A Quantitative Rabbit Model of Vaccinia Keratitis

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PURPOSE. The goal of this study was to use multiple quantitative disease measures to evaluate the effect of various viral inocula on the development of vaccinia keratitis in rabbits.

METHODS. Trephined eyes of female rabbits were infected with 10^4 , 10^5 , 10^6 , or 10^7 plaque-forming units (pfu) of the Dryvax strain of the vaccinia virus and scored daily for disease for 14 days according to a modification of the MacDonald-Shadduck scoring system. Ocular viral titers and vaccinia-specific antibody titers were determined by plaque assay and ELISA, respectively.

RESULTS. The amount of virus used for infection affected the severity of disease, with 10^4 pfu eliciting milder keratitis after delayed onset compared with higher amounts of virus. At inocula above 10^5 pfu the course and severity of corneal disease was not significantly different. The time to reach peak titers was delayed in the 10^4 group but peak titers were similar in all groups. Severe conjunctival chemosis interfered with scoring in animals infected with 10^6 or 10^7 pfu. Virus-specific antibody titers were similar in all groups at day 14. Body weights decreased less than 10% in all groups.

CONCLUSIONS. The course of vaccinia keratitis in rabbits paralleled that in humans. A viral inoculum of 10^5 pfu/eye was determined to be optimal for use in further studies of vaccinia keratitis. (*Invest Ophthalmol Vis Sci.* 2010;51:4531–4540) DOI:10.1167/iovs.09-5106

In 1971, the United States Public Health Service recommended that routine childhood vaccination against smallpox be halted, in part because of the high risk of morbidity and mortality associated with the vaccine relative to the likelihood of disease occurrence.¹ Smallpox was officially declared to be eradicated in 1980, 3 years after the last naturally occurring

case was diagnosed.² Vaccination requirements were waived for health care workers in 1976, international travelers in 1982, and military personnel in 1990. However, vaccinations of health care workers and military personnel resumed in 2001 because of concerns about the potential use of smallpox for bioterrorism. Adverse reactions to smallpox vaccination are common, and range from mild fever and muscle pain to systemic infection, encephalitis, myocarditis, and death.^{3–9} A recent study found that greater than one third of the study participants missed work due to mild-to-moderate symptoms after vaccination.¹⁰

One common adverse reaction to smallpox vaccination is ocular vaccinia, resulting from accidental transfer of vaccinia virus (VACV) from the inoculation site to the eye. Ocular vaccinia occurred in approximately 1 to 4 recipients per 40,000 primary vaccinations during the smallpox eradication effort and can manifest as blepharitis, conjunctivitis, iritis, and keratitis.^{9–11} Of these, corneal involvement is the most severe and can result in vision loss. In humans, vaccinia keratitis (VACVK) generally occurs in conjunction with conjunctivitis and blepharitis and begins as a fine granular opacification of the cornea. The disease can progress through the development of opacities in the superficial stroma to ulceration, endothelial keratitis, and diffuse interstitial keratitis.¹² Corneal vascularization and uveal involvement (aqueous flare) are common.¹² The estimated rates of keratitis during eradication efforts ranged from 6% to 30% of ocular vaccinia cases, depending on reporting conditions.^{12,13}

Rabbits have previously been used to model both the pathologic course^{14–17} and treatment of vaccinia keratitis,^{18–23} and the current clinical guidelines for treating vaccinia keratitis in humans are based on the findings in these studies.²⁴ However, the extent to which vaccinia keratitis in rabbits resembles the disease as it presents in humans has not been determined, and the disease parameters that have been monitored are limited in scope. This poses challenges for evaluating current and future therapies for vaccinia keratitis in a comprehensive manner. Information available in the literature does not allow relative comparison of treatment efficacy due to considerable variation in concentration of initial viral inoculum between studies. The establishment of a quantitative scoring system would greatly improve studies of the pathogenesis of VACVK and the testing of therapeutic modalities.

A standardized concentration of viral inoculum would also greatly improve therapeutic intervention and virulence studies. The optimal virus inoculum would satisfy four main criteria: (1) It would result in a mean keratitis score near the middle of the scoring range; (2) it would result in disease in 90% to 100% of the animals; (3) it would result in disease in 90% to 100% of the animals within 48 hours after infection, to reduce potential variability in the onset of therapy; and (4) it would minimize the number of animals with conjunctival chemosis severe enough to make scoring keratitis difficult. We present the most complete quantitative analysis of the course of vaccinia kerati-

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Supported by grant number AGRDTD 05/09/07 from the Centers for Disease Control via Cangene, Corp. and National Institutes of Health Grant P30 EY016665.

Submitted for publication December 21, 2009; revised February 23, 2010; accepted March 6, 2010.

Disclosure: S. Altmann, Cangene Corp. (F); A. Emanuel, Cangene Corp. (F, E, R); M. Toomey, Cangene Corp. (F); K. McIntyre, Cangene Corp. (F); J. Covert, Cangene Corp. (F); R.R. Dubielzig, Cangene Corp. (F); G. Leatherberry, Cangene Corp. (F); C.J. Murphy, Cangene Corp. (F); S. Kodihalli, Cangene Corp. (F, E, R); C.R. Brandt, Cangene Corp. (F)

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tis in rabbits reported to date and determine the optimal viral inoculum for inducing vaccinia keratitis for therapeutic testing.

MATERIALS AND METHODS

Cells and Viruses

Vero cells (CCL-81; American Type Culture Collection [ATCC], Manassas, VA) and HeLa cells (ATCC, CCL-2) were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin (30-002-CI; Mediatech, Manassas, VA). Virus was propagated as described previously.²⁵ Briefly, Vero cells were infected with the New York City Department of Health Laboratories strain of VACV (ATCC, VR-1536) at a multiplicity of infection (MOI) of 0.01 in DMEM supplemented with 2% fetal bovine serum. The cells and supernatants were harvested when the cytopathic effect reached 90% to 100%, the cells were frozen and thawed three times to release bound virus, and the nuclei were pelleted by centrifugation. The supernatants were then combined and layered onto a cushion of 36% sucrose in HEPES-buffered Hanks' balanced salt solution (H-HBSS, CC-5024; Lonza, Mapleton, IL) and centrifuged at 20,000g for 80 minutes in an rotor (model SW28; Beckman, Fullerton, CA). The pelleted virus was then titered on HeLa cells, resuspended in H-HBSS at a concentration of 1×10^9 pfu/mL, and stored in 100- μ L aliquots at -80°C until use. The amount of endotoxin in the virus preparations was determined with an endotoxin assay (ToxinSensor Chromogenic LAL, cat. No. L00350; GenScript, Piscataway, NJ). The concentration was 0.0159 U/mL, which translates to 0.08 ng. The inoculum size was 50 μ L, and so each eye received 0.004 ng of endotoxin.

Animals

Three- to 5-month-old female rabbits Hra:[(NZW)SPF] weighing 3.09 to 3.87 kg, were obtained from Harlan (Indianapolis, IN). Forty naive animals were randomly assigned to one of four experimental groups. All procedures were in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, the Office of Laboratory Animal Welfare, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Animal Inoculation and Disease Scoring

All animal studies were conducted under BL3 conditions. On day 1 of the study, animals were anesthetized with ketamine hydrochloride (up to 30 mg/kg; Lloyd Laboratories, Shenandoah, IA) and midazolam hydrochloride (1 mg/kg; Abraxis Pharmaceutical Products, Schaumburg, IL) via intramuscular injection into a caudal thigh muscle. If needed, a booster dose of ketamine was given at 2 to 5 mg/kg. The right eye of each rabbit was trephined to a depth of approximately 250 μ m with a 7.5-mm diameter Hessburg-Barron vacuum trephine (Jedmed, St. Louis, MO). After trephination, 20 μ L of the appropriate viral inoculum (group 1, 1×10^4 pfu; group 2, 1×10^5 pfu; group 3, 1×10^6 pfu; and group 4, 1×10^7 pfu) was pipetted directly into the trephine wound. Although trephination is not physiological, it increases reproducibility and reduces the number of animals needed. Disease parameters were scored according to a modification of the MacDonald-Shaddock Scoring System (Table 1) once daily for the duration of the study. Slit lamp evaluations were performed by a board-certified veterinary ophthalmologist using a handheld biomicroscope (model SL-15; Kowa Company, Ltd., Tokyo, Japan) at high (15 $\times$) magnification. Indirect ophthalmoscopy was performed on days 1 and 14 with an indirect headset (VantagePlus; Keeler Instruments Inc., Broomall, PA) in combination with a panretinal 2.2 lens (Volk Optical Inc. Mentor, OH). A pachymeter (AccuPach V; Accutome Inc., Malvern, PA) was used to determine the thickness of the corneas. Proparacaine hydrochloride sterile ophthalmic solution, USP (Akorn, Inc. Buffalo Grove, IL) was placed in each eye as needed for evaluation and before obtaining pachymetry readings. The fluorescein stain solution was prepared with one fluorescein strip (Ful-Glo Fluorescein

Sodium Ophthalmic Strip, USP; 1.0 mg; Akorn, Inc.) for every 1 mL of 0.9% sodium chloride injection, USP (Baxter Health Care Corp., Deerfield, IL).

Photography

Digital photographs were taken of both eyes before inoculation of the virus and on day 14. Photographs of the inoculated eye were taken on days 2 through 13. A single-lens reflex digital camera (model D100; Nikon, Tokyo, Japan) and a 105-mm 1:2.8-D lens (AF Micro Nikkor; Nikon) fitted with a ring flash (Macro Speedlight SB-29s; Nikon) and a 52-mm filter (Skylight 1A; Promaster, Fairfield, CT) was used for standard color imaging. Fluorescein images were taken with a single-lens reflex digital camera (model D200; Nikon) and the 105-mm 1:2.8-D lens fitted with a flash system (R1; Speedlight SB-R200 wireless remote flash, SX-1 ring, SZ-1 filter cover, blue AWB flash filter, and Sy-1-62 yellow lens filter; Nikon Corp., Tokyo, Japan). This setup optimized imaging of the fluorescein dye.

Virus Isolation

Corneal swabs were collected from the right eye once before inoculation of the virus and on days 2, 3, 4, 6, 8, 10, 12, and 14 after infection. Samples were stored in HBSS at -80°C until they were titered. Viral titers were determined by serial dilution and plaque assay on HeLa cells.²⁵

Detection of Anti-VACV Antibodies by ELISA

Blood was collected once before inoculation of the virus (baseline) and on days 7 and 14, from the jugular vein (baseline and day 7) and via cardiac puncture after induction of anesthesia via intramuscular ketamine and midazolam and immediately before euthanatization (day 14). To prepare microtiter plates for the assay, 20 μ L of sucrose-gradient purified VACV in carbonate buffer (50 μ g/mL protein in 0.125 M sodium bicarbonate and 0.042 M sodium carbonate) were plated in a 96-well microtiter plate and incubated in a humidified chamber at 4°C for at least 24 hours. Infectious particles were inactivated after binding using 10% formaldehyde. The plates were then blocked for 30 minutes in PBS+0.05% Tween-20, 5% powdered skim milk, 2% normal goat serum, and 2% bovine serum albumin, washed three times in PBS with 0.05% Tween-20 (PBST), and incubated with serially diluted test sera for 1 hour at 37°C in a humidified chamber. The plates were then washed in PBST and incubated with horse radish-peroxidase conjugated goat anti-rabbit IgG which detects all isotypes (1:1000 dilution, sc-2030; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at 37°C in a humidified chamber. The plates were developed (SigmaFAST OPD kit; P9187; Sigma-Aldrich, St. Louis, MO), and the reaction was halted by the addition of 3 M HCl. The absorbance was read at 490 nm using a microplate reader (EL₈₀₀ NB Universal; BioTek Instruments, Winooski, VT). Samples were considered positive if the absorbance values were more than 3 SD greater than the negative control.

Histology

The eyes, eyelids, and any extraocular lesions present were fixed in 4% paraformaldehyde and then embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically with a light microscope (BX51; Olympus America Inc., Center Valley, PA). The following areas of the eye were graded as described in Table 2: haired skin (eyelids), including meibomian glands; palpebral conjunctiva (including the palpebral surface of the third eyelid); bulbar conjunctiva (including the bulbar surface of the third eyelid); and cornea.

Immunohistochemistry

Ocular sections were deparaffinized in xylene and then rehydrated in graded ethanol. The sections were incubated for 30 minutes in 3% hydrogen peroxide to reduce endogenous peroxidase activity and washed in PBS for 5 minutes. Sections of the infected and uninfected eyes from the same rabbit were paired during staining for control

TABLE 1. Modified MacDonald-Shadduck Scoring System

Corneal Opacity

- 0 Normal cornea. Appears with the slit lamp as having a bright grey line on the epithelial surface and a bright grey line on the endothelial surface, with a marblelike grey appearance of the stroma.
- 1 Some loss of transparency. Only the anterior half of the stroma is involved as observed with an optical section of the slit lamp. The underlying structures are clearly visible with diffuse illumination, although some cloudiness can be readily apparent with diffuse illumination.
- 2 Moderate loss of transparency. In addition to involving the anterior stroma, the cloudiness extends all the way to the endothelium. The stroma has lost its marblelike appearance and is homogeneously white. With diffuse illumination, underlying structures are clearly visible.
- 3 Involvement of the entire thickness of the stroma. With optical section, the endothelial surface is still visible. However, with diffuse illumination, the underlying structures are just barely visible (to the extent the observer is still able to grade flare, iritis, and note lenticular changes).
- 4 Involvement on the entire thickness of the stroma. With the optical section, cannot clearly visualize the endothelium. With diffuse illumination, the underlying structures cannot be seen. Cloudiness removes the capability for judging and grading aqueous flare, iritis, and lenticular changes. Can see posterior margin of the cornea, but cannot score by slit lamp examination.
- 5 Cannot see posterior margin of cornea.
- 6 Corneal perforation.

Corneal Opacity (% Area)

- 0 Normal cornea with no area of cloudiness.
- 1 1% to 25% area of stromal cloudiness.
- 2 26% to 50% area of stromal cloudiness.
- 3 51% to 75% area of stromal cloudiness.
- 4 76% to 100% area of stromal cloudiness.

Corneal Vascularization

- 0 No corneal vascularization (pannus).
- 1 Vascularization is present but vessels have not invaded the entire corneal circumference. Where localized vessel invasion has occurred, they have not penetrated beyond 2 mm.
- 2 Vessels have invaded 2 mm or more around the entire corneal circumference.
- CVL Cannot visualize limbus.

Conjunctival Congestion

- 0 Normal. May appear blanched to reddish pink without perilimbal injection (except at 12 and 6 o'clock positions) with vessels of the palpebral and bulbar conjunctiva easily observed.
- 1 A flushed reddish color predominantly confined to the palpebral conjunctiva with some perilimbal injection but primarily confined to the lower and upper parts of the eye from the 4 and 7 and 11 and 1 o'clock positions.
- 2 Bright red color of the palpebral conjunctiva with accompanying perilimbal injection covering at least 75% of the circumference of the perilimbal region.
- 3 Dark, beefy red color with congestion of both the bulbar and the palpebral conjunctiva along with pronounced perilimbal injection and the presence of petechia on the conjunctiva. The petechiae generally predominate along the nictitating membrane and the upper palpebral conjunctiva.

Conjunctival Chemosis and Swelling

- 0 Normal or no swelling of the conjunctival tissue.
- 1 Swelling above normal without eversion of the lids (can be easily ascertained by noting that the upper and lower eyelids are positioned as in the normal eye); swelling generally starts in the lower cul-de-sac near the inner canthus.
- 2 Swelling with misalignment of the normal approximation of the upper and lower eyelids; primarily confined to the upper eyelid so that in the initial stages the misapproximation of the eyelids begins by partial eversion of the upper eyelid. In this stage, swelling is confined generally to the upper eyelid, although it exists in the lower cul-de-sac (observed best with the slit lamp).
- 3 Swelling definite with partial eversion of the upper and lower eyelids is essentially equivalent. This can be easily ascertained by looking at the animal head-on and noticing the positioning of the eyelids; if the eye margins do not meet, eversion has occurred.
- 4 Eversion of the upper eyelid is pronounced with less pronounced eversion of the lower eyelid. It is difficult to retract the lids and observe the perilimbal region.

Conjunctival Discharge

- 0 Normal. No discharge.
- 1 Discharge is above normal and present on the inner portion of the eye but not on the lids or hairs of the eyelids. One can ignore the small amount that is in the inner canthus if it has not been removed before the study began.
- 2 Discharge is abundant, easily observed, and has collected on the lids and around the hairs of the eyelids.
- 3 Discharge has been flowing over the eyelids and has wet the hairs substantially on the skin around the eyes.
- CS Cannot score due to corneal opacity.

Corneal Staining (% Area)

- 0 No area of fluorescein staining.
- 1 1% to 25% area of fluorescein staining.
- 2 26% to 50% area of fluorescein staining.
- 3 51% to 75% area of fluorescein staining.
- 4 76% to 100% area of fluorescein staining.

TABLE 2. Histology Scoring Scale

	Score				
	0	1	2	3	4
Extent of ulceration	None	Microscopic focus	Multiple areas of microscopic focus or one focus >1 mm	≥1 Ulcerated surface area	Global or near global ulceration
Quality of epithelium	Normal	Focal metaplasia	Regional metaplasia	Global metaplasia	NA
Extent of inflammation	None	Microscopic or local	Regional	Global	Global and effacing
Corneal stroma	Normal	Superficial and regional (≤200 μm)	>200 μm, <50% thickness of cornea	Evidence of deep ulceration	Perforation
Meibomian gland	Normal	Microscopic inflammation	Regional inflammation	Regional inflammation with local effacing	Global effacing inflammation

purposes. Immunohistochemical staining was performed (Vectastain Elite ABC system, PK-6102; Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. A monoclonal antibody specific for VACV was used as the primary antibody (1:100 dilution, ab48569; Abcam, Cambridge, MA). The signal was developed for 30 minutes with a peroxidase substrate kit (NovaRed; SK-4800; Vector Laboratories). The slides were counterstained (hematoxylin QS, H3404; Vector Laboratories), mounted (Vectamount H-5000; Vector Laboratories), and visualized and scored with an inverted microscope (Axiovert 200M; Carl Zeiss, Thornwood, NY). Slides were scored for the presence or absence of viral antigen in the cornea, eyelids, trabecular meshwork (TM; angle), iris, ciliary body, lens, retina, and choroid.

Statistical Analysis

Wilcoxon rank-sum tests were performed on keratitis onset and peak keratitis data. ANOVA on ranks analysis was performed on viral titer, chemosis, and corneal opacity scores (SigmaPlot ver. 11.0; Systat Software, Inc., Chicago, IL).

RESULTS

Ophthalmic Findings

Infected eyes were examined by slit lamp daily and scored using a modification of the MacDonald-Shadduck scoring system (Table 1). Quantitative results for the various disease parameters are shown in Figures 1 and 2. The onset of keratitis was initially defined as a score of 1.0 for either corneal clouding or fluorescein staining. The study found that corneal clouding occurred earlier and was therefore the earliest indicator of the onset of keratitis. The progression of keratitis over the course of the study is shown in the representative color and fluorescein photographs of representative rabbits from each group in Figure 3. Schematic illustrations depicting the key slit lamp findings throughout the progression of keratitis using an inoculum of 1×10^5 pfu are shown in Figure 4.

The development of corneal opacity was first seen between days 2 and 4 in groups 2, 3, and 4 (Fig. 1A). The onset of corneal opacification was delayed in group 1 compared with that in groups 2 to 4 and occurred between days 3 and 5. This

FIGURE 1. Quantification of clinical vaccinia keratitis. Infected eyes were examined daily by slit lamp and scored according to a modification of the MacDonald-Shadduck Scoring System (Table 1). (A) Severity of corneal opacity. (B) Percentage of corneal surface with stromal cloudiness. (C) Percentage of corneal surface staining positive for epithelial disruption with fluorescein. (D) Extent of corneal vascularization. The data points represent the mean results in 10 animals per group. Group 1 (1×10^4 pfu; ●), group 2 (1×10^5 pfu; ○), group 3 (1×10^6 pfu; ▼), and group 4 (1×10^7 pfu; △).

FIGURE 2. Quantification of macroscopic disease during vaccinia keratitis. Macroscopic symptoms of disease in infected eyes were scored daily. Points represent the mean of 10 animals per group. (A) Corneal thickness. Corneal thickness was measured daily with a pachymeter. Conjunctival chemosis (B), conjunctival congestion (C) and conjunctival discharge (D) were scored according to Table 1. The data points represent the mean results in 10 animals per group. Group 1 (1×10^4 pfu; ●), group 2 (1×10^5 ; ○), group 3 (1×10^6 pfu; ▼), and group 4 (1×10^7 pfu; △).

delay was statistically significant (group 1 vs. groups 2–4) according to the Wilcoxon rank-sum test ($P_r < 0.0001$). Beginning on day 2, groups 2, 3, and 4 exhibited comparable corneal opacity scores throughout the study, with clouding progressing through day 14 ($P < 0.05$ ANOVA on ranks; Fig. 1A). Corneal opacity in group 1 was significantly less severe than that observed in groups 3 and 4 throughout the study ($P < 0.05$, ANOVA on ranks) and significantly less severe than that observed in group 2 on days 3, 4, 11, and 12 (Fig. 1A). The percentage of the cornea exhibiting cloudiness reached 75% to 100% on day 3 for groups 2, 3, and 4, whereas group 1 did not reach 75% to 100% until day 7 (Fig. 1B). Advanced opacification of the cornea precluded the scoring of aqueous flare, aqueous cell, iritis, lens, vitreous cell, and retinal involvement in all groups.

Fluorescein staining was used to determine the extent to which the corneal epithelium was disrupted by infection. The onset of epithelial disruption was observed in groups 2, 3, and 4 on day 2. As with the other clinical signs, onset was delayed in group 1 (Fig. 1C). Groups 3 and 4 displayed the most severe epithelial damage, with the disruption peaking on day 8 at mean scores of 3.8 and 3.9, respectively, and then gradually decreasing through day 14 (Fig. 1C). Group 2 had a mean score of 3.3 and peaked on day 8, whereas group 1 had the least severe epithelial disease and peaked in severity on day 9 at a mean score of 1.5 (Fig. 1C).

Corneal vascularization, which began on days 7 and 8 for all groups (Fig. 1D), increased gradually through day 14. Group 1 displayed slightly less extensive vessel invasion than the other groups, with a mean score of 1.8 on day 14 versus a mean score of 2 for the other three groups (Fig. 1D). It should be noted that between days 5 and 10, only 20% of the rabbits could be scored for vascularization in group 4, and 40% from group 3, due to extensive conjunctival chemosis that interfered with the observation of the perilimbal region. Statistical analysis was therefore not conducted of corneal vascularization in these groups.

Corneal thickening was observed in all groups, with group 1 again displaying delayed onset and the least severe disease

compared with groups 2 to 4 (Fig. 2A). Conjunctival chemosis began on day 2 in all groups, and peaked at day 6 in group 1, with a mean score of 2.9; day 8 in group 2, with a mean score of 3.4; and day 7 in groups 3 and 4, with mean scores of 3.8 and 3.9, respectively (Fig. 2B). Group 1 experienced significantly milder chemosis than group 4 throughout the study and group 3 for all days except days 2, 6, 8, and 9 ($P < 0.05$, ANOVA on ranks). The mean conjunctival congestion score for group 1 was 2.1 to 2.2 from days 5 through 9, whereas groups 2, 3, and 4 peaked on day 8 with scores of 2.4, 2.8, and 2.9, respectively (Fig. 2C). All four groups reached a peak score for conjunctival discharge of 3. However, peak scores for group 1 were delayed until day 6 compared with day 4 for the other groups (Fig. 2D). Anisocoria developed in the animals in a dose-dependent manner, as well; all group 4 animals had anisocoria by day 2, whereas 100% of the animals in groups 2 and 3 had anisocoria on day 3. Anisocoria in 100% of group 1 animals was not observed until day 7 (data not shown).

The progression of keratitis, the onset of which was defined as a corneal opacity score of 1.0, in representative rabbits from each group is shown in Figure 3. The initial slit lamp findings were multiple faint gray punctuate foci of decreased translucency within the epithelium adjacent to the trephine wound (Fig. 4A). The gray foci did not prevent the passage of light but evidenced diminished optical clarity best seen by alternating indirect and retroillumination. With the progression of keratitis, the punctuate lesions enlarged, coalesced, and extended laterally away from the trephination. Epithelial lesions continued to spread away from the trephine wound, coincident with frank epithelial loss (positive fluorescein staining; Fig. 4B). At day 6, multiple lesions, often bifurcating, were evident with retroillumination (Fig. 4C).

Histology

Paraformaldehyde-fixed sections of both infected and uninfected eyes were stained with hematoxylin and eosin and examined for evidence of inflammation in the eyelids, the palpebral conjunctiva, the bulbar conjunctiva, the meibomian

FIGURE 3. Progression of vaccinia keratitis. The progression of disease was recorded with digital photography. Light images (**A, C, E, G**) were taken with a single-lens reflex digital camera and 105-mm 1:2.8-D lens fitted with a ring flash and 52-mm filter. Images of fluorescein staining (**B, D, F, H**) were taken with a single-lens reflex digital camera and 105-mm 1:2.8-D lens fitted with a flash system. Images shown are time courses of one representative rabbit per group over the course of the study. Note the accelerated course of disease in the 10^6 and 10^7 groups compared with lower inocula. Group 1 (1×10^4 pfu; **A, B**), group 2 (1×10^5 pfu; **C, D**), group 3 (1×10^6 pfu; **E, F**), and group 4 (1×10^7 pfu; **G, H**).

glands, and the cornea. Sections were scored according to the scale presented in Table 2. Higher doses of virus inoculum led to increased inflammation and ulceration and a decrease in the quality of the epithelium in all features examined (data not shown). Considered in aggregate, these data indicate that, with increasing virus inoculum, there is a more rapid onset of clinical signs, an increase of the severity of inflammation and corneal epithelial disruption, and a shorter period to reach the peak severity for each given clinical/histologic finding.

Viral Titers

Figure 5 shows the viral titers detected in corneal swabs on days 2, 3, 4, 6, 8, 10, 12, and 14 of the study. Virus was detected in all

groups on day 2 of the study. Group 1 displayed a lower titer of 5×10^2 on day 2 (~ 2 logs lower) compared with that in group 4, but this difference was not significant ($P > 0.05$ ANOVA on ranks). Group 2 displayed an intermediate titer of 10^4 . Viral titers in groups 2, 3, and 4 peaked at 10^7 pfu/mL on day 3, while group 1 titers peaked at 10^7 pfu/mL on day 6. Peak viral titers were not significantly different between any of the groups ($P > 0.05$ ANOVA on ranks). Virus did not clear in any of the groups by day 14.

Virus Localization

Immunohistochemistry was used to determine whether viral antigens were present in infected and noninfected (control)

FIGURE 4. Slit lamp biomicroscopy findings. Typical slit lamp biomicroscopy findings during infection with 1×10^5 pfu of vaccinia virus are shown. (Ai) The earliest findings were an epithelial keratitis characterized by a graying of the epithelium. In many rabbits very fine grayish oval nodules were seen at the trephine wound with a decreasing gradient of haze extending out from this point (*inset*). On retroillumination the epithelium adjacent to the trephine wound could appear irregular and wavy. (Aii) Fluorescein staining (*green*) was limited to small foci over the trephine wound. (Aiii) Fine slit view. Changes were limited to the epithelium. The trephine cut was evident in the anterior stroma. (B) The epithelial irregularities were multifocal and extended 1 to 2 mm beyond the trephine wound. The individual opacities had a rice grain shape (*inset*). Some of the adjacent opacities gave the appearance of a chain of pearls. (Ci) Multiple lesions, often bifurcating, and epithelial irregularities were present. Lesions were most evident on retroillumination. The epithelial changes were located away from the trephine wound, although in some animals discrete

FIGURE 5. Viral titers. Corneal swabs of infected eyes were taken on the indicated days and stored at -80°C . Samples were then titrated by plaque assay on HeLa cells. Points represent the mean of 10 animals per group titrated in duplicate. Group 1 (1×10^4 pfu; ●), group 2 (1×10^5 ; ○), group 3 (1×10^6 pfu; ▼), and group 4 (1×10^7 pfu; ▽).

eyes at day 14 of the study. The results are shown in Table 3. The corneas and eyelids of all infected samples in all four groups tested positive for viral antigen which was consistent with the viral titers. Occasional foci of staining were observed in the trabecular meshwork (angle), iris, ciliary body, retina, and choroid. Staining was localized to focal regions that displayed viral cytopathic effect and apparent immune cell invasion. There was a trend toward more foci of infection and more intense staining in the cornea and eyelids as the inoculum increased, which was consistent with the increase in corneal epithelial disruption observed at higher inocula. Occasional staining in the corneas and eyelids of noninfected eyes was observed in all groups, but was usually restricted to a single focus in the entire tissue and did not appear to correlate with the amount of inoculum, most likely because of autoinoculation by the animals. These results indicate that viral antigen can be consistently detected in infected corneas and eyelids at 14 days after infection, and that, although the virus is largely restricted to these structures, it can spread to other structures and to the uninfected eye.

Serology

The rabbits were screened for the presence of anti-VACV antibodies preinfection on days 7 and 14. No rabbits were positive for anti-VACV antibodies before infection. On day 7, one animal each in groups 1 and 2, four animals in group 3, and two animals in group 4 had detectable levels of VACV-specific antibodies. By day 14, VACV-specific antibodies were detected in all animals at comparable titers (Fig. 6). These data indicate that rabbits produce a virus-specific antibody response to corneal challenge with VACV that was independent of viral inoculum at 1×10^4 pfu or higher.

Body Weight

Mean body weights for each group decreased through day 8 with decreases of 2.6%, 5.2%, 4.9%, and 7.2% from baseline

epithelial foci persisted over the trephine wound (*inset*). (Cii) Fluorescein staining (*green*) extended outward from the trephine area in some animals. The fluorescein positive zone was surrounded by epithelium with altered translucency on retroillumination. T, trephine wound; E, corneal epithelium; S, corneal stroma.

TABLE 3. Immunohistochemistry Results*

Group	Eye	Cornea	Eyelids	Angle	Iris	Ciliary Body	Lens	Retina	Choroid
1	Infected	100	100	10	30	20	0	10	10
	Control	10†	50	0	0	10	0	0	0
2	Infected	100	100	30	20	10	0	10	10
	Control	20	40	0	0	0	0	0	0
3	Infected	100	100	0	10	0	0	0	0
	Control	0	60	0	0	0	0	0	0
4	Infected	100	100	10	0	20	0	0	10
	Control	20†	60	0	0	10	0	0	0

Data are the percentage of samples positive for vaccinia virus antigen. Slides were viewed at $\times 20$ magnification.

* All groups had eight animals, thus eight infected and eight noninoculated eyes were examined.

† Cornea absent from one section.

weights for groups 1, 2, 3, and 4, respectively. This corresponded to a decrease in food and water consumption beginning on day 2, as well as decreased fecal and urine output. By day 11, increases in body weight were noted only in group 2 relative to day 1, whereas decreases were still 0.9%, 1.4%, and 2.6% from day 1 in groups 1, 3, and 4, respectively (data not shown).

Extraocular Lesions

On day 9, an ulcerated lip lesion was noted on one group 1 animal. Lip lesions were also noted on one additional group 1 animal, one group 2 animal, and two group 4 animals during gross examination at necropsy (data not shown). The lips are a common site of secondary poxvirus lesions in rabbits,²⁴ most likely due to grooming activity.

DISCUSSION

To date, most of the studies of vaccinia keratitis have focused on testing possible therapeutic regimens for efficacy. These studies have relied primarily on epithelial disruption as an

indicator of keratitis severity.^{14,19–21,23} More persistent damage from vaccinia keratitis, such as severe corneal opacification, the percentage of the cornea exhibiting cloudiness, corneal vascularization, and conjunctivitis (chemosis and congestion) have largely been ignored. In the few studies in which corneal opacification or vascularization was recorded, the data were reported qualitatively as either presence or absence, with the duration of the signs, but disease severity was unreported.^{14,19} A few of these studies also determined viral titers in infected eyes.^{19,21,23} Proper evaluation of therapeutics and mechanisms of pathogenesis require quantitative analyses. We describe a quantitative model for VACV ocular infection that includes the severity and duration of corneal epithelial disruption, corneal opacification, the percentage of the cornea exhibiting opacification, corneal vascularization, viral titers, and the antibody response to infection, as well as secondary signs such as corneal thickness, ocular discharge, conjunctival chemosis, and congestion. The results show that the course of disease in rabbits closely parallels that in humans.¹² This comprehensive analysis of the course of vaccinia keratitis in rabbits provides the context necessary for a more

FIGURE 6. Antibody responses. The box-and-whisker plots represent the reciprocal range of VACV-specific antibodies present in each test group. The central box represents the median titer, the box boundaries represent the 25th and 75th percentiles, and the whiskers represent the 10th and 90th percentiles. Blood was collected once before infection (day 0) and on days 7 and 14. Antibodies against whole virus particles were detected with horseradish peroxidase-conjugated secondary antibody and *o*-phenylenediamine dihydrochloride substrate. Samples were considered positive if the absorbance values were more than 3 SD greater than on day 0. (A) Group 1 (1×10^4 pfu); (B) group 2 (1×10^5 pfu); (C) group 3 (1×10^6 pfu); and (D) group 4 (1×10^7 pfu).

thorough analysis of both the pathogenesis of vaccinia keratitis and the evaluation of therapeutic interventions.

Four criteria were used for the determination of the optimal amount of virus: (1) keratitis scores should be in the midrange of the scale (e.g., 3.0), (2) the onset of keratitis should occur within 48 hours of infection, (3) keratitis should develop in 90% to 100% of the animals, and (4) chemosis and congestion should not interfere with scoring corneal disease. Based on the data, we determined that a virus inoculum of 1×10^5 pfu met the criteria and is the optimal amount of virus for studying the pathogenesis and therapy of vaccinia keratitis in rabbits. Corneal opacity and epithelial damage both peaked near the middle of the scoring system (2.9 and 3.3, respectively; Figs. 1A, 1B), allowing for the analysis of treatments that either exacerbated or ameliorated disease. Disease developed within 48 hours of infection in all animals, which would reduce variability in the response to therapy in drug studies. Finally, conjunctival chemosis in most animals infected with 1×10^5 pfu of virus was not severe enough to interfere with scoring. In addition, in the 1×10^5 group, keratitis developed in 90% to 100% of the rabbits. In the 1×10^4 group, the onset of keratitis was significantly delayed, whereas at viral inocula higher than 1×10^5 conjunctival chemosis was severe enough to interfere with the scoring of corneal disease. Based on our evaluation as 1×10^5 pfu as optimal for inducing vaccinia keratitis, we have illustrated the key slit lamp findings throughout the progression of keratitis using this inoculum (Fig. 4).

Viral keratitis can be caused by adenoviruses, arboviruses, herpesviruses, morbilliviruses, myxoviruses, paramyxoviruses, and poxviruses.^{26–32} Herpes simplex virus keratitis (HSV keratitis) is the leading cause of infectious blindness in developed countries,³² and the clinical presentation of vaccinia keratitis and HSV keratitis are very similar^{12,22,33,34} but there are differences. In HSV, stromal disease and blindness are usually the result of repeated reactivation events from latency, whereas vaccinia virus keratitis is an acute infection.³⁵

The humoral immune response was not affected by differences in the amount of virus inoculum. Virus-specific antibodies reached detectable levels in all animals by day 14, which is consistent with previous reports.¹⁷ The titer of the antibody response was also comparable between groups, which suggests that the immune response was similar. Several studies have shown that the development of virus-specific antibodies is important for controlling primary acute poxvirus infections.^{36–38} Antibodies are also important for long-term protection against poxvirus infections, and titers higher than 1:32 have been shown to provide resistance to smallpox infection in humans.³⁹ The antibody titers in all four groups at day 14 were higher than 1:32.

In summary, we have demonstrated that vaccinia keratitis in rabbits has a clinical course that parallels that in humans and that the severity of the disease exhibits a dose response relative to the amount of the virus inoculum. Based on our results, an inoculum of 1×10^5 pfu is optimal for use in future studies of vaccinia keratitis in rabbits. The results of this study validate the use of the rabbit as a model for vaccinia keratitis and provide a more complete and quantitative context for studying the disease pathogenesis and evaluating therapeutic interventions.

Acknowledgments

The authors thank Janice Lokken for tissue preparation, sectioning, and staining; Monica Sauter for the endotoxin analysis; and the numerous undergraduates who assisted with animal husbandry, drug administration, and other aspects of the work.

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