2004

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Background. Severe complications may arise as a result of virus dissemination after smallpox (live vaccinia virus) vaccination, particularly in immunocompromised individuals. We developed a new mouse model for studying the effects of antiviral agents on progressive vaccinia virus infections.

Methods. Hairless mice were treated with cyclophosphamide (100 mg/kg/day) every 4 days starting 1 day before vaccinia virus exposure to wounded skin. Primary lesions progressed in severity, satellite lesions developed, and the infection eventually killed the mice.

Results. Topical treatment with 1%-cidofovir cream (twice daily for 7 days) was much more effective in reducing the severity of primary lesions and the number of satellite lesions than was parenteral cidofovir treatment (100 mg/kg/day, given every 3 days). Both forms of treatment delayed death. Topical drug treatment markedly reduced virus titers in the skin and snout, whereas parenteral treatment did not, suggesting that the latter treatment resulted in lower drug exposure to skin. Topical treatment starting 9 days after infection delayed death by 10 days, compared with treatment with placebo. Combining topical and parenteral cidofovir treatments provided the greatest reduction in lesion severity and prolongation of life.

Conclusions. Topical cidofovir treatment was superior to parenteral treatment. This new animal model may be useful in evaluation of the efficacy of treatment regimens against complications from smallpox vaccination.
models that have been employed in the past to study antiviral treatments have not mimicked well many of the progressive vaccinia conditions described above. The majority of the models have been developed using normal animals. Lethal infections have been induced by intranasal inoculation of vaccinia or cowpox viruses [6, 7]. Other antiviral studies have focused on non-lethal infections of the skin, tails, or eyes of animals [12]. Some studies have used severe combined immunodeficient (SCID) mice infected parenterally or intranasally [13, 14]. One report indicated that SCID mice infected intradermally on the tail develop progressively worse tail lesions and later die from a systemic infection and that cidofovir combined with vaccinia immune globulin (VIG) was beneficial in treatment [15].

Over the years, we have studied infections in mice that have been immunosuppressed using repeated cyclophosphamide injections. We found that, with either murine cytomegalovirus (MCMV) or murine gamma herpesvirus 68, the infections progressed and the animals died [16, 17]. The use of cyclophosphamide for such studies was originally reported by Selgrade et al. [18], who studied MCMV infections in mice. Because cyclophosphamide-induced immunosuppression is not permanent, the mice have to be treated every few days to maintain a profound immunosuppressed state. In this condition, the animals responded to MCMV infection in a manner similar to SCID mice [19]. Worthington et al. [20] showed that mice treated with cyclophosphamide died from an otherwise nonlethal vaccinia virus infection induced by intracutaneous virus inoculation. Treatment of the animals with antibody prevented death.

We wanted to develop a model in which exposed skin could be infected with vaccinia virus, progressive lesion development would ensue, and viral dissemination to other areas of the skin would occur. Hairless animals were targeted, to avoid problems associated with frequent depilation. Two strains of hairless mice are readily available, athymic nude (T cell immunodeficient) and normal hairless mice. In addition, there are normal hairless rats and hairless guinea pigs commercially available. A number of vaccinia virus strains exist that could be used for infection. In our preliminary studies, we found that athymic nude mice infected with the IHD, Lederle, and WR strains of vaccinia virus developed lesions; however, the lesions did not continue to get progressively larger beyond a certain size, and few satellite lesions appeared before the mice died from systemic infection (authors’ unpublished data). In the same studies, normal hairless mice developed lesions that quickly resolved, and the mice recovered fully from the infections. Hairless mice immunosuppressed with cyclophosphamide developed disseminated disease when infected with the WR strain of vaccinia virus and later died. Lesser degrees of severity were seen with infection with the IHD and Lederle strains of vaccinia virus. With these initial findings, we began studying the effects of cidofovir treatment, administered topically and/or parenterally, on vaccinia virus (WR strain) cutaneous infections in immunosuppressed hairless mice. This report describes the results of these experiments.

MATERIALS AND METHODS

Mice. Female, specific pathogen–free, SKH-1 hairless mice (24 g), 7–8 weeks old, were obtained from Charles River Labs. The mice were quarantined 48 h before use and were maintained on Teklad Rodent Diet (Harlan Teklad) and tap water at the Laboratory Animal Research Center of Utah State University (Logan), which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Large numbers of these mice were not available from the supplier; thus, generally 7–8 mice were used per group (held until death), depending on the total number required for each experiment.

Virus. Vaccinia virus (WR strain) was purchased from the American Type Culture Collection. The virus was propagated in African green monkey kidney (MA-104; Cambrex Bio Science) cells for use in these studies.

Compound. Cidofovir was provided by Mick Hitchcock of Gilead Sciences. Dermovan (Owen Laboratories) was purchased from a local pharmacy for preparation of the topical cidofovir cream [21]. Cidofovir was dissolved in water at a concentration of 25 mg/mL and then combined with Dermovan to prepare the 1%–cidofovir cream formulation. Dermovan combined with water served as the placebo control. Saline was used to dissolve cidofovir (100 mg/kg/day) for intraperitoneal (ip) injection. Saline served as the placebo control for ip treatments.

Experiment design. Mice were anesthetized with ketamine (100 mg/kg) by ip injection. They were scratched sufficiently to penetrate the dermal layer in the hip and shoulder areas on 1 side of the body. The area of each scratched site was 25 mm2 (5 mm × 5 mm) and consisted of 4–5 scratches in 1 direction. A 25-μL volume of vaccinia virus (containing 2.5 × 103 pfu) was placed on each wound area and remained there while the mice rested under the influence of the anesthesia. Immunosuppression was accomplished by ip treatment of the mice with cyclophosphamide (100 mg/kg/day) every 4 days, starting 1 day before virus challenge, until all the mice died. Creams (50–100 μL) were applied with a spatula to each lesion site twice daily for 7 days, starting at different times after virus challenge. As they formed, satellite lesions were treated in a similar manner. Cidofovir was administered by ip injection every 3 days, starting at different times after virus challenge, through day 21.

Three parameters were used to evaluate disease progression. (1) Deaths were recorded daily for as long as the mice survived, which was <40 days in these experiments. We treated uninfected hairless mice with cyclophosphamide for up to 45 days, and the mice remained healthy. (2) The primary lesion area was measured in square millimeters (length times width). Satellite lesions that merged with the primary lesion were not included in the mea-
urement of the primary lesion area. (3) The number of satellite lesions per mouse was counted each day. Satellite lesions could occur anywhere on the body. Mice were tagged by toe clipping and were accounted for individually. Their final scores for primary lesion area and number of satellite lesions were maintained, for counting purposes, through day 21 of the experiment.

**Virus titers from infected tissues.** Determinations of virus titers were made from tissues obtained on different days after infection. Homogenization and plaque-assay titrations were performed as described elsewhere [7].

**Statistical evaluation.** The 2-tailed Fisher’s exact test was used to evaluate increases in number of survivors. The 2-tailed Mann-Whitney U test was used to analyze differences in the mean number of days until death, reductions in primary lesion area, reductions in number of satellite lesions, and differences in virus titers in tissue.

**RESULTS**

**Development of animal model.** Experiments were initially conducted using normal hairless mice infected on the skin with vaccinia virus. In those studies, the mice developed self-limiting primary lesions that peaked in severity around days 5–7 after infection. High virus titers (up to 10^6 pfu/g) were detected in skin tissues. The lesions quickly healed after that time and did not disseminate. None of the mice died during infection. A pilot antiviral study was conducted using normal mice and ip cidofovir treatment on days 1 and 4 after virus challenge. In that experiment, the peak in severity of virus lesions occurred on day 5 after infection. On that day, the mean size of the lesion area for the cidofovir-treated group was 26 ± 12 mm², compared with 57 ± 26 mm² for the placebo-treated group, and virus titers in skin lesions of the cidofovir-treated and placebo-treated groups were 8.6 ± 0.4 and 8.8 ± 0.2 log_{10} pfu/mL, respectively. Thus, parental cidofovir treatment had minimal effect on the infection. Because of the short duration of this self-limiting infection in normal mice, we did not pursue other forms of antiviral treatment or conduct in-depth studies. Instead, we began investigating ways to prolong the infection and to enhance its severity, which led to studying the effect of immunosuppression (particularly by means of cyclophosphamide) on the severity of the vaccinia virus (WR strain) infection.

When the mice were treated every 4 days with cyclophosphamide, to induce immunosuppression, a different pattern of infection emerged than that seen in normal mice. Primary lesions became progressively larger over time, and many satellite lesions formed during the late stage of the infection, before death. Figure 1 shows these immunosuppressed mice on different days after infection. On day 5, swelling was evident under the skin of the infection sites located on the hip and shoulder regions, but a full breakout of the lesion had not yet occurred (figure 1A). By day 9, poxlike vesicles were fully formed, but no satellite lesions were evident (figure 1B). By day 18, numerous satellite lesions had developed, and the primary lesions were more severe. In figure 1C, satellite lesions appear to be clustered mostly around the primary lesion area. Numerous disseminated satellite lesions had developed (figure 1D), including many on the abdominal surface (not visible in photograph). The front paw also was swollen in this mouse. Swelling of ≥1 paw may occur in either the front or the hind limbs and on either side of the body. Many mice developed lesions around the mouth, probably as a result of rubbing and/or chewing the primary lesion area. Because the mice were immunosuppressed, they ultimately died from the infection. By day 18 (figure 1C and 1D), the mice had started to lose weight and had become sedentary in their behavior. They died within a few days after the photographs were taken.

Development of virus titers was determined in the mice after cutaneous infection (figure 2). Virus titers developed very rapidly in the skin, reaching and persisting at concentrations >10^6 pfu/g. Later, the mice developed high virus titers in their snouts, which is believed to have been due to transmission of infection from the primary lesion area as a result of the mice rubbing the lesions with their snouts. The lesions began to irritate the mice, and the mice reacted by scratching them with their paws and biting them. Virus titers progressively increased in the lungs over time, which may represent a progression of infection from snout to lungs by means of inhalation. The virus infection eventually underwent dissemination to the other visceral organs and the brain.

**Topical and ip cidofovir treatment.** Studies were conducted to determine the efficacy of cidofovir against vaccinia virus cutaneous infections in immunosuppressed mice when treatment began 1 day after infection. Topical treatments given twice daily for a week were compared with ip treatments given every 3 days through day 21 after infection (figure 3). The ip-treatment regimen was similar to that found to be effective against a cowpox virus respiratory infection in SCID mice [6]. Both the topical and the ip treatments significantly delayed the time to death (P < .001), compared with time to death in the placebo-treated groups (figure 3A). Parenteral cidofovir treatment was less effective in reducing primary lesion areas (figure 3B) and numbers of satellite lesions (figure 3C) than was topical application. However, both treatments caused significant reductions in lesion severity, compared with lesion severity in the placebo-treated groups. As part of the experiment, virus titers in various tissues were determined on day 14 after infection (figure 4). Topical treatment caused dramatic reductions in virus titers in the skin and snout. A lesser but statistically significant effect was seen for virus titers in the liver. In contrast, ip treatment had little, if any, effect in reducing virus titers in the skin and snout. However, reductions in virus titers in the lungs, kidney, and liver were seen with ip treatments.
**Effects of delayed cidofovir treatment.** The effect of starting cidofovir treatment at different times after infection was studied next. Topical and ip treatments began 1, 3, or 5 days after infection and continued for a total of 7 days (topical) or through day 21 (ip) (figure 5). Results of topical treatments starting on day 1 were similar to those shown in figure 3, with regard to impact on survival, scores for primary lesion area, and numbers of satellite lesions. Parenteral treatment was not as effective in delaying the time to death as was topical treatment (figure 5A). In 2 additional studies in which treatment started 1 day after infection, topical treatment also was more effective in delaying death than was ip treatment (data not shown). Treatments by the ip route reduced scores for primary lesion area and numbers of satellite lesions significantly, but the overall effect was not as good as that of topical treatment (figure 5B and 5C). A delay in the start of treatment until day 3 or 5 resulted in reduced efficacy, but significant reductions in parameters for lesion severity were still seen. Topical treatments started on day 3 were not as effective in delaying death as those started on day 1. Parenteral treatments started on day 1, 3, or 5 were similarly effective in delaying death.

**Topical cidofovir treatment starting on day 9.** Primary skin lesions were well formed by day 9 after infection (figure 1B), before initiation of treatment. The impact of cidofovir on this type of infection was studied by using 3%- and 1%-cidofovir topical cream, compared with placebo cream. Treatments were given twice daily for 7 days. Mean (±SD) time until death for the 3 treatment groups (n = 8 for each group) was 22.5 ± 4.9 (P < .01), 20.3 ± 3.5 (P < .01), and 14.6 ± 2.3 days, respectively. Because the primary lesions were large before the start of treatment, no reductions in lesion size were seen. On day 17 after infection, the mean (±SE) number of satellite lesions per mouse was 14.1 ± 2.2, 25.0 ± 3.2, and 23.0 ± 3.0 lesions, respectively. Because of mouse-to-mouse variability in the number of satellite lesions, the results for the 3% cidofovir–treated group, compared with the placebo-treated group, were not statistically significant.

**Combination of topical and ip treatments.** Topical and
ip treatments both had an impact on reduction of mortality in the model, although topical treatments were superior in reducing parameters of lesion severity. A study was conducted to determine whether the combination of topical and ip treatments would be more effective than either treatment alone, when treatment started 5 days after infection. For this study, groups consisted of 8–10 mice. Mice treated topically with 1%-cidofovir cream for 1 week lived a mean (± SD) of 19.8 ± 3.2
Figure 5. Efficacies of topical and intraperitoneal (ip) cidofovir treatments when started 1, 3, or 5 days after infection of immunosuppressed hairless mice with vaccinia virus (WR strain). Topical treatments were given twice daily for 7 days, and ip treatments were administered once daily every 3 days, ending on or before day 21 after infection. Data are mean + SE (n = 7). A, Percentage survival; B, mean size of primary lesion area (in square millimeters); C, mean number of satellite lesions. ●, Cidofovir (1%), topical treatment; ○, placebo, topical treatment; ■, cidofovir (100 mg/kg/day), ip treatment; □, placebo, ip treatment. *P < .05; **P < .01; ***P < .001.

days (P < .05), compared with 16.3 ± 2.9 days for the placebo-treated group. Mice that received ip treatment every 3 days (100 mg/kg/day), through day 20, lived 21.4 ± 6.7 days (P < .05), compared with 15.3 ± 2.7 days for the respective placebo-treated group. The combination of cidofovir cream plus parenteral drug treatment increased survival to 28.3 ± 5.7 days (P < .001). On day 17 after infection, mice receiving combination treatment, cidofovir cream alone, and placebo cream had scores for primary lesion area (± SE) of 9.6 ± 2.7 (P < .001), 10.0 ± 3.5 (P < .001), and 34.2 ± 3.7 mm², respectively. For these groups, the numbers of satellite lesions (± SE) on day 17 were 4.4 ± 1.1 (P < .01), 8.0 ± 1.7, and 14.7 ± 3.4 lesions, respectively. The difference in numbers of satellite lesions between the combination-treated group and the cidofovir cream–treated group was not statistically significant. Thus, the combination treatment was judged to be no better than topical treatment alone in reducing the severity of primary lesions and the numbers of satellite lesions, but the impact on mortality was definitely superior with the combination treatment.

Less-frequent topical cidofovir treatments. In the previous studies, we arbitrarily chose to administer topical cidofovir treatments twice daily. Cidofovir is known to have a long intracellular half-life [22]; thus, fewer treatments per day also may be beneficial. A study was initiated to evaluate the efficacy of 1%-cidofovir cream given once daily, once every other day, or once every 3 days, starting 5 days after infection (table 1). Daily treatment resulted in the largest effect on survival and the best reduction of primary lesion area. Treatment every other day was not as effective for survival but did significantly reduce primary lesion area, compared with results for the respective
ically applied cidofovir treatment was superior to parenteral treatment slowed disease progression and may have helped the placebo-treated group. Treatment every 3 days delayed death by 3 days (although the results were not statistically significant) but did not reduce primary lesion areas. In this experiment, none of the treatments reduced the number of satellite lesions per mouse.

**Toxicity of topical cidofovir treatment and cyclophosphamide.** Topical treatments proved to be safe in terms of not inducing weight loss during treatments. The 3%-cidofovir formulation caused skin irritation (reddening), which was more pronounced in the shoulder region than on the hip. The twice-daily application caused a buildup of the dried cream on the skin, which was more noticeable in the 3% cidofovir–treated group than in the 1% cidofovir–treated group but was not noticeable in the placebo–treated group. For this reason, the majority of the studies were conducted with the 1%-cidofovir formulation. A dose-response study that included a 0.5%-cidofovir formulation indicated that this dose was not as effective in treating vaccinia lesions as was the 1%-cidofovir formulation (data not shown). Administration of ip treatments every 3 days had previously been shown to be nontoxic to mice [3]. Cyclophosphamide was administered to uninfected hairless mice every 4 days for 45 days, without evidence of toxic effects. The mice gained weight over that time period and appeared to be healthy.

### DISCUSSION

The results demonstrate the utility of a new immunosuppressed-mouse model for the quantitative study of the progression of vaccinia virus cutaneous lesions, satellite-lesion formation, and mortality. Infection began with rapid development of virus titers in skin and was followed within days by virus replication in the snout and lungs, which led to dissemination to other vital organs and the brain. An important finding was that topically applied cidofovir treatment was superior to parenteral cidofovir treatment in terms of inhibiting the size of primary cutaneous lesions and reducing the number of satellite lesions. These results were explained by the fact that virus titers in skin lesions were markedly reduced (1000-fold) by topical treatment but were reduced <10-fold by parenteral treatment. Because topical treatment reduced virus titers in the primary lesions, we believe that this treatment also markedly reduced virus titers in the snout. Transmission of virus from primary lesion to snout was most likely occurred, since the placebo-treated mice were observed to bite and scratch lesions in the hip area.

The best efficacy was observed when topical treatments were initiated 1 day after infection, and efficacy decreased when treatments started on day 3 or 5 after infection. Topical treatments started as late as 9 days after infection delayed the time to death but did not help resolve preexisting lesions. Parenteral treatments also delayed the time to death, relative to that in the placebo–treated groups. Since the treatment regimens used were different (twice daily for 7 days for topical treatment vs. every 3 days, through day 21, for parenteral treatment), direct comparisons of the efficacy of the 2 treatments, in terms of prevention of death, may not be warranted. During the experiments, topical treatments were terminated well before parenteral treatments. It has been reported that human patients with molluscum contagiosum infection had detectable levels of cidofovir in their blood and excreted in their urine [21]. In particular, wounded skin or skin with viral lesions may permit internalization of drug. Topical cidofovir treatment was highly likely to have introduced some drug systemically in the mouse model, which provided protection against internal infection.

In the first week of vaccinia virus infection in immunosuppressed hairless mice, the majority of the virus burden was in the skin. Thus, severely reducing that virus burden by topical treatment slowed disease progression and may have helped the
mice live longer. Parenteral treatment was not effective in reducing the virus burden in the skin. It seems logical that topical treatment of vaccinia complications in humans would be similarly more effective than parenteral treatment, owing to a concentration effect. The drug has already been used to topically treat molluscum contagiosum and orf virus infections [21, 23]. More-serious infections that become systemic may be treated by a combination of topical and parenteral routes, as was demonstrated in one of the present experiments. Treatment with VIG combined with an antiviral drug also may be more effective, as has been previously reported for mice [18]. A single case of progressive vaccinia in a patient with chronic lymphocytic leukemia was treated with the combination of VIG and ribavirin [24], and the patient’s condition improved. However, conclusions regarding the efficacy of this combination therapy cannot be drawn from the results for a single case. In the future, information on the use of an antiviral agent combined with VIG may be obtained by use of our experimental immunosuppressed-mouse model.

The mice used in the present study were not adversely affected by topical treatment with 1%-cidofovir cream. Treatment with 3%-cidofovir cream caused skin reddening and crusting of the material on the skin. This also was evident during treatment of molluscum contagiosum infection in humans [21]. The 1%-cidofovir formulation appeared to be well tolerated by the mice. Topical treatments may not necessarily be safe for humans, because of potential of the drug to cause kidney damage [25] and because the drug has been shown to be absorbed into the body by the topical route [21].

The application of a topically applied antiviral agent may not be practical for severe smallpox and monkeypox virus infections, because the lesions are distributed over the entire body. Perhaps more importantly, lesions also may be distributed internally throughout the oropharynx and alimentary canal. However, topical treatment should definitely be considered for lo-

ternally throughout the oropharynx and alimentary canal. How-

References


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