed to an aerosolized dose of ~5 × 105, 5 × 104, or 5 × 103 pfu of virus.

For inl challenge, virus suspended in PBS was placed dropwise on the nares with a micropipette tip. The LD50 of the virus was determined by inoculating groups of 10 mice inl with 104–106 pfu of virus, observing them for 21 days, and analyzing survival with a linear regression program (SPSS, Chicago). The age dependence of susceptibility was tested by inoculating 4-, 5-, 6-, and 7-week-old mice with 2 × 103 pfu of virus.

In vivo drug evaluation. Mice were challenged inl with 2 × 106 pfu or by aerosol with ~5 × 105 pfu of CPV, treated with cidofovir or PBS by sc injection at various time points, and then weighed daily for 21 days and observed for illness and death. Drug efficacy was also tested in SCID mice challenged inl with CPV and treated with either a dose (100 mg/kg) of cidofovir or PBS on the day of infection or treated repeatedly at intervals of 3, 4, 5, or 6 days, beginning on day 0. On day 20, all surviving mice were killed, and lung virus titers were determined. Drug toxicity was assessed by treating 12 uninfected mice with 100 mg/kg drug; exsanguinating 3 of them on each of posttreatment days 1, 3, 5, and 9; determining the serum levels of urea nitrogen, creatinine, aspartate aminotransferase, and alanine aminotransferase, using a Kodak 250 chemistry analyzer (Eastman Kodak, Rochester, NY); and comparing values with those of 5 untreated mice.

Postmortem examination and tissue virus titration. For pathology studies, groups of mice were infected by aerosol or inl, as described above, and treated with cidofovir at various time points or mock-treated with PBS. In some experiments, groups of 10 drug- or placebo-treated mice were observed for illness and death after infection, while on each day or every second or third day, 3–5 mice from parallel groups were killed, and tissues were removed for histologic examination and virus titration. Lung samples were collected from some inl or aerosol-infected mice 4–6 h after challenge. Whole blood and tissue specimens were removed aseptically. Portions of both lungs and of the liver, spleen, and brain were weighed, frozen, and then thawed and ground in a mortar with sterile sand and growth medium. The resulting suspension was clarified by low-speed centrifugation and titrated by plaque assay, as described below. Collection and processing of tissues for histology, histochemistry, and immunohistochemistry have been described elsewhere [25].

Virus titration. Vero cells were plated in 6-well dishes and used within 48 h of confluence. Virus suspensions were frozen and thawed, vortexed, sonicated, and diluted serially 10-fold in EMEM. Growth medium was removed from 6-well dishes, and 0.2-mL volumes of serially diluted virus were added and allowed to incubate for 2 h, and then the medium was replaced. At 4–5 days after
infection, an equal volume of crystal violet–formalin stain (1.3 g/L crystal violet, 5% ethanol, 3% buffered formalin) was added to each well. After 20 min, the wells were washed 3 times in water and air-dried. Plaques were counted, and virus titers were calculated.

Neutral red uptake assay of antiviral activity. Cidofovir was dissolved in EMEM, serially diluted 3-fold, and added to the medium of confluent Vero E6 cells in 96-well microtiter plates. At each drug concentration, 3 wells were then infected with 1000 pfu of CPV, and 3 were left uninfected for toxicity determination. Six days after infection, neutral red dye was added to a final concentration of 0.01%, and the plates were returned to the incubator for 2 h. The wells were then rinsed, retained stain was solubilized with an ethanol–0.01 M NH₄H₂PO₄ solution (pH 3.5), and the optical density was measured at a wavelength of 540 nm. Data were analyzed by use of a curve-fitting program (Molecular Devices, Menlo Park, CA) to determine the IC₅₀ for the drug.

Vaccination with VV or CPV. Groups of 10 3-week-old mice were vaccinated by scarification of the base of the tail through a 10-μL droplet of PBS containing 10⁶ pfu of VV or CPV or were mock-vaccinated with PBS and then treated with 100 mg/kg cidofovir at various time points with respect to vaccination, or they were mock-treated and challenged inl with CPV at various time points with respect to vaccination. In 1 experiment, groups of mice were vaccinated and treated with cidofovir at various time points or mock-treated with PBS, and the approximate area of the resulting skin lesions (maximum width × maximum length) was measured on day 4 after vaccination.

Results

Dose-ranging studies. Preliminary experiments revealed that a series of daily doses of 20, 5, or 1 mg/kg cidofovir, begun on the day of infection, protected mice from lethal inl CPV infection (J.W.H., unpublished data). As little as 5 mg/kg cidofovir given on day 0 protected nearly all mice against death, but higher doses were required to ensure survival when treatment was delayed to postinfection days 1–3. A single injection of 100 mg/kg cidofovir was highly protective throughout this time period. This dose caused no signs of illness or increase in serum urea nitrogen, creatinine, aspartate aminotransferase, or alanine aminotransferase in uninfected mice over 9 days following treatment.

Features of aerosol CPV infection. An aerosol dose of ~5 × 10⁶ pfu of CPV killed all mice (mean weight, 12 g) in 1 experiment and 13 of 20 mice (mean weight, 17 g) in a second experiment. Further experiments have confirmed that younger mice are more susceptible to aerosol infection. Visible illness (ruffled fur, slowed activity) and weight loss began on day 5, and deaths occurred by day 12 after infection. Exposure of 12-g mice to 5 × 10⁴ pfu caused transient mild illness and weight loss but no deaths, whereas 5 × 10² pfu of virus or of aerolized growth medium alone produced no signs of disease.

Mice infected with 5 × 10⁵ pfu of CPV developed diffuse, bilateral viral pneumonitis. Grossly, the lungs showed multi-
focal-to-coalescing areas of dark red consolidation by day 6, which involved the majority of the visceral pleural surface by day 10. Lung weights began to increase on day 4 and exceeded 3 times normal weight by the time of death (see below). The most common pathologic lesions were located in small bronchioles, notably at the bronchioloalveolar junction, indicating that aerosolized virus reached the most distal portions of the airways (figure 1). Individual lesions showed features consistent with cell-to-cell spread, including centrifugal expansion from a necrotic center, epithelial degeneration and necrosis, intra- and extracellular edema, exfoliation, and hemorrhage. The inflammatory cell infiltrate consisted predominantly of neutrophils and histiocytes.

Viral antigen was detected in bronchiolar epithelial and smooth-muscle cells, macrophages, and alveolar septal lining cells by day 2 and in fibroblasts, vascular smooth-muscle cells, and alveolar macrophages at later time points (figure 1). Eosinophilic intracellular viral inclusion bodies were evident by day 4. The expansion of these bronchiolar lesions over time caused edema and accumulation of cellular debris and fibrin within the lumina, leading to airway occlusion, alveolar consolidation, bronchopneumonia, and hemorrhage (for additional findings, see [25]).

Pulmonary virus titers reached $10^9$ pfu/g by day 6 (figure 2). Although virus was not detected in samples of whole blood, hematogenous dissemination clearly took place, because virus was detectable in the liver and spleen beginning on day 6 and persisted through day 12. Titers did not exceed $10^6$ pfu/g. Many aerosol-infected mice developed multiple small ulcerated pocks on the tail, feet, and ears beginning 10–12 days after infection.

**Cidofovir therapy for aerosol CPV infection.** One dose of 100 mg/kg cidofovir was highly protective against death from aerosol CPV infection. In an experiment in which all 10 control mice died 10–12 days after challenge, all 20 mice treated on day 0 or 4 and 9 of 10 mice treated on day 2 survived infection. Treatment on day 6, when the mice were already ill and losing weight, rescued 5 of 10 animals from death. Mice treated on day 0 or 2 remained active but went through a period of weight loss beginning on day 4, 5, or 6; they eventually recovered the lost weight (figure 3A). Delay in treatment until day 4 or 6 resulted in development of visible illness (ruffled fur, slowed activity) and greater loss of body weight. The lung weights of...
mock-treated mice quadrupled by day 12 after infection, reflecting bronchopneumonia (figure 3B). Treatment on day 0 prevented any increase in lung weight, whereas treatment on day 2, 4, or 6 was progressively less protective.

Therapy on day 0 resulted in a slower increase in pulmonary virus titers, and a 10- to 100-fold decrease in peak titers, relative to those in mock-treated animals (figure 4A). Delay in treatment to day 2 diminished these effects (figure 4B). Treatment on day 4 or 6 resulted in a marked reduction in lung virus titers on day 6 or 9, respectively, but titers in all treated groups rose to $10^7$ pfu/g on day 12. Lung titers of all mice were still in the range of $10^6$–$10^7$ pfu/g on day 18; the highest levels were seen in animals treated on day 6.

Pulmonary disease was examined by microscopy in drug-treated mice and mock-treated controls on day 9 after infection. Mock-treated mice showed innumerable infected bronchioles throughout the lungs, with peribronchial hemorrhage and bronchopneumonia [25]. Treatment on day 0 prevented the development of all features of infection, except for occasional small foci of virus-induced mucosal epithelial degeneration, with intra- and intercellular edema. These involved only a portion of the bronchiolar circumference, with a minimal inflammatory response and no hemorrhage. A delay in treatment to day 2 or 4 resulted in a progressive increase in the number and extent of bronchiolar mucosal lesions and in the severity of inflammation, degeneration, necrosis, airway obstruction, alveolar consolidation, and peribronchovascular hemorrhage. The principal difference between the lungs of day 2- and day 4-treated mice and mock-treated controls was that in the former, pathologic abnormalities remained essentially confined to the bronchioles, whereas in mock-treated animals, disease progression led to alveolar consolidation and bronchopneumonia.

Even though treatment on day 6 resulted in 50% survival, pulmonary lesions in this group were indistinguishable from those of mock-treated animals. The lungs of surviving drug-treated mice examined on day 19 showed extensive perivascular cuffing by chronic inflammatory infiltrates, which were composed of macrophages, lymphocytes, and plasma cells. Bronchiolar epithelium adjacent to foci of inflammation showed residual mucosal injury, with necrotic debris within alveolar ducts and bronchioles; this finding was more prominent in groups treated at later time points.

**Features of inl CPV infection.** The LD$_{50}$ of CPV for 10- to 12-g mice infected by the inl route was $\sim 5 \times 10^4$ pfu. Infection with $2 \times 10^6$ pfu (100 LD$_{50}$) caused weight loss beginning on day 3 or 4, with eventual loss of up to 25% of initial body weight. The mean time to death was $\sim$8 days. Older mice were less susceptible to the same dose of virus: by 7 weeks, only half succumbed to a dose of $2 \times 10^6$ pfu.

Microscopic features of inl infection were consistent with those described by Thompson et al. [21]. All untreated animals developed extensive infection of the upper respiratory tract, and most developed bronchitis and bronchopneumonia, as revealed by increased lung weight and histopathologic changes (for additional details, see [25]). The extent of pulmonary disease was much more variable than that observed after aerosol infection, probably as a result of nonuniform distribution of virus following inl inoculation. Most pulmonary virus titers among inl infected animals were in the range of $10^7$–$10^8$ pfu/g by day 4 and remained elevated until death (not shown). Virus was not detected in samples of whole blood, but titers in the spleen reached $10^6$ pfu/g by day 4 and then declined to $<10^5$ pfu/g on days 5–8. The liver showed a similar rise and fall of virus titers, but the highest values did not exceed $10^5$ pfu/g. Virus was not detected in the brain. Skin lesions (pocks) did not develop in mock-treated mice dying from inl infection.

**Cidofovir therapy for inl CPV infection.** A single sc inoculation of 100 mg/kg cidofovir was highly protective, both for prophylaxis and as therapy. This dose, administered from 4 days
before to 3 days after intranasal infection, prevented death in 80%–100% of mice in a series of experiments (figure 5A). The same dose protected 35%–50% of mice when given on day 4 and 10%–20% when given on day 6. Remarkably, 1 dose of cidofovir on day −16 prevented death in 4 of 10 and 5 of 10 animals in duplicate experiments. Many mice that survived as a result of treatment on postinfection day 3 or later developed pocks on the tail, paws, and ears, beginning at about day 10. Treatment on day 0 prevented the weight loss that began on day 4 in mock-treated mice (figure 5B). Some weight loss was seen in animals treated on day 0, and more significant loss was seen in animals treated at later time points. In an experiment comparing the effect of 1 or 2 doses of 100 mg/kg cidofovir, all 20 mice treated on day 0 or on days 0 and 5 survived infection, as did all 10 mice treated on day 2 and 9 of 10 mice treated on days 2 and 7. All 10 mock-treated animals died. As shown in figure 5B, the addition of a second dose of drug had no effect on weight loss, indicating that an early large dose of cidofovir was sufficient to provide the entire benefit of therapy.

Pulmonary pathology in mice treated on day 0 was compared with that in mice treated on day 0 or in mock-treated mice. Animals treated on day 0 showed an almost complete absence of pulmonary disease, with only occasional locally affected bronchi or bronchioles (or both), epithelial degeneration and necrosis, small viral inclusions, and a minimal inflammatory cell infiltrate. Mice treated on day 2 had a slightly greater number of pulmonary lesions, located predominantly in the hilar region, that showed a notable increase in the extent and severity of epithelial degeneration and necrosis, larger viral inclusions, and more extensive infiltration with neutrophils and macrophages. However, like mice treated on day 0, day 2–treated mice had minimal or no peribronchovascular hemorrhage.

All mice treated on day 0 had markedly lower pulmonary virus titers (or no detectable virus) than mock-treated controls during the first week after infection. After the end of the first week, the lung virus titers of drug-treated mice increased, approaching 10^5 pfu/g on day 8 and 10^6 pfu/g on day 12, before plummeting to undetectable levels on day 14 (not shown). A second dose of drug on day 5 did not prevent the later increase in virus titers. An identical pattern was seen in an earlier experiment. All animals appeared healthy and continued to gain weight during the course of the infection. To determine whether increasing virus titers on days 8–12 resulted from the outgrowth of drug-resistant virus, we examined virus recovered from the lungs of 6 mice on days 10 and 12 for sensitivity to cidofovir by a neutral red uptake assay. All 6 isolates proved to be as susceptible to the drug as the original challenge virus (data not shown).

**Cidofovir therapy of intranasal infection in SCID mice.** SCID mice were far more susceptible than immunocompetent animals to intranasal infection; the LD₅₀ was ~100 pfu. To determine whether an intact immune system was required for successful cidofovir therapy, we infected SCID mice with the same quantity of virus used to challenge normal mice and treated them either on day 0 or with repeated doses every 3–6 days, beginning on day 0, with 100 mg/kg cidofovir. As shown in figure 5C, treatment on day 0 delayed death by only a few days. Repeating the dose every 3 days delayed the first death to day 14, but 7 of 10 mice thus treated were dead by day 20. Those treated every 4–6 days began dying earlier, and only 1 or 2 in each

![Figure 5](https://academic.oup.com/jid/article-abstract/181/1/10/890814/09 April 2019)

**Figure 5.** Intranasal cowpox virus (CPV) challenge. *A.* Effect of single subcutaneous inoculation of 100 mg/kg cidofovir on survival after intranasal (IN) infection. Crosses, mean % survival in 2–6 experiments, except for days −6 and −4, when 1 experiment was done. Vertical bars, SDs. *B.* Effect on mean body weight of groups of 10 mice treated with 100 mg/kg cidofovir on indicated days or mock-treated with PBS on day 0. *C.* Effect of drug treatment on course of IN CPV infection in SCID mice. Mice were treated with 100 mg/kg cidofovir on day 0 only or every 3 or every 6 days, beginning on day 0, or were mock-treated with PBS on day 0.
group were still alive on day 20. CPV was still present in the lungs of all surviving mice on day 20, with titers ranging from $5 \times 10^4$ to $2 \times 10^5$ pfu/g. Prolonged survival of cidofovir-treated SCID mice did not lead to development of skin pocks, indicating the role of the cellular immune response in the formation of such lesions.

**Effect of cidofovir treatment on vaccine efficacy.** Tail scarification with $10^6$ pfu of VV, performed either 21 or 8 days before inl CPV challenge, completely prevented illness, weight loss, and death. However, vaccination on day $-6$, $-2$, or 0 protected only 50%–60% of animals, and vaccination 2 days after challenge did not alter the course of illness. Treatment with 100 mg/kg cidofovir on day $-1$ or 0, with respect to vaccination, decreased the size of VV tail lesions, reducing their mean area to values equal to those of sites scarified with PBS alone, whereas treatment on day 2 or 4 had no effect (data not shown). All drug-treated, vaccinated mice were protected against inl challenge on day 21.

In contrast to tail scarification with VV, which produced a mild, localized reaction, tail scarification with the same quantity of CPV produced a much larger, more severe lesion involving the entire base of the tail. All mice with such lesions became ill and lost weight, and 4 of 10 died, apparently from disseminated virus infection. Survivors appeared healthy by day 21. A single injection of cidofovir on day $-1$ to 4, with respect to scarification, had little or no effect on the size of the resulting tail lesions but prevented illness and death. All drug-treated, CPV-vaccinated mice were protected against later inl challenge.

**Discussion**

Our experimental approach to the treatment of lethal orthopoxvirus infection was based on two considerations. The first was the need to simulate the type of exposure that would occur during close contact with a patient with smallpox or monkeypox or during a hostile attack with an aerosolized biological warfare agent. We therefore infected mice either by small-particle aerosol or inl with CPV. Second, we took advantage of the remarkably long tissue half-life of cidofovir by administering a high dose of drug only one time. This treatment strategy would be ideal for the circumstances under which natural infection with monkeypox occurs (i.e., in rain forest areas of Africa). Given the limited medical facilities in the region, it would be desirable to be able to treat patients and their close contacts with a single dose of an antiviral drug at the time of clinical presentation. Similarly, in a biological warfare scenario, it would be advantageous to be able to treat each person exposed to an aerosolized orthopoxivirus only once. Immediate treatment of an entire population with a single large dose of an antiviral drug could create a uniform barrier to viral replication and discourage the selection of drug-resistant mutants. If early warning of an impending attack provided time for prophylactic self-administration of a long-acting medication, for instance, by means of an aerosol inhaler, this would provide an even more effective barrier to infection.

Cidofovir was strongly protective against lethal orthopoxvirus infection in mice. Therapy for inl or aerosol-infected animals gave generally similar results, indicating that either model could be used for routine in vivo drug testing. Studies of drug prophylaxis showed that a single 100-mg/kg dose protected mice when given as long as 16 days before virus challenge, a duration of activity unparalleled by any other antiviral medication. Similar effects of cidofovir have been observed in other animal models. For example, Soike et al. [26] found that a single injection of 50 mg/kg cidofovir was as effective as 5 daily doses of 10 mg/kg or 10 daily doses of 5 mg/kg for treating simian varicella infection of the respiratory tract in nonhuman primates. In the current therapy for human CMV retinitis, the drug is administered iv every 1–2 weeks [8]. No evidence of renal or hepatic toxicity was observed after a 100-mg/kg dose of cidofovir. (Previous studies in mice have used doses as large as 250 mg/kg/day [12].)

The existence of such medications as cidofovir and of related compounds now under development may offer the possibility of treating severe human orthopoxvirus infections with only 1 or a few inoculations of drug. Cidofovir’s long half-life is a result of its unique metabolic processing. The compound is taken up by cells through pinocytosis rather than by an active transport mechanism. Once inside the cell, it is converted to cidofovir monophosphate and diphosphate by cellular rather than viral enzymes. Viruses sensitive to cidofovir thus cannot become resistant through mutational loss of the ability to phosphorylate the drug. HPMPC diphosphate forms a reversible adduct with choline, constituting, in effect, an intracellular drug depot. An in vitro half-life of the choline adduct of as long as 87 h has been measured [27–29]. Cidofovir diphosphate competes with dCTP for the active site of the viral DNA polymerase, disrupting replication through incorporation into viral DNA or through chain termination. Viral DNA polymerases are more sensitive than the corresponding cellular enzymes to inhibition by cidofovir [30].

Unfortunately, cidofovir also has disadvantages that limit its potential use. It is poorly absorbed by mouth and may induce local fibrosis if injected. It is therefore administered iv in clinical practice [31]. Treatment carries a small risk of nephrotoxicity, which can be minimized through concomitant hydration and administration of probenecid [32]. It is hoped that new phosphonate nucleoside analogues, chemically modified to enhance oral uptake, will prove to be less toxic but equally efficacious [33–36]. One such compound, bis(pivaloyloxymethyl)-9-(2-phosphonylmethoxyethyl)-adenine (adefovir), is currently in phase II/III human clinical trials for therapy for HIV, hepatitis B, and other chronic infections [33].
The pulmonary lesion causing death from aerosolized CPV appeared to be multifocal infection of distal bronchioles with subsequent cell-to-cell spread of virus around the circumference of the mucosa, resulting in edema and necrosis, sloughing of cells, airway occlusion, peribronchiolar hemorrhage, and bronchopneumonia. Early drug treatment decreased the rate of virus accumulation and reduced peak pulmonary virus titers 10- to 100-fold relative to those in mock-treated mice. Therapy appears to slow the rate of cell-to-cell spread of infection, preventing bronchiolar obstruction and its lethal consequences. The long-term pathologic sequelae of surviving aerosol CPV infection are the subject of an ongoing study (M.B. and M.M., unpublished data).

Our findings in part resemble those of the only previously published study of aerosol infection of mice with an orthopoxvirus, in which Roberts [37] examined the spread of ectromelia virus. He observed that bronchiolar mucosal cells and alveolar macrophages were the first cells infected and that the illness progressed through expansion of individual bronchiolar mucosal lesions. An increase in the number of infected alveolar macrophages and their migration through lymphatic vessels to hilar lymph nodes were followed by generalized infection and death. Other investigators have induced pulmonary disease through infection of rabbits with aerosolized rabbitpox and of monkeys with aerosolized variola virus [38, 39]. In both cases, obstructive bronchiolar mucosal lesions, alveolar edema, and hemorrhage were observed, resembling the findings in our study. Murine CPV infection thus appears to be an appropriate model for evaluating antiviral drug therapy for virulent orthopoxvirus infections of primates.

By using CPV as the challenge virus, we chose an agent that is more virulent than VV for immunocompetent mice [20, 40]. The relative virulence of CPV and VV for mice resembles that of variola and VV for humans. VV produces a localized lesion in mice, as it does in humans, and protects mice against CPV, as it protects humans against smallpox. In mice, simultaneous cidofovir treatment had little or no measurable effect on the size of lesions from tail scarification with VV and did not interfere with vaccination efficacy. This suggests that humans exposed to a virulent orthopoxvirus could be treated with an antiviral drug and vaccinated with VV at the same time without significantly decreasing the protective effect of vaccination.

Intradermal inoculation of CPV in mice is analogous to cutaneous inoculation of variola in humans. The latter practice (variolation) produced a severe local lesion, with a mortality rate of ~1%, but provided solid protection against smallpox, which had a case fatality rate as high as 30%-40% [1]. Similarly, tail scarification with CPV resulted in a severe local lesion and was lethal for some mice, but survivors were protected against subsequent lethal intral challenge. Cidofovir treatment at the time of tail scarification prevented the lethal complications of CPV inoculation but did not interfere with induction of a protective immune response. This result suggests that cidofovir might also be effective for treating the complications of VV vaccination, such as ocular infection, that occasionally occur in immunocompetent humans.

There is little experience with the treatment of orthopoxvirus infections in immunodeficient persons, because both global smallpox eradication and the discontinuation of vaccination preceded the arrival of the human immunodeficiency virus pandemic. The most severe complication of human vaccination is uncontrolled VV replication, which takes place in the setting of an underlying immunodeficiency disorder. A single report describes the arrest of progressive VV infection in an immunodeficient patient by treatment with ribavirin and vaccinia immune globulin [41]. On the basis of our data, it is reasonable to expect that cidofovir would be at least as effective as ribavirin in this situation.

However, our data from SCID mice suggest that an intact immune system may be required for cidofovir therapy to control infection with a virulent orthopoxvirus. A dose of drug that prevented CPV illness in immunocompetent mice only prolonged by 1–2 days the survival of SCID mice challenged with the same quantity of virus. Even repeated treatment could not rescue the animals from death. This finding suggests that cidofovir therapy might fail to control monkeypox or variola infection in immunodeficient humans. Our result differs from the earlier findings of Neyts et al. [9] and de Clercq et al. [12], who were able to eliminate herpes simplex virus infection in athymic nude mice and could indefinitely suppress VV infection in SCID mice. The difference in outcome is probably related to the greater virulence of CPV for mice.

In summary, infection of mice with CPV by the aerosol or intral route provides a model for initial testing of therapy for lethal orthopoxvirus infection of the respiratory tract. Aerosol exposure of mice to a virulent orthopoxvirus represents a worst-case scenario in that it replicates a disease that does not occur in nature but that would inevitably result if a non-immune human were unwittingly exposed to a small-particle aerosol of virulent virus. Inhalation of such an aerosol resulted in the simultaneous development of “pocks” throughout the lungs, producing innumerable foci of bronchiolar mucosal edema, small-airway obstruction, alveolar consolidation, and hemorrhage. Mice treated with 1 dose of cidofovir survived this severe challenge, apparently as a result of a 10- to 100-fold reduction in peak lung virus titers. Current studies of cidofovir treatment of nonhuman primates exposed to large quantities (as much as 1000 LD$_{50}$) of aerosolized monkeypox are providing similar results: treatment with cidofovir can rescue animals from an overwhelming viral challenge, providing the drug is started within a few days after exposure (J.W.H., unpublished data). Cidofovir given iv is currently approved only as therapy for CMV infection in humans, but we believe that the drug would also be effective for treating at least the early stages of human smallpox or monkeypox.
The length of the postexposure “window” for successful therapy is the major remaining question.

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References