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# Rabbitpox Virus and Vaccinia Virus Infection of Rabbits as a Model for Human Smallpox▽

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**The threat of smallpox release and use as a bioweapon has encouraged the search for new vaccines and antiviral drugs, as well as development of new small-animal models in which their efficacy can be determined. Here, we reinvestigate a rabbit model in which the intradermal infection of rabbits with very low doses of either rabbitpox virus (RPV) or vaccinia virus Western Reserve (VV-WR) recapitulates many of the clinical features of human smallpox. Following intradermal inoculation with RPV, rabbits develop systemic disease characterized by extensive viremia, numerous secondary lesions on the skin and mucocutaneous tissues, severe respiratory disease, death by 9 days postinfection, and, importantly, natural aerosol transmission between animals. Contrary to previous reports, intradermal infection with VV-WR also resulted in a very similar lethal systemic disease in rabbits, again with natural aerosol transmission between animals. When sentinel and index animals were cohoused, transmission rates approached 100% with either virus, with sentinel animals exhibiting a similar, severe disease. Lower rates of transmission were observed when index and sentinel animals were housed in separate cages. Sentinel animals infected with RPV with one exception succumbed to the disease. However, the majority of VV-WR-infected sentinel animals, while becoming seriously ill, survived. Finally, we tested the efficacy of the drug 1-*O*-hexadecyloxypropyl-cidofovir in the RPV/rabbit model and found that an oral dose of 5 mg/kg twice a day for 5 days beginning 1 day before infection was able to completely protect rabbits from lethal disease.**

Poxviruses have received added attention in recent years because of the perceived increased threat of bioterrorism and concerns about release of variola virus into the environment (12). These concerns have spurred the search for new smallpox vaccines with fewer side effects than the current vaccine (Dryvax), as well as antiviral drugs. As a first step toward deployment, both vaccines and antiviral drugs must show efficacy in animal models of orthopoxvirus disease. The ideal animal model would recapitulate the essential features of human disease, such as a high level of lethality following inoculation with low levels of virus; produce generalized dissemination with secondary lesions; and result in animal-to-animal spread and an overall clinical disease similar to that seen in smallpox.

None of the current models emulate all aspects of variola in humans. Ectromelia virus infection of mice is one of the most sensitive models, leading to death in some mouse strains at very low doses of virus, but ectromelia infections are hepatotropic and generate a major degree of liver necrosis, a feature not observed in humans infected with smallpox (6). Mouse models using vaccinia virus (VV) inoculated intratracheally or intranasally generally require high levels of virus ($10^4$ to $10^5$ PFU) to generate lethal disease (19, 21). Nonhuman primate models using monkeypox virus or variola virus likewise require very high doses of virus, on the order of $10^7$ to $10^9$ PFU, delivered intravenously or intranasally (10, 13, 24). A monkeypox virus model using ground squirrels is under development (26) and shows disseminated lethal disease following a very low inoculum by intradermal or respiratory routes of infection. However, natural aerosol spread of the virus, typical of smallpox infections, has not been reported in these models.

Rabbitpox virus (RPV), an orthopoxvirus now known to be closely related to VV (17), was isolated from a rabbit colony outbreak in Utrecht, The Netherlands (5, 14), and has over 95% sequence similarity to VV (17). Historically, in the 1960s, rabbits were commonly used to study the pathogeneses and immune responses of various poxvirus isolates, including VV and RPV (2, 16, 27). However, the RPV/rabbit model has been largely ignored in recent times.

The early rabbit model studies reported that RPV produced a disseminated lethal disease in rabbits at very low inoculum and, unlike other orthopoxvirus models, produced a natural aerosol transmission of the virus between animals (7–9). In contrast to the highly virulent disease caused by RPV, VV strains, including Western Reserve (VV-WR), were reported to produce a local lesion with few clinical symptoms in rabbits (25).

The original purpose of this study was to compare RPV and VV-WR infections in rabbits and to identify regions of the RPV genome associated with increased pathogenicity in rabbits compared to VV-WR by generating RPV/VV-WR chimeras with increased or decreased pathogenicity. However, upon reexamination of both wild-type RPV and VV-WR in rabbits, it was found that intradermal inoculation of either virus produced lethal, systemic disease and exhibited all the aspects desired of a small-animal model for variola virus in humans. We have also reconfirmed the transmissibility of RPV between

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rabbits, which provides a model of natural aerosol transmission of variola virus between humans.

To illustrate the utility of the rabbit model, we evaluated the efficacy of the drug 1-*O*-hexadecyloxypropyl-cidofovir (HDP-CDV) (CMX001) (15), a lipid-conjugated form of cidofovir, in protecting rabbits from intradermal RPV challenge. HDP-CDV is orally bioavailable and is actively transported from the gut into cells due to the presence of the lipid moiety and has been demonstrated to have reduced renal toxicity compared to cidofovir (1, 3). This drug has proven effective in mouse models of orthopoxvirus disease, such as cowpox, VV, and ectromelia virus infections (11, 20, 22). We show that the drug is similarly effective when tested in the rabbit model of disease.

## MATERIALS AND METHODS

**Cells and viruses.** RPV strain Utrecht was obtained from the American Type Culture Collection (ATCC). Two isolates of VV-WR were used in this study. The first was kindly provided by Richard Condit, University of Florida (designated VV-$WR_C$), originating from the ATCC, and the other was from Bernard Moss, NIH (designated VV-$WR_M$).

All virus strains were grown and titered in green monkey kidney (CV-1) cells, which were maintained in minimum essential medium with Earle's salts (GIBCO, Grand Island, NY) supplemented with 5% fetal bovine serum (GIBCO), 2 mM glutamine, 50 U/ml penicillin G, 50 μg/ml streptomycin, 340 mM sodium pyruvate, and 0.1 mM nonessential amino acids (Mediatech, Herndon, VA).

**Housing and infection of rabbits.** Nine-week-old (3- to 4-lb) or 6-month-old (9- to 10-lb) female New Zealand White rabbits were obtained from Myrtle's Rabbitry (Thompsons Station, TN) or Charles River Laboratories (St Constant, Canada). The animals were housed in standard single cages at 20°C on a 12 h light/12 h dark regime and fed ad libitum on standard rabbit pellets. A sterile Implantable Programmable Temperature Transponder chip (Biomedic Data Systems, Delaware) was injected subcutaneously at the base of the neck to transmit data for animal identification and body temperature.

Infection was performed by bilateral shaving of both thighs of the rabbit and sterilization with an isopropanol wipe. Virus was diluted in phosphate-buffered saline (PBS) to the appropriate dose and injected intradermally in the middle of the shaved area using a 27-gauge needle. The animals were infected bilaterally with half of the total dose of virus in 100 μl PBS delivered to each thigh. Mock-infected rabbits were injected with PBS alone. Following infection, the animals were closely monitored twice daily for temperature, weight, respiration rate, and clinical signs of disease. Rabbits were euthanized upon the onset of severe respiratory distress (labored, extremely slow, or open-mouth breathing), a temperature of greater than 42°C, or anorexia. Weight loss was calculated as the percentage of a rabbit's weight change compared to uninfected, age-matched control animals.

For virus transmission studies, six rabbits were cohoused in a 160- by 80-cm cage with shredded paper bedding and fed ad libitum. One rabbit was infected intradermally with RPV or VV-$WR_C$ as described previously (the index animal) and housed with the other rabbits (sentinel animals). Upon onset of severe respiratory disease, the index animal was euthanized and the sentinel animals were placed in a clean cage. For noncontact transmission studies, the large cage was divided into 50- by 80-cm and 95- by 80-cm sections separated by a 15-cm gap with two layers of stainless steel mesh. Two rabbits were intradermally infected with virus and placed in the smaller section of the cage, while four uninfected sentinel rabbits were placed in the larger section. A 15-cm desk fan was used to promote airflow from the infected rabbits toward the sentinel animals.

All animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

**HDP-CDV administration and efficacy studies.** Dry powdered HDP-CDV (Chimerix Inc., North Carolina) was dissolved in 10% glucose in water to the required concentration. The drug was fed to the animals by syringe to the mouth, which the rabbits consumed readily. Placebo and uninfected animals received 10% glucose solution only, in the same volume. The animals were dosed with 1 mg/kg of body weight or 5 mg/kg of HDP-CDV twice a day (BID) (8 a.m. and 4 p.m.) for a total of 5 days from 1 day before infection. For RPV challenge, rabbits were prepared as described above. One day after the initial dose of the drug, the animals were intradermally challenged with 250 PFU of RPV bilaterally (500 PFU per animal) on the flanks and monitored as described above.

## RESULTS

**Infection of 9-week-old rabbits with RPV.** To establish the basic parameters of infection and to confirm the earlier results, a total of 25 New Zealand White rabbits were infected with 1,000 PFU of RPV (500 PFU bilaterally) by intradermal injection. The timing, description, and course of disease in these animals are displayed in Fig. 1. A raised red swelling was evident at the inoculation site within 1 to 2 days postinfection (p.i.). This lesion continued to grow in diameter and thickness, developing a pustular, black, and necrotic center between 3 and 5 days p.i. By 7 or 8 days p.i., the lesion at the inoculation site was usually between 8 and 10 cm in diameter, covering the entire thigh of the rabbit with skin up to 2 cm thick. A hard black scab with pustular edges several centimeters in diameter covered the center of the lesion (Fig. 2D).

Clinical signs of overt disease first become evident between 3 and 4 days p.i. Rabbits exhibited an increase in temperature to 40 to 42°C and weight loss, both of which continued until death (Fig. 1A). By 8 days p.i., RPV-infected rabbits generally demonstrated weight loss of 23% prior to death compared to uninfected, age-matched control animals (Fig. 1B).

Secondary lesions were first observed on the fifth day of infection, indicating spread of the virus from the inoculation site (Fig. 1D). The secondary lesions usually first appeared on the eyelids, beginning as red areas 3 to 5 mm in diameter. These progressed over several days to become protuberant crimson swellings, sometimes developing black scabby coverings. Discharge from the eyes was common, ranging from mild clear discharge to profuse mucopurulent discharge. Secondary lesions were also common on other mucocutaneous tissues, such as the nose, lips, tongue, mouth, genitals, and anus. By 6 to 8 days p.i., some rabbits also exhibited a generalized rash of raised red lesions across the skin, most notably on the ears (Fig. 2F), neck, and trunk.

Respiratory symptoms were first evident as a decrease in the resting respiration rate of the animals from a baseline of 120 to 200 breaths per minute to below 80 breaths per minute. This coincided with constriction and frank lung sounds becoming evident. At 7 or 8 days p.i., most animals exhibited severe respiratory symptoms (Fig. 1D), including profuse mucopurulent discharge from the nostrils, often tinged with blood (Fig. 2E), and very slow (<40 breaths per minute), labored breathing. At this stage, the animals frequently exhibited open-mouth breathing. However, open-mouth breathing did not always coincide with profuse nasal discharge. Several animals exhibited this extreme respiratory distress with no discharge. Upon necropsy, the lungs of most RPV-infected rabbits were dark and marbled, displaying large hemorrhagic regions. This severe respiratory distress was the most common reason for euthanasia. RPV was 100% lethal in 9-week-old rabbits by 9 days p.i. in these experiments (Fig. 1C).

**Infection of 9-week-old rabbits with VV-$WR_C$.** A total of 24 rabbits were infected intradermally with 1,000 PFU of the Condit isolate of VV (VV-$WR_C$), with the clinical data and course of disease summarized in Fig. 3. VV caused a disease very similar to rabbitpox in rabbits yet was distinguishable from

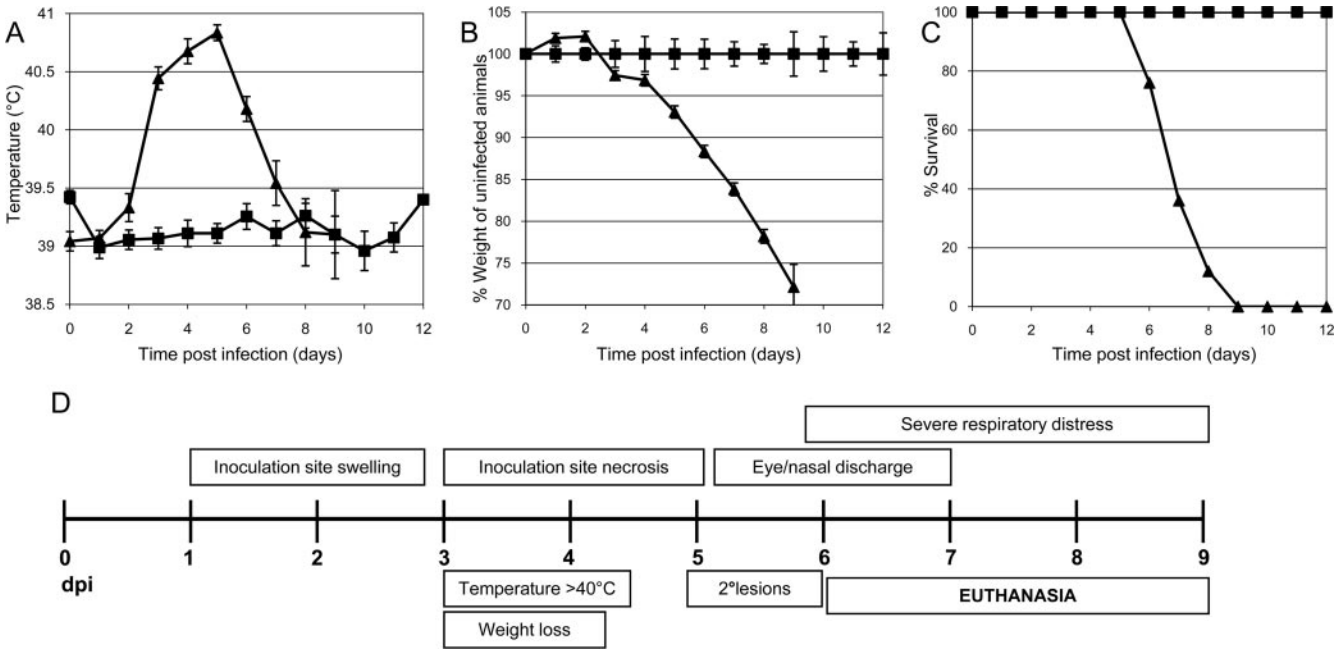


FIG. 1. Pathology of disease in 9-week-old rabbits infected intradermally with 1,000 PFU of RPV. (A) Average temperatures of animals. (B) Average weights of animals normalized against uninfected and age-matched controls. (C) Survival curves. (D) Time line description of the first appearance of key clinical signs of disease. The boxes indicate the range of days in which these symptoms were first observed. ■, mock- infected rabbits ($n = 9$); ▲, RPV-infected rabbits ($n = 25$). The error bars indicate standard errors of the mean. Morbidity dictated that all animals be euthanized by 9 days p.i.

that of RPV. As seen with RPV, the first sign of disease was a small red area of dermal swelling at the site of virus injection. By 5 days p.i., this lesion had developed a black, necrotic center with pustular regions. The lesion continued to grow in size until 8 days p.i., when it was up to 8 cm in diameter and encompassed the entire thigh of the rabbit, with up to 2 cm of thickness (Fig. 2G).

Between 3 and 5 days p.i., the rabbits displayed an increase in temperature to above 40°C (Fig. 3A), which coincided with weight loss (Fig. 3B), both of which continued for the duration of the infection. Secondary lesions first appeared 2 to 3 days after the temperature spike on mucocutaneous sites, such as the eyelids, nose, mouth, genitals, and anus. By 6 to 8 days p.i., secondary lesions also began to develop on the skin at sites such as the ears and trunk. VV-$WR_C$ infection often resulted in more prominent and numerous dermal secondary lesions on the ears (Fig. 2I) and body of rabbits than was noted for RPV infections. Respiratory symptoms first appeared between 5 and 7 days p.i., initially as a mild, clear discharge from the nose; slight constriction and frank lung noise; and a decrease in the resting breathing rate to below 80 breaths per minute. These respiratory signs increased rapidly in severity, so that usually within 24 to 48 h (7 to 9 days p.i.), the animals had developed severe lung sounds, profuse mucopurulent discharge (Fig. 2H), and breathing at less than 40 breaths per minute, often with labored, open-mouth breathing, and were euthanized. Of the 24 rabbits infected with VV-$WR_C$, two survived (Fig. 3C) and began to recover from 9 days p.i. (Fig. 3D), as indicated by a return to normal body temperature, return of appetite and weight gain, and the scabbing and regression of the primary and secondary lesions. Survivors were rarely, if ever, noted with RPV infections of animals of this age.

The systemic lethal disease caused by VV-$WR_C$ in rabbits was unexpected. Previous reports had not indicated that this strain of VV was particularly virulent in rabbits (23, 25). To determine if this phenomenon was specific to the particular VV-WR isolate used (VV-$WR_C$ from R. Condit), rabbits were also infected with a range of doses ($10^3$ to $10^6$ PFU) of a second Western Reserve isolate obtained from Bernard Moss (VV-$WR_M$). The disease caused by this isolate was very similar to that from the Condit isolate, resulting in a generalized lethal disease (data not shown). Rabbits were also sourced from a different supplier (Charles River) to determine if the particular genetic stock used was more susceptible to VV infection. The results seen were comparable in all animals regardless of source (data not shown).

**RPV infection in 6-month-old rabbits.** We investigated the course of disease in sexually mature rabbits (6 months old) to determine if the lethality of orthopoxvirus infections was dependent on the age of the rabbit. Five rabbits were infected intradermally with 1,000 PFU of RPV and monitored daily.

The development of disease in 6-month-old rabbits infected with 1,000 PFU of RPV is shown in Fig. 4 and was generally similar, but not identical, to that noted for the younger rabbits. Following RPV infection, a red swelling developed at the inoculation site within 3 days p.i. As seen with younger rabbits, this lesion increased in diameter and developed a black, necrotic center by 6 days p.i. The lesion continued to grow in size, and by 8 days p.i. was 8 to 10 cm in diameter. The rabbits exhibited a temperature spike at 3 or 4 days p.i. and began to

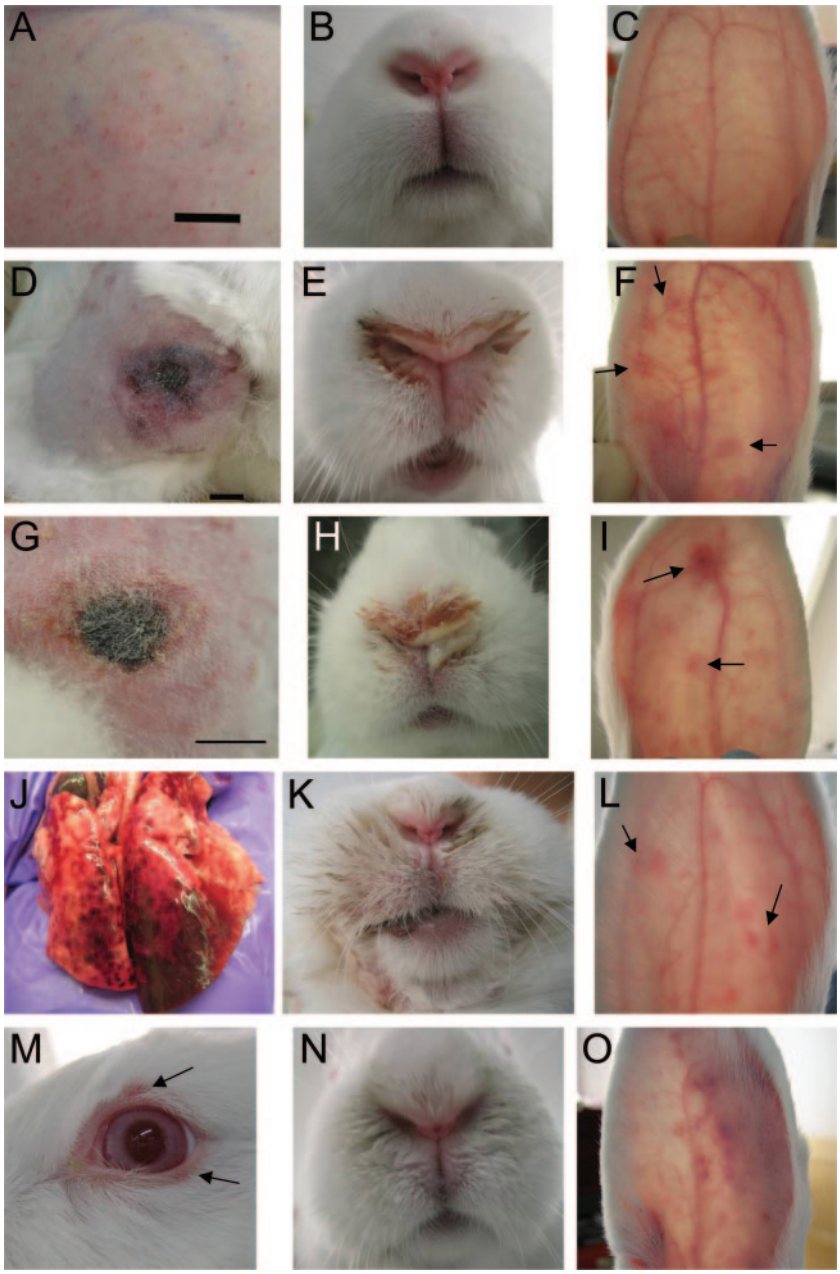


FIG. 2. Images of mock-infected rabbits (A to C), RPV-infected rabbits (D to F), VV-$WR_C$-infected rabbits (G to I), and sentinel rabbits infected by contact exposure to RPV-infected (J to L) or VV-$WR_C$-infected (M to O) rabbits. (A to C) A mock-infected rabbit 7 days after intradermal injection with PBS showing the primary inoculation site (A), nose and mouth (B), and ear pinnae (C). (D to F) A 9-week-old rabbit 7 days after intradermal infection with 1,000 PFU of RPV showing the primary inoculation site (D), mucopurulent discharge from the nostrils (E), and secondary lesions on the ear pinnae (F, arrows). (G to I) A 9-week-old rabbit, 8 days after intradermal infection with 1,000 PFU of VV-$WR_C$ showing the primary inoculation site (G), profuse mucopurulent discharge from the nostrils (H), and secondary lesions on the ear pinnae (I, arrows). (J to L) An RPV-infected sentinel animal 13 days after infection of the index animal showing hemorrhagic lungs (J), profound swelling of the face and neck with nasal discharge (K), and secondary lesions in the ear pinnae (L, arrows). (M to O) A VV-$WR_C$-infected sentinel animal 15 days after infection of the index animal showing secondary lesions on the eyelids (M, arrows), mucopurulent nasal discharge (N), and confluent secondary lesions in the ear pinnae (O). The bars on the images are 1 cm in length.

exhibit weight loss at 4 or 5 days p.i. (Fig. 4A and B), which continued until the end of the experiment. However, weight loss was not as severe as that seen in younger rabbits infected with RPV or VV-$WR_C$. This may be attributed to the uninfected adult rabbits no longer gaining weight at the rate seen in 9-week-old animals. Secondary lesions were first observed from 4 days p.i., appearing at sites such as the eyelids, nose, mouth, genitals, and skin, and developed in a fashion similar to that of lesions in younger animals. Two animals developed severe respiratory disease at 7 days p.i. and were euthanized. Two other animals exhibited respiratory disease for 3 more days until they were euthanized at 10 days p.i. One RPV-

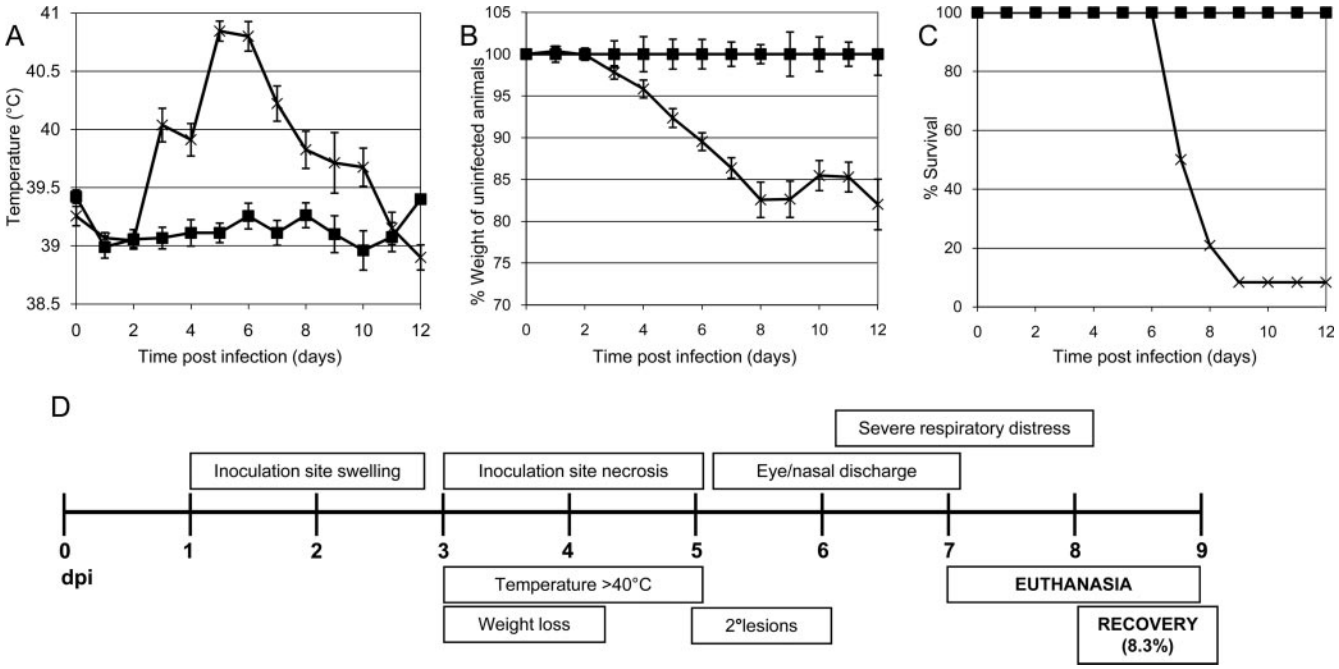


FIG. 3. Pathology of disease in 9-week-old rabbits infected intradermally with 1,000 PFU of VV-$WR_C$. (A) Average temperatures of animals. (B) Average weights of animals normalized against uninfected and age-matched controls. (C) Survival curves. (D) Time line description of the first appearance of key clinical signs of disease. The boxes indicate the range of days in which these symptoms were first observed. ■, mock-infected rabbits ($n = 9$); ▲, VV-$WR_C$-infected rabbits ($n = 24$). The error bars indicate standard errors of the mean.

infected animal developed few secondary signs of disease, limited to a relatively small, 3-cm lesion at the inoculation site and only five secondary lesions on the eyelids, lips, and anus. This was the only animal in this study to survive intradermal infection with RPV.

**VV-WR infection in 6-month-old rabbits.** The development of disease in 6-month-old rabbits infected with 1,000 PFU of VV-$WR_C$ is shown in Fig. 4. Again, the disease was similar to that observed for VV-$WR_C$ in younger animals. A red swelling developed at the inoculation site by 2 to 3 days p.i. This grew in size to be comparable to RPV lesions and lesions seen on younger VV-$WR_C$-infected rabbits, up to 12 cm in diameter with a black, necrotic, and pustular center. Increase in body temperature to above 40°C was noted as early as 4 days p.i. and as late as 7 days p.i. in these rabbits (Fig. 4A and D). Secondary lesions were first noted at 5 days p.i. on mucocutaneous and skin sites of all animals. Mild weight loss occurred in VV-$WR_C$-infected rabbits (Fig. 4B) and was not as pronounced as that seen in the majority of RPV-infected rabbits of this age. All animals developed mild to severe respiratory disease, as seen by a decrease in respiration rate, nasal discharge, and labored breathing. Sixty percent developed severe respiratory distress and were euthanized at 10 days p.i. Two of these animals appeared to resolve the infection, as suggested by the regression of secondary lesions, an increase in appetite, and a return to normal body temperature (from 9 days p.i.). However, they then developed severe secondary respiratory infections that worsened, and these animals were euthanized. In contrast to RPV infection, 6-month-old rabbits appeared to be markedly more resistant to VV-$WR_C$ than 9-week-old rabbits, although all of the animals developed generalized disease.

**Rabbit-to-rabbit transmission of RPV.** Transmission of virus from animal to animal is a key parameter of variola virus infections of humans. We first investigated the ability of RPV to be naturally transmitted from an infected (index) rabbit to uninfected cage mates, as had been previously reported (2). In the first experiment, a single rabbit intradermally infected with 1,000 PFU of RPV was cohoused in direct contact with five sentinel rabbits. The RPV-infected index animal became morbidly ill and was removed from the cage and euthanized at 8 days p.i., and the sentinel animals were placed in a clean cage. All sentinel animals developed disease beginning 10 to 12 days after the infection of the index animals. The time course of disease development in the sentinel rabbits is shown in Fig. 5. The first sign of infection in the sentinel rabbits was a temperature spike, which was observed between 10 and 12 days p.i. in all sentinel animals and coincided with weight loss (Fig. 5A, B, and D). Secondary lesions appeared on sites such as the eyelids, nose, lips, skin, and genitals 1 to 2 days after this temperature spike (Fig. 2L), which developed as seen previously on rabbits intradermally infected with RPV, beginning as a red mark or swelling and progressing to become raised, crimson lesions with black necrotic centers.

Respiratory symptoms first became evident between 11 and 13 days p.i., initially visible as nasal discharge and increased lung noise. These symptoms progressed very quickly, and within 2 days of their first appearance, RPV-infected sentinel rabbits were extremely ill, displaying severe respiratory distress. Three rabbits began to develop pronounced swelling of the neck and face at 12 days p.i., which by 13 days p.i. was severe, constricting the animals' ability to breathe and move their jaws (Fig. 2K). Animals were euthanized between 13 and

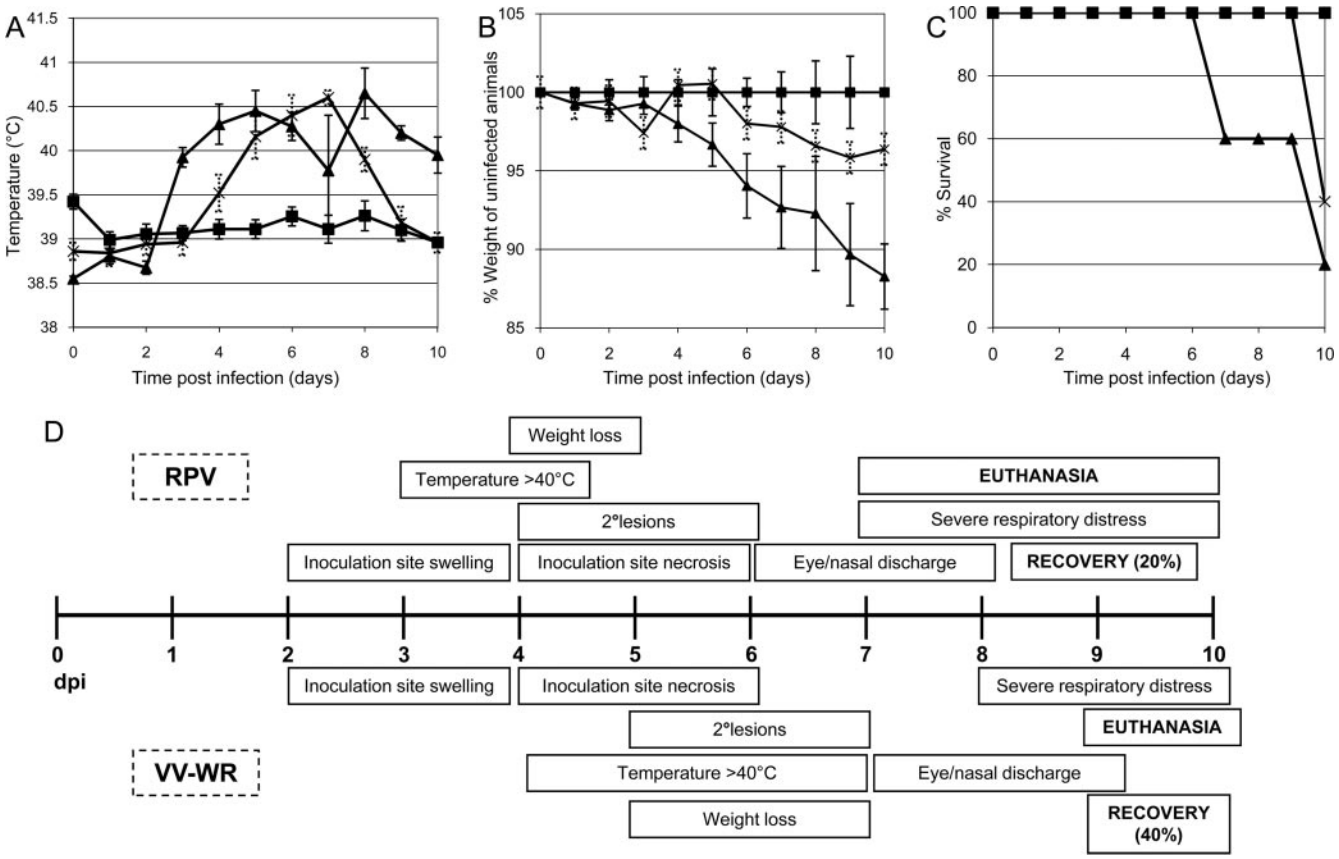


FIG. 4. Comparative pathology of disease in 6-month-old rabbits infected intradermally with 1,000 PFU of RPV or VV-$WR_C$. (A) Average temperatures of animals. (B) Average weights of animals normalized against uninfected and age-matched controls. (C) Survival curves. (D) Time of the first appearance of key clinical signs of disease. The boxes indicate the range of days in which these symptoms were first observed. ■, mock-infected rabbits ($n = 2$); ▲, RPV-infected rabbits ($n = 5$); ×, VV-$WR_C$-infected rabbits ($n = 5$). The error bars indicate standard errors of the mean.

17 days after the infection of the index animal. The lungs of animals that succumbed to severe respiratory distress were very marbled and hemorrhagic (Fig. 2J), as seen in rabbits infected intradermally. All 11 of the sentinel rabbits housed in direct contact with an RPV-infected rabbit became infected with RPV. However, one sentinel animal survived even after developing severe generalized disease (Fig. 5C). If one assumes that once infected the sentinel animals develop disease with kinetics similar to those in intradermally infected rabbits, then transmission may occur between 5 and 8 days after infection of the sentinel animals or about the time that secondary lesions occur and respiratory symptoms develop.

While there was no evidence of any primary lesions in sentinel animals that could suggest spread by contact rather than aerosol, we performed a second experiment in which the sentinel animals had no direct contact with the index infected animal. Two rabbits intradermally infected with 1,000 PFU of RPV were separately housed and separated from four sentinel rabbits by a 15-cm empty space with wire mesh walls. A small fan moved air in a direction from index to sentinel animals. The index animals developed the typical severe respiratory symptoms and were euthanized 8 days after infection, and the sentinel animals were again placed in a clean cage. The noncontact group housing study was performed in quadruplicate with a total of 16 sentinel animals. Disease developed in some, but not all, of the sentinel animals. The transmission rates for the four replicate experiments were 0, 25, 25, and 75% (mean, 31.3%; five infected animals). Figure 5 displays the course of disease of infected animals only. As seen with contact housing, the first indication of infection was a temperature spike to above 40°C between 10 and 12 days after the infection of the index rabbits (Fig. 5A), followed by weight loss (Fig. 5B) of over 25% by 16 days p.i. compared to uninfected controls and the appearance of secondary lesions on sites such as the eyelids, nose, lips, genitals, and skin within 2 days (Fig. 5D). All five animals that became infected developed severe respiratory disease and were euthanized between 15 and 17 days p.i. None of these rabbits exhibited the neck and facial swelling seen in a subset of animals infected by direct contact with index animals; however, the total number of animals in the experiment was small.

The course of disease in sentinel animals infected by direct contact or in separate cages followed the same general course, except that the appearance of symptoms was delayed by 1 or 2 days in animals in the noncontact exposure experiments. This is clearly seen in Fig. 5, where temperature increases (Fig. 5A), weight loss (Fig. 5B), and symptom appearance (Fig. 5D) all

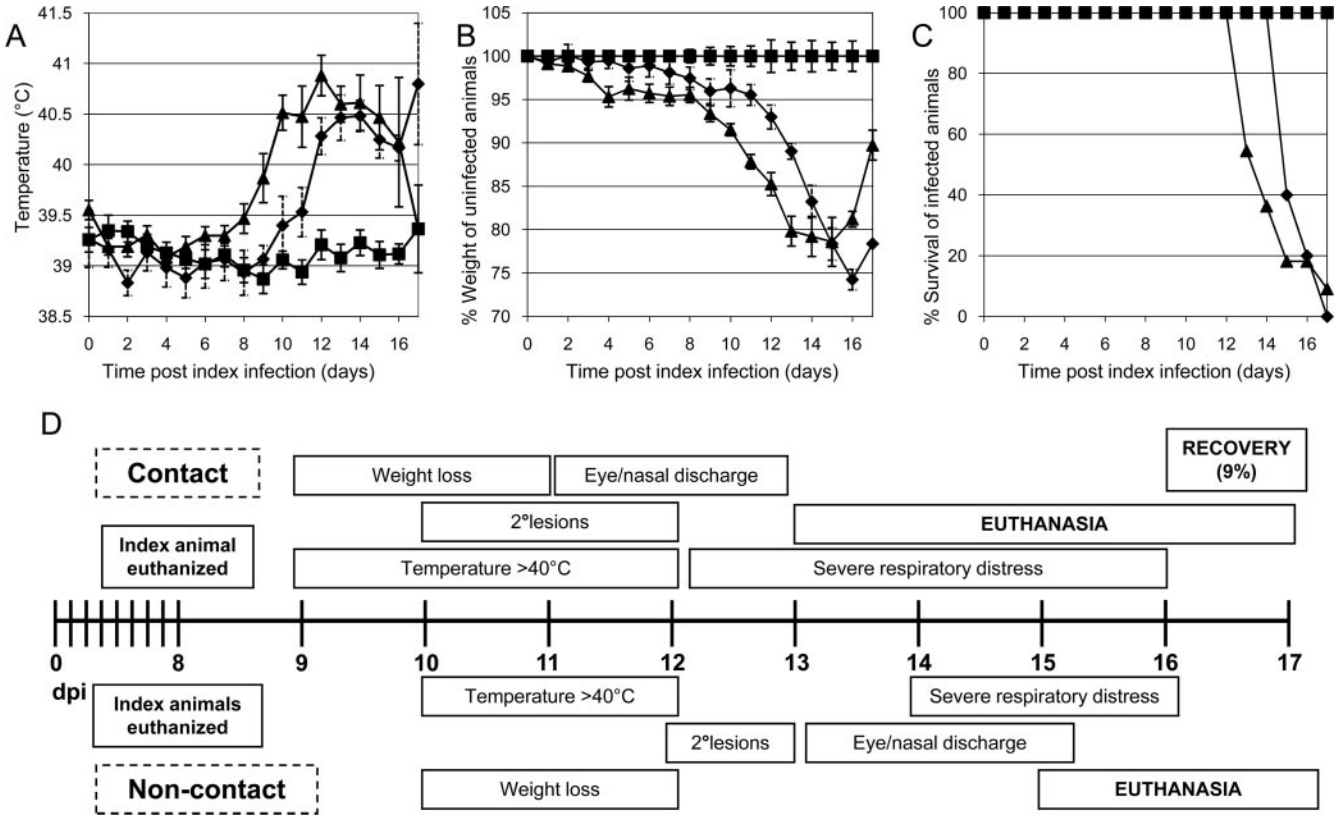


FIG. 5. Comparative pathology of disease in 9-week-old sentinel rabbits infected by exposure to rabbits intradermally infected with 1,000 PFU of RPV. (A) Average temperatures of infected sentinel animals. (B) Average weights of infected sentinel animals normalized against uninfected and age-matched controls. (C) Survival curves of infected animals. (D) Time line description of the first appearance of key clinical signs of disease. The boxes indicate the range of days in which these symptoms were first observed in infected sentinel animals. ■, uninfected sentinel rabbits ($n$ = 11); ▲, RPV-infected by direct contact ($n$ = 11); ◆, RPV infected without contact ($n$ = 5). The error bars indicate standard errors of the mean.

follow very similar profiles but are delayed slightly in animals infected without contact.

Following direct or aerosol infection, the presence of virus within organs and tissues distant from the site of inoculation within the rabbits has been described and is taken as evidence for generalized dissemination (2). In agreement with these earlier descriptions, we found that animals infected by the aerosol route exhibited the described spread of the virus throughout the animals, as expected from earlier reports (Table 1). Generally high titers of the virus in the lung, the presumed initial site of the aerosol infection, were observed. Relatively high titers of the virus in ovaries and secondary lesions were also observed, providing a clear indication of dissemination and spread of the virus from an initial site of infection. Generally, all animals but one (the sole rabbit surviving infection) yielded significant amounts of virus from these two sites. We also examined the liver, spleen, regional lymph nodes, and adrenal glands for virus in the aerosol-infected animals. While virus could be recovered from each of these sites, not all animals yielded virus from every site.

The time to death for individual animals from the first day of displaying a fever over 40°C was between 4 and 5 days for all but one infected sentinel, the same as seen for the vast majority of animals intradermally infected with RPV. This delay in onset of disease may reflect a smaller dose of infecting virus when the animals were physically separated.

**Rabbit-to-rabbit transmission of VV-WR$_c$.** Both contact and noncontact rabbit-to-rabbit transmission experiments were re-

TABLE 1. Titers of RPV detected in various organs of sentinel rabbits exposed to virus-infected index animals

| RPV transmission type | Lung | | Secondary lesion | | Ovary | |
|---|---|---|---|---|---|---|
| | No. of positive/ measured samples | Mean titer ± SEM[b] | No. of positive/ measured samples | Mean titer ± SEM[b] | No. of positive/ measured samples | Mean titer ± SEM[b] |
| Contact | 9/10[a] | 5.37 ± 0.38 | 8/8 | 6.07 ± 0.42 | 10/10 | 4.89 ± 0.52 |
| Noncontact | 6/6 | 4.67 ± 1.09 | 6/6 | 6.18 ± 0.64 | 5/6 | 6.39 ± 0.56 |

[a] The sole animal in which virus was not detected was recovering from infection at the time of sampling.
[b] The numbers represent the geometric mean of the $\log_{10}$ titer measured from an individual rabbit in PFU/g tissue. Titers were performed at the time of sacrifice due to extreme signs of illness.

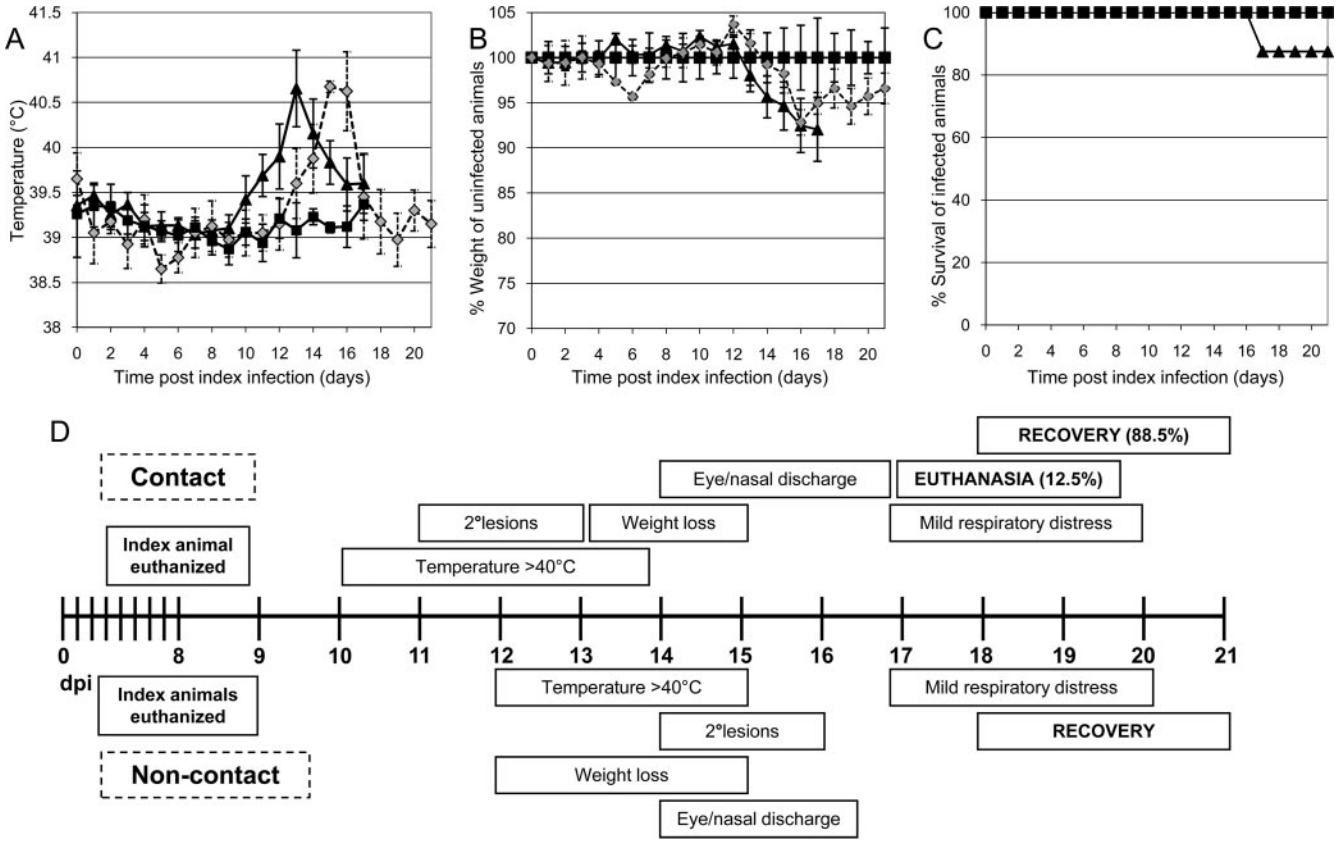


FIG. 6. Comparative pathology of disease in 9-week-old sentinel rabbits infected by exposure to rabbits intradermally infected with 1,000 PFU of VV-$WR_C$. (A) Average temperatures of infected sentinel animals. (B) Average weights of infected sentinel animals normalized against uninfected and age-matched controls. (C) Survival curves of infected animals. (D) Time line description of the first appearance of key clinical signs of disease in infected sentinel animals. The boxes indicate the range of days in which these symptoms were first observed. ■, uninfected sentinel rabbits ($n = 4$); ▲, VV-$WR_C$-infected by direct contact ($n = 8$); ◇, VV-$WR_C$-infected without contact ($n = 4$). The error bars indicate standard errors of the mean.

peated using index animals infected with 1,000 PFU of VV-$WR_C$, using the same setup described above for RPV transmission. The course of disease in infected sentinel animals is summarized in Fig. 6. As was seen with RPV transmission, all sentinel animals that were housed in direct contact with VV-$WR_C$-infected rabbits became infected with VV-WR (100% transmission). The first sign of infection was a temperature spike, which appeared between 10 and 13 days after infection of the index animal (Fig. 6A), followed by the appearance of secondary lesions (Fig. 6D). In contrast to sentinel rabbits infected with RPV, only one sentinel rabbit infected with VV-$WR_C$ succumbed to lethal disease at 17 days p.i. after developing more severe respiratory disease. The remaining seven sentinel animals developed very mild and transient respiratory disease, indicated by slightly lower breathing rates, mild nasal discharge (Fig. 2N), and lung noise. The severity of secondary signs of disease was also markedly less than that seen in RPV transmission studies, with most animals exhibiting fewer than 10 secondary lesions (Fig. 2M), although several animals had more widespread lesion development, especially in the ears (Fig. 2O) and on the body.

Consistent with a less virulent VV-$WR_C$-mediated aerosol infection, virus was always readily recovered from secondary lesions, but recovery from organs, including the lung, ovaries, liver, spleen, lymph node, and adrenal gland, was much less reproducible. Since these animals were in the recovery phase of infection at the time of sample collection, much of the virus would have been cleared.

By 18 to 20 days p.i., the animals began to recover from infection, as indicated by the regression of secondary lesions, loss of nasal discharge, and a return to normal body temperature. It is clear from this experiment that while VV and RPV cause similar diseases, VV is somewhat less pathogenic when transmitted to sentinel animals.

Similar results were found following noncontact transmission of VV-$WR_C$ (Fig. 6). This study was repeated in duplicate with a total of eight sentinel animals, with transmission rates of 25 and 75% (mean, 50%: four infected sentinels). Following euthanasia of the index animals at 8 days p.i., the first sign of infection in sentinel rabbits was a temperature spike first seen between 12 and 15 days p.i. This fever lasted no longer than 5 days. Secondary lesions, slight weight loss, and mild respiratory symptoms followed within a few days, and infected rabbits began to recover within 6 days of the appearance of the temperature spike.

VV-$WR_C$ was transmitted with a frequency similar to that of RPV by both contact and noncontact exposure but was generally nonlethal, with only 1 of the 13 infected animals develop-

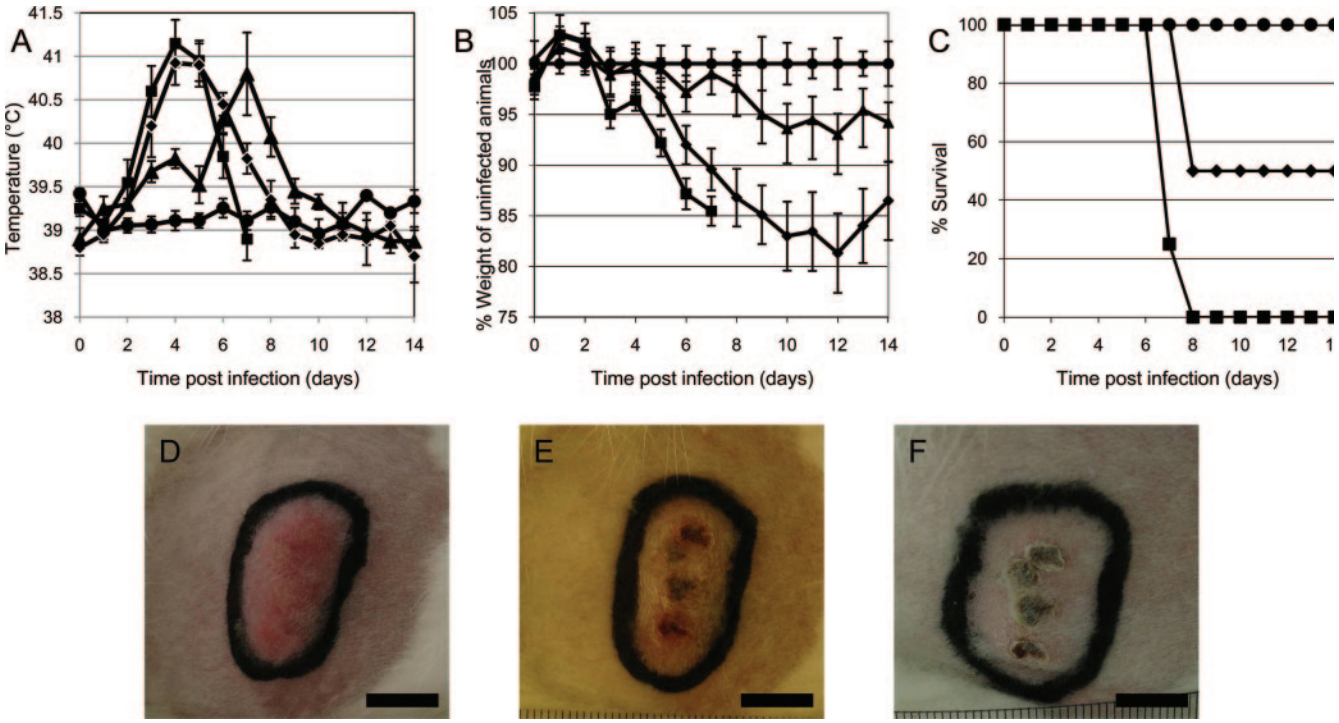


FIG. 7. Comparative pathology of disease in 9-week-old rabbits treated with HDP-CDV and infected with 500 PFU of RPV. (A) Average temperatures of infected and control animals. (B) Average weights of infected animals normalized against uninfected and age-matched controls. (C) Survival curves of infected animals. (D to F) Images of the primary inoculation site of a rabbit treated with 5 mg/kg of HDP-CDV from −1 to 3 days p.i. at 4 days p.i. (D), 8 days p.i. (E), and 12 days p.i. (F). ■, placebo-treated rabbits ($n = 4$); ▲, 5 mg/kg HDP-CDV-treated rabbits ($n = 4$); ◆, 1 mg/kg HDP-CDV-treated rabbits ($n = 4$); ●, uninfected control animals ($n = 4$). The bars on the images are 1 cm in length. The primary lesion areas are demarcated by the circles. The error bars indicate standard errors of the mean.

ing severe respiratory distress and only slight weight loss (<10%) evident in those animals that did become infected (Fig. 6B). As seen in RPV transmission studies, the appearance of symptoms in sentinel rabbits infected by noncontact exposure was delayed by 1 or 2 days compared to animals housed in direct contact (Fig. 6D).

**Treatment of rabbits with HDP-CDV.** To evaluate the efficacy of HDP-CDV in the rabbit model, groups of four animals were orally administered 1 mg/kg or 5 mg/kg of HDP-CDV dissolved in 10% glucose in water or a placebo solution (10% glucose in water). The animals were treated BID (8 a.m. and 4 p.m.) from 1 day before challenge with 500 PFU of RPV for a total of 5 days (until 3 days p.i.). The pathogeneses of RPV infection that we described following 500 or 1,000 PFU were found to be virtually identical (data not shown).

The first sign of infection in all groups of animals was a red swelling at the inoculation site between 1 and 3 days p.i. Placebo-treated (infected but untreated) and 1-mg/kg-treated animals exhibited a temperature spike to above 40°C at 3 days p.i. (Fig. 7A), which was not evident in animals that received 5 mg/kg until 6 days p.i. (3 days after the drug was withdrawn) (Fig. 7A). By 6 days p.i., placebo-treated animals were all very ill and had developed respiratory disease. This progressed to more severe respiratory disease, and the placebo-treated animals were euthanized at 7 or 8 days p.i. (Fig. 7B).

Animals treated with 1 mg/kg of HDP-CDV developed numerous secondary lesions, first evident at 6 days p.i. Half of these animals also developed more serious respiratory disease and were euthanized at 8 days p.i. (Fig. 7B). The remaining animals were quite ill and exhibited mild respiratory disease, a large primary lesion up to 7 cm in diameter, weight loss of 19% compared to uninfected control rabbits (Fig. 7B), and numerous secondary lesions on the eyelids, body, nose, mouth, and ears. However, by 11 days p.i. the surviving animals began to recover and were significantly improved by the end of the experiment at 14 days p.i.

Animals treated with 5 mg/kg of HDP-CDV exhibited little in the way of disease during challenge. The lesion at the inoculation site remained much smaller than those seen on placebo- or 1 mg/kg-treated rabbits throughout the challenge. By 4 days p.i., a red swelling up to 1.5 cm in diameter was evident on all animals (Fig. 7D). By 8 days p.i., these lesions were only slightly larger and had begun to scab over (Fig. 7E). At this time, these animals exhibited little in the way of secondary signs of disease, limited to a mild, transient fever in most animals (Fig. 7A), and fewer than five secondary lesions per animal at sites such as the ears and eyelids. By 12 days p.i., swelling and redness at the inoculation site had largely regressed and the lesions had scabbed over (Fig. 7F). Although these animals did not gain weight at the rate of uninfected control animals (Fig. 7B), they continued to gain weight during the course of infection, as opposed to placebo- and 1 mg/kg-treated rabbits, which began to lose weight from 3 to 4 days p.i. until death or 12 days p.i. All animals that had received 5 mg/kg of HDP-CDV survived challenge (Fig. 7C) with few or no secondary signs of disease.

Animals treated with 1 mg/kg of HDP-CDV that succumbed to disease were found to have high titers of virus in most organs, including the lungs, with titers of up to $7.6 \times 10^7$ PFU/g at the time of death, comparable to placebo-treated animals, while virus was detected only in the scab material over the inoculation site in animals that survived until the end of the study at 14 days p.i. (data not shown).

## DISCUSSION

We have confirmed that the intradermal infection of rabbits with RPV results in a highly lethal systemic disease characterized by generalized dissemination of the virus from the dermal site of inoculation to ultimately produce secondary lesions on mucocutaneous tissues and the skin and severe respiratory disease resulting in death. The pathology of RPV infection in the rabbits we observed corresponds well to that described in earlier studies (2). Thus, intradermal infection of rabbits provides an excellent small-animal model for human smallpox and is the only orthopoxvirus model in which natural aerosol transmission of the virus to sentinel animals, resulting in lethal systemic disease, has been reported.

The rabbit is an unusually sensitive animal model for RPV or VV-$WR_C$ infections. Lethal infections of a young (roughly 17-g) BALB/c mouse generally requires $10^4$ to $10^5$ PFU of virus introduced intranasally (20). Although we typically use 1,000 PFU in a 1.7- to 2.2-kg rabbit to ensure experimental reproducibility, we have shown that rabbits uniformly succumb to 50 PFU of either VV-WR or RPV following intradermal injection. This extreme sensitivity to the virus likely explains why aerosol transmission is so prevalent in the rabbit model, as so little virus is required to initiate a lethal infection.

Based on the literature, the severe disease seen upon infection of rabbits with VV-WR was completely unexpected. There are numerous studies of infection of the rabbit with VV that describe only minimal disease (23, 25). Indeed, infection of rabbits with VV is commonly used to produce anti-vaccinia virus polyclonal immune sera. Intradermal infection of rabbits with VV-WR at levels up to $10^9$ PFU has been previously reported to cause only relatively minor disease (23, 25). Similarly, respiratory infections of rabbits with doses of up to 250 PFU of an undefined strain of VV were reported to be nonlethal (27). In comparison, we observed 92% lethality for VV-$WR_C$-infected rabbits within 9 days with an intradermal dose of only $10^3$ PFU.

In earlier studies of the respiratory infection of rabbits by VV, virus was not detected in other organs and 100% of the infected animals survived, despite virus titers in the lungs reaching levels comparable to those of RPV (27). Similarly, VV transmission by contact and aerosol has been reported previously but was recorded as mild or asymptomatic (8). The differences between the rather mild symptoms generated by VV infection observed in the earlier studies and our results are most likely due to the fact that the VV strains used in the older studies represented a mixed population of viruses derived from animal infections resulting from repeated serial passage rather than being derived from a cloned isolate of a virulent strain.

Serial passage of virus stocks quickly accumulates variants with multiple deletions and rearrangements of genes, leading to attenuation of the virus (18). We have used only carefully maintained virus stocks of known genetic background for our experiments. Our RPV Utrecht strain was originally obtained in 1976 as a lyophilized preparation from Joe Sambrook, who obtained it from Frank Fenner while a student in that laboratory. The virus was then plaque purified twice by us on pig kidney cells before expansion into small seed stocks. We routinely prepare virus from a seed stock grown on pig kidney cells. Our strain of RPV was completely sequenced by Mark Buller and is considered the reference strain of RPV. VV-$WR_C$ was obtained by Richard C. Condit from the ATCC as passage 64 of the Norman Salzman strain originally supplied by Joel Dalrymple in 1962 as a brain homogenate in 20% rabbit serum. Upon receipt, the Condit strain was plaque purified twice on mouse L cells, grown and plaque purified twice on BSC-40 cells, and grown at low multiplicity on BSC-40 cells and used thereafter as seed stock (R. Condit, personal communication; 4).

A second possibility to explain the high level of pathogenicity of VV-$WR_C$ in rabbits might have been the youth of the animals. Therefore, we examined both RPV and VV-$WR_C$ infections in sexually mature rabbits at 6 months of age. We found that although not quite as susceptible to disease as younger animals, older rabbits still developed generalized disease and severe illness when infected with RPV and VV-$WR_C$. RPV was lethal in the majority of older rabbits (80%), although half of those that died survived until 10 days p.i., which was somewhat longer than seen with 9-week-old animals, comparable to previous reports (2). Similarly, we found VV-$WR_C$ was only 60% lethal in older rabbits compared to 92% in 9-week-old rabbits. However, all of the older animals developed obvious generalized disease that had not been reported previously.

The original discovery of RPV in rabbit colonies was suggested to originate from a strain of VV selected by animal passage from a strain of VV used at the time (2). Our results suggest that VV-WR and RPV are quite similar biologically, consistent with sequenced genomes, which show them to be closely related (17), with RPV containing differences in only a few genes with possible roles in host range and immunomodulation.

Although VV-$WR_c$ was found to be an extremely virulent strain in our model, we have found that intradermal infections of rabbits with Dryvax produced no symptoms of disease but fully protected the rabbits against an intradermal lethal challenge with RPV (M. Adams and R. W. Moyer, unpublished results). The virulence of other VV strains, such as Lister and Copenhagen, can also be easily evaluated in the rabbit model.

Animals cohoused with either VV-$WR_C$- or RPV-infected animals developed disease with 100% efficiency some 5 to 8 days later than the index animals. We believe that illness in the cohoused sentinel animals was most likely the consequence of aerosol infection. None of the cohoused sentinel animals that were in direct contact with an infected index rabbit displayed any external signs of lesions before the onset of fever. The only skin lesions observed were those in the ears and elsewhere, which appeared after or simultaneously with fever and other clinical symptoms. These lesions most likely represented secondary lesions associated with the extensive viremia that normally occurs in infected rabbits. The high rate of transmission suggests that the infectious dose required to generate clinical

disease via the projected aerosol route is very low, on the order of a few virus particles. Previous studies have shown that aerosol infection with 15 PFU was 86% lethal (16, 27), and the authors suggested that only 25% of this dose would have been retained in the lungs. Respiratory exposure via aerosol is further supported by the severe neck and facial swelling seen in three of the RPV-infected sentinel animals, suggesting that the initial site of infection in these animals was in the upper respiratory tract. This swelling was never seen in animals infected intradermally.

When sentinel and index animals were housed in separate cages, we again observed infection of the sentinel animals, albeit at a lower frequency. The disease and symptoms were virtually identical to those observed for intradermal infection but were somewhat delayed compared to the animals in direct contact, consistent with more frequent exposure and higher infectious doses of virus in the case of the cohoused sentinel animals. From a practical point of view, we propose that cohousing rabbits provides a model for natural poxvirus aerosol transmission with nearly 100% efficiency.

Our experiments with RPV transmission are completely consistent with the older literature. RPV was previously found to be highly transmissible by contact at 4 to 7 days p.i. (50 to 100% transmission) and achieved 50% transmission between 15-cm-separated animals at 4 to 5 days p.i. (2). Other studies determined that RPV could only be recovered from the air in rooms with infected rabbits at 6 and 7 days p.i. (27).

We used the rabbit model described here to evaluate the drug HDP-CDV, which has proven highly effective in mouse models of orthopoxvirus disease (12, 21, 23). We found that a dose of 5 mg/kg BID for 5 days from 1 day before infection was able to protect rabbits from almost all signs of RPV infection. However, doses of 1 mg/kg offered only partial protection, with 50% mortality following a challenge of 500 PFU of RPV, and surviving animals exhibited moderate generalized signs of infection, including mild respiratory disease. However, all rabbits treated with this dose and challenged with only 100 PFU of RPV survived (data not shown), suggesting the efficacy of 1 mg/kg BID against lower virus inocula. Further studies have shown that 5 mg/kg BID is able to prevent mortality in rabbits when treatment is commenced as late as 4 days p.i. (M. M. Adams and R. W. Moyer, unpublished data).

These results agree well with those found in intranasal and aerosol challenge models with ectromelia virus in mice, where 2 mg/kg given once a day from 0 to 4 days p.i. resulted in only 0 to 20% mortality, while a dose of 8 mg/kg protected all mice from death (12). HDP-CDV was more effective in the rabbit challenge model than in mouse models using cowpox and VV strains, such as WR and IHD, using comparable doses of the drug (12, 21). However, studies analogous to those described in this paper involving pre- and postinfection dosing with HDP-CDV in these challenge models have not been published.

These results further support the efficacy of HDP-CDV in preventing mortality and morbidity in orthopoxvirus animal models and suggest that this orally bioavailable drug should be pursued as a possible treatment for smallpox in humans.

In summary, because of the sensitivity of the rabbit to intradermal infection by either RPV or VV-$WR_C$, followed by the onset of lethal disease that includes recapitulation of most of the critical features of human smallpox (general dissemination of virus, secondary lesions, respiratory involvement, and lethal animal-to-animal aerosol transmission), the rabbit provides an excellent model for testing the efficacy of both antiviral drugs and vaccines.

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