Kinetics of Immune Cell Infiltration in Vaccinia Virus Keratitis

Sharon Altmann,¹ Megan Toomey,² Brittany Nesbit,³ Kim McIntyre,³ Jill Covert,^{3,4} Richard Redd Dubielzig,^{3,5} Gary Leatherberry,³ Elizabeth Adkins,^{3,5} Christopher J. Murphy,^{3,4,5,6} and Curtis R. Brandt^{1,2,3,5}

PURPOSE. Vaccinia virus keratitis leading to blindness is a severe complication of smallpox vaccination. The clinical manifestations of vaccinia virus keratitis are similar to those of herpes simplex virus keratitis, a well-studied immunopathologic disease. Vaccinia virus keratitis is likely to involve an immunopathologic component, but little is known about the pathogenesis of the disease. The goal of this study was to determine type and kinetics of immune cell infiltration in the cornea during vaccinia virus keratitis.

METHODS. Rabbit eyes were trephined and inoculated with 1×10^5 pfu of the Dryvax strain of the vaccinia virus. On days 2, 4, 7, 10, 14, and 28 after infection, the animals were scored for clinical disease and eye sections were stained for B cells, CD4⁺ cells, CD8⁺ cells, and neutrophils. The eyelid, ciliary body, cornea, iris, iridocorneal angle, and choroid were examined.

RESULTS. Corneal vaccinia virus challenge resulted in the infiltration of B cells, CD4⁺ cells, CD8⁺ cells, and neutrophils into the cornea and eyelids. Neutrophils were the predominant cell type on days 2 and 3 after infection, whereas CD4⁺ cells were the predominant cell type detected in corneas on days 4 through 10. CD8⁺ cells and B cells peaked on day 10, but at lower levels than CD4⁺ cells and neutrophils.

CONCLUSIONS. These results suggest that sequential migration of neutrophils, then CD4⁺ cells, plays an important role in vaccinia virus keratitis. (*Invest Ophthalmol Vis Sci.* 2010;51:4541–4548) DOI:10.1167/iops.09-5107

Vaccinia virus (VACV) ocular infections are common adverse reactions to smallpox vaccinations and may occur in as many as 1 to 4 recipients per 40,000 vaccinees.^{1,2} Ocular manifestations include blepharitis (chemosis), conjunctivitis, iritis, and keratitis. Corneal involvement, which occurs in 6% to

30% of ocular VACV cases, is the most serious complication and can result in vision loss.^{3,4} Disease severity can range from mild punctate superficial keratitis to blinding stromal opacification and corneal perforation.³ Corneal epithelial disruption occurs early on in the course of disease, and corneal vascularization is common. Since the cessation of civilian vaccination programs in the United States in 1971⁵ and the formal declaration of the eradication of smallpox in 1980,⁶ little work has been done to characterize the pathologic course of Vaccinia virus keratitis (VACVK). Vaccination of military personnel and first responders resumed in 2001 as a result of the potential threat of a deliberate release of smallpox, leading to a renewed interest in the course of this disease.

The clinical presentation of VACVK is similar to that of herpes simplex virus type I keratitis (HSVK).^{3,7} In HSVK, an immunopathologic disease, several cell types have been implicated. Neutrophils infiltrate the cornea by 48 hours after infection⁸ and have been shown to limit virus replication early in infection,^{9,10} but may exacerbate HSVK after virus has been cleared.^{9,11} Infiltration of the cornea by CD4⁺ and CD8⁺ cells occurs by day 4 after infection.⁸ Depletion studies have shown that CD4⁺ cells are the principal mediators of HSVK.^{12–15} The role of CD8⁺ cells in the immunopathology of HSVK is uncertain. Some evidence suggests that CD8⁺ cells help clear virus from the eyes without contributing to lesion formation^{16,17} and may limit immunopathogenic disease.¹⁸ Others report that CD8⁺ cells can mediate mild HSVK in the absence of CD4⁺ cells.¹⁹

B cells infiltrate the cornea by day 14 after infection,²⁰ but their contribution to HSVK is uncertain. Although one group has reported that BALB/c mice depleted of B cells exhibited reduced mortality, reduced virus shedding, and delayed HSVK onset,²¹ others have reported prolonged viral persistence, increased susceptibility to HSVK, and death in BALB/c mice deficient for B-cell production.²² The reason for the discrepancies between the reports is unclear, but may be related to differences in virus strains. Passive immunization of mice either before or after HSV infection, particularly with antibodies raised against glycoproteins B and D, reduced stromal but not epithelial keratitis.^{23–25} These reports indicate that a virus-specific antibody response plays an important role in controlling HSVK.

The immune cell response to corneal VACV challenge, however, is poorly characterized. The similarities between the clinical presentations of VACVK and HSVK suggest that neutrophils, CD4⁺ cells, CD8⁺ cells, and B cells may also be involved in the pathogenesis of VACVK. The goal of this study was to characterize the infiltration of neutrophils, CD4⁺ cells, CD8⁺ cells, and B cells into the eye in response to corneal VACV challenge.

From the Departments of ¹Medical Microbiology and Immunology, ²Ophthalmology and Visual Sciences, and ⁵Surgical Sciences, and the ³Comparative Ophthalmic Research Laboratories, University of Wisconsin-Madison, Madison, Wisconsin; and the ⁴Department of Ophthalmology and Vision Sciences, School of Medicine, and the ⁶Department of Surgical and Radiological Sciences, School of Veterinary Medicine, University of California, Davis, Davis, California.

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Corresponding author: Curtis R. Brandt, Department of Ophthalmology and Visual Sciences, 6630 Medical Sciences Center, 1300 University Avenue, Madison, WI 53706; crbrandt@wisc.edu.

MATERIALS AND METHODS

Cells and Viruses

Vero cells (American Type Culture Collection, Manassas, VA [ATCC], CCL-81) 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). High titer viral stocks were prepared as described previously.²⁶ Briefly, Vero cells were infected with the New York City Department of Health Laboratories strain of VACV (ATCC, VR-1536) at an 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 then frozen and thawed three times, 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 a rotor (model SW28; Beckman, Fullerton, CA). The pelleted virus was then titered on HeLa cells, resuspended in H-HBSS at a concentration of 10^9 pfu/mL, and stored in 100- μ L aliquots at -80°C until use. Endotoxin levels in the virus preparations was determined (ToxinSensor Chromogenic LAL Endotoxin Assay Kit; cat. no. L00350; GenScript, Piscataway, NJ). The concentration was 0.0159 endotoxin units (EU) per mL (0.08 ng/mL). The inoculum size was 50 μ L; each eye received 0.004 ng of endotoxin.

Animals

Female rabbits Hra: [(NZW)SPF] were obtained from Harlan (Indianapolis, IN). 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

On day 1 of the study, the 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 trephinated to a depth of approximately 0.25 mm with a 7.5-mm diameter Hessburg-Barron vacuum trephine (Jedmed, St. Louis, MO). After trephination, 20 μ L of virus in H-HBSS (10^5 pfu/eye) was placed in the trephine wound. Ocular disease was scored according to a modification of the MacDonald-Shadduck Scoring System, as described in the accompanying paper in this issue,²⁷ on days 2, 4, 7, 10, 14, and 28 of the study. Briefly, corneal opacity was scored from 0 to 6, with 6 representing corneal perforation. The percentage of opacity was scored 0 to 4 with 4 representing 75% and 100% of the cornea involved. Fluorescence staining of the epithelium was scored 0 to 4 based on quartile percentages of the surface. Vascularization was scored 0 to 2, with 2 representing extension into the cornea by 2 mm or more around the entire circumference. Chemosis of the conjunctiva was scored from 0 to 4, with 4 being eversion of the eyelids. Conjunctival congestion was scored from 0 to 3 with 3 representing a dark red color. Finally, conjunctival discharge was scored 0 to 3 with 3 representing wetting of the fur around the eye. Slit lamp evaluations were performed with a handheld biomicroscope (model SL-15; Kowa Co., Ltd., Tokyo, Japan) at high (15 $\times$) magnification. Proparacaine hydrochloride sterile ophthalmic solution, USP (Akorn, Inc. Buffalo Grove, IL) was placed in each eye, as needed for evaluation. Two animals were euthanatized on days 2, 14, and 28, and three were euthanatized on days 4, 7, and 10, and their eyes were enucleated for analysis.

Virus Isolation

Corneal swabs were collected from the right eye immediately before ocular examination. The samples were stored in HBSS at -80°C until

FIGURE 1. Disease scores. On the days they were euthanatized, the animals were scored for disease with a modified MacDonald-Shadduck scoring system.²⁷ (A) Corneal disease. (B) Conjunctival disease. (C) Viral titers. Corneal swabs were taken from the animals before examination and titered in duplicate in HeLa cells.

they were titrated. Titers were acquired by serial dilution and plaque assay on HeLa cells.

Immunofluorescent Microscopy

Infected and control eyes were fixed in 4% paraformaldehyde and then embedded in paraffin. Sections from one (day 2, 7, and 28) or two (day 4, 10, and 14) rabbits per day were deparaffinized in xylene and then rehydrated in graded ethanol. The sections were incubated with 0.05% trypsin and 0.1% CaCl_2 in water for 13 minutes at 37°C and 10 minutes at room temperature, then rinsed 3×10 minutes in 100 mM glycine and 2×2 minutes in PBS before being blocked for 20 minutes at room temperature with 10% FBS in PBS. The sections were stained for 1 hour at room temperature with a 1:100 dilution of mouse αCD79a (NB 600-557; Novus Biologicals, Littleton, CO), mouse αCD4 (MA1-81754; Affinity BioReagents, Golden, CO), mouse αCD8 (MA1-82,699; Affinity BioReagents), or mouse α neutrophil defensin 5 (HM4008; Hycult Biotechnology, Uden, The Netherlands) in blocking buffer. The sections were rinsed twice in PBS, stained for 30 minutes with a 1:200 dilution of AlexaFluor 594 goat α -mouse IgG (A11032; Invitrogen, Carlsbad, CA), rinsed, and mounted (Vectamount, H-5000; Vector Laboratories, Burlingame, CA). Control slides were prepared as just described from infected day-10 eyes, and were stained with primary or

secondary antibodies only. The sections were viewed at $40\times$ magnification with a fluorescence microscope (Axioplan 2; Carl Zeiss Meditec, Göttingen, Germany), and the number of positive cells was counted in five fields of view per structure. Images were also taken at $20\times$ magnification, to demonstrate cell distribution throughout the tissue.

RESULTS

Clinical Disease

Infected eyes were examined on days 2, 4, 7, 10, 14, and 28 after infection and scored for clinical disease. Corneal opacity was first detected on day 2 after infection in all animals, peaked at a score of 2 on day 7, and remained at that level throughout the study (Fig. 1A). The percentage area of cornea that was opacified reached 75% to 100% on day 7 and remained at that level for the remainder of the study (Fig. 1A). Epithelial damage, as detected by fluorescein staining, reached a maximum score of 2 on day 7 (Fig. 1A). Corneal vascularization was first detected on day 10 in all animals and involved the entire perimeter of the cornea by day 14.

FIGURE 2. Neutrophil kinetics. Ocular sections from control and infected eyes were stained for neutrophil defensin 5. Five images per ocular structure were taken at $40\times$ magnification and the number of positive cells present was counted. Data for one representative rabbit are shown. (A) Control eye. (B) Infected eye. (C) Fluorescent imaging on day 10. Arrows: representative positive cells. E, epithelial surface of the cornea. Bar, 50 μm .

Conjunctival chemosis peaked on day 7 after infection (score, 3.7) and decreased to mild swelling (score, 1.5) by days 14 and 28 (Fig. 1B). Conjunctival congestion and discharge both plateaued on days 3 through 10 (scores of 3 for both) before beginning to heal (Fig. 1B). Virus titers in infected eyes peaked on day 4 after infection at 1×10^7 pfu/mL and had nearly cleared by day 15 after infection (Fig. 1C). No infectious virus was detected at day 28.

Neutrophil Infiltration

Infiltrating immune cells were not uniformly distributed through the ocular tissues. As such, the total number of positive cells detected in two (iridocorneal angle) or five (all other structures) random microscope fields per tissue are reported herein. Quantitation of neutrophil infiltration on days 2, 4, 7, 10, 14, and 28 after infection in various tissues of a representative rabbit is shown in Figures 2A and 2B. Representative examples are shown in Figure 2C. Neutrophils were detectable at low levels in control eyelids during the study, with a maximum of 27 cells detected on day 2 (Fig. 2A). In contrast, in infected eyelids, neutrophil infiltration peaked at 169 cells

detected on day 4 (Fig. 2B). The peak number of neutrophils was detected in the infected corneas on day 10 (81 cells). Neutrophils were also detected in the iridocorneal angle of infected eyes on days 7 (29 cells) and 10 (42 cells) after infection (Fig. 2B). Neutrophils were found primarily in the epithelial layer of the eyelids, both in control and infected eyes. In infected corneas, neutrophils localized more toward the epithelial than the endothelial surface. Peak neutrophil infiltration of the cornea and eyelids on day 10 was confirmed in all rabbits, although infiltration of the iridocorneal angle was not detected in one animal (data not shown).

CD4⁺ Cell Infiltration

CD4⁺ cells were present at minimal levels in control eyes, with a maximum of 0.2 cells/field detected in corneas (Fig. 3A). In infected eyes, CD4⁺ cells were first detected in the corneas on day 4 after infection, with a peak of 153 cells detected by day 7. Cell counts dropped to 37 cells detected by day 14 and were not detected on day 28 (Fig. 3B). CD4⁺ cells were detected in the eyelids and iris on day 10 (76 and 23 cells, respectively) and in the iridocorneal angle, beginning on day 4 and peaking on day

FIGURE 3. CD4⁺ cell kinetics. Ocular sections from control and infected eyes were stained for CD4. Five images per ocular structure were taken at 40 $\times$ magnification and the number of positive cells present was counted. Data from one representative rabbit are shown. (A) Control eye. (B) Infected eye. (C) Fluorescent imaging on day 10. Arrows: some of the positive cells. E, epithelial surface of the cornea. Bar, 50 μ m.

10 (92 cells; Fig. 3B). On day 10, CD4⁺ cells were distributed throughout the stroma of both the eyelid and cornea (Fig. 3C).

CD4⁺ cell infiltration of the cornea followed a similar timeline in a second rabbit, whereas infiltration of the eyelid was detected on all days examined (4, 10, and 14) with peak infiltration on day 14 (data not shown). Limited infiltration of the iridocorneal angle was observed in one rabbit.

CD8⁺ Cell Infiltration

Few, if any, CD8⁺ cells were present in control eyes during the study (Fig. 4A). In infected corneas, CD8⁺ cells peaked with 81 total cells detectable on day 10 (Fig. 4B). CD8⁺ cell infiltration of the infected eyelid oscillated between 12 and 46 cells detected from days 2 through 14, peaking at 46 cells on day 4. CD8⁺ cells were also detected in the angle on day 10 (15 cells; Fig. 4B). On day 10, CD8⁺ cells were found in the stroma of the eyelid, throughout the iridocorneal angle, and, primarily, localized near the trephination wounds in the cornea (Fig. 4C). Peak CD8⁺ cell infiltration of the cornea and the iridocorneal angle on day 10 was confirmed in all rabbits. Infiltration of the eyelids remained between one and three cells per field of view on days 4, 10, and 14.

B-Cell Infiltration

Few, if any, B cells were detected in control eyes during the experiment (Fig. 5A). In infected eyes, B cells appeared in the cornea beginning on day 4 after infection and peaked with 115 cells detected on day 10. Some infiltration was also detected in the eyelids on days 2, 4, and 7 after infection, with a peak of 22 cells detected on day 4 (Fig. 5B). B cells localized to the stroma of eyelids and closer to the epithelial surface of the cornea than to the endothelial surface (Fig. 5C).

Infiltration of the Retina

Because of a high level of autofluorescence, we were unable to observe infiltration by neutrophils, CD4⁺ cells, CD8⁺ cells, and B cells into the retina.

DISCUSSION

Vaccinia virus keratitis is a serious complication of smallpox vaccination than can result in vision loss. Clinically, the disease has similarities to HSVK but little is known about pathogenic mechanisms in vaccinia keratitis. We present the first charac-

FIGURE 4. CD8⁺ cell kinetics. Ocular sections from control and infected eyes were stained for CD8. Five images per ocular structure were taken at 40 $\times$ magnification and the number of positive cells present was counted. Data from one representative rabbit are shown. (A) Control eye. (B) Infected eye. (C) Fluorescent imaging on day 10. Arrows: some of the positive cells. E, epithelial surface of the cornea. Bar, 50 μ m.

FIGURE 5. B-cell kinetics. Ocular sections from control and infected eyes were stained for CD79a. Five images per ocular structure were taken at 40 $\times$ magnification and the number of positive cells present was counted. Data from one representative rabbit are shown. (A) Control eye. (B) Infected eye. (C) Fluorescent imaging on day 10. Arrows: some of the positive cells. E, epithelial surface of the cornea. Bar, 50 μ m.

terization of the kinetics of immune cell infiltration into the cornea and other ocular structures in response to corneal VACV infection. Neutrophils were the first cell type to be detected in infected corneas, with 20 cells detected on day 2 after infection, and were the only cell type detected in infected corneas on day 28 (Fig. 2B). This early infiltration occurred concurrently with the onset of conjunctival disease (Fig. 1B). The primary cell type identified in infected corneas on days 4 through 10 were CD4⁺ cells, which peaked on day 7 at 153 cells detected. Virus titers also peaked between days 4 and 7 and were largely undetectable by day 14 (Fig. 1C). Neutrophils, CD8⁺ and B cells exhibited peak infiltration of infected corneas on day 10 (81, 81, and 115 cells per field of view, respectively).

The infiltration of neutrophils into both the cornea and the eyelids was concurrent with the onset of corneal and conjunctival disease (Figs. 1A, 1B), suggesting that neutrophils may contribute to the early stages of ocular disease. Neutrophils and other leukocytes have been shown to be among the earliest and most prevalent cell types to respond to intradermal, but not intranasal, VACV challenge with peak infiltration on day 10.^{28,29} How these cells contribute to the clearance of VACV in

vivo or to the pathologic course of the disease is unknown, although in HSVK neutrophils have been shown to limit virus replication early in infection.^{9,10} Neutrophils are among the earliest cells to infiltrate corneas during human adenovirus 37 keratitis in mice,³⁰ suggesting that neutrophils may play important roles in the pathologic course of keratitis, caused by several unrelated viruses.

CD4⁺ cell infiltration of the cornea peaked on day 7 (Fig. 3B). Previous work in other laboratories has shown that CD4⁺ cells are essential for the development of a VACV-specific antibody response.³¹ This CD4⁺ T-cell-dependent antibody response has been shown to be necessary for clearing VACV in intraperitoneally infected mice.³¹ Mice depleted of either CD4⁺ T cells or B cells also failed to control virus replication.³¹ As B cells were also found in the infected corneas on days 4 and 7 (Fig. 5B), it seems likely that the CD4⁺ cells and perhaps B cells contribute to viral clearance during VACVK. CD8⁺ cells, which are not believed to contribute to VACV clearance during systemic infections,³¹ were not detected in infected corneas until day 7 (Fig. 4B). However, as the viral titer dropped three logs between days 7 and 10 (Fig. 1C), it is possible that CD8⁺ cells contribute to viral clearance during ocular VACV infec-

tions. Both CD4⁺ and CD8⁺ cells are capable of generating a VACV-specific CTL response.^{31,32} However, CD8⁺ cells have been reported to account for most of the CTL activity and most of the IFN- γ produced in response to primary vaccination with VACV.^{31,33}

Corneal vascularization was first detected on day 10 after infection (Fig. 1A), the same day that infiltration by neutrophils, CD8⁺ cells, and B cells peaked, suggesting that some combination of those cell types may have contributed to the onset of angiogenesis.

The kinetics of immune cell infiltration in response to VACVK shared several similarities to what has been reported for HSVK. Neutrophils are the first cells to respond to HSV challenge and are believed to restrict viral replication early on in infection.^{8–10} Depletion of circulating neutrophils from rabbits resulted in significant reduction in damage to the cornea stroma during HSV infection, suggesting that neutrophils play a significant role in the pathogenesis of HSVK.¹¹ During VACVK, the onset of corneal and conjunctival disease occurred concurrently with neutrophil infiltration into the cornea and eyelids. This result suggests that neutrophils play a similar role in early tissue damage during VACVK, as they do in HSVK.

During HSVK, neutrophils display a biphasic infiltration pattern.^{9,34} The first infiltration into the corneal stroma occurs in response to cytokine release by epithelial cells, which peaks within 24 hours of infection in immunologically competent mice,^{8,9} whereas the second infiltration requires virus replication³⁵ and the presence of CD4⁺ T cells.⁹ This biphasic pattern was not seen in response to VACV challenge (Fig. 5B). Our data showed that VACV replication peaked on day 4 after infection, with at least 10³ pfu/mL of infectious virus still detectable as late as day 10 after infection. In contrast to this finding, during HSVK, virus is largely cleared by day 7.¹⁰ Since VACV actively replicates longer in the cornea than does HSV, it is possible that the wave of neutrophils infiltrating the cornea in response to VACV replication overlaps the wave that infiltrates in response to T-cell signaling.

CD4⁺ cells are the principle mediators of HSVK^{12,13,15} and have a peak presence in the cornea between days 5 and 7 after infection.⁸ In HSVK, viral clearance during the acute phase of infection is believed to be mediated by CD8⁺ cells,^{16,17} which infiltrate the cornea with approximately the same kinetics as do CD4⁺ cells.⁸ During VACV infection, CD8⁺ cells were not detected until day 7, 3 days after CD4⁺ cells were detected (Fig. 4B versus Fig. 3B). As CD8⁺ cells appeared after the onset of viral clearance (Fig. 1C), it is unclear what role the cells play in VACV clearance from the cornea.

In summary, we have shown that corneal challenge with VACV results in the infiltration of neutrophils, CD4⁺ cells, CD8⁺ cells, and B cells into the cornea and eyelids. The kinetics of cellular infiltration are similar, but not identical with, infiltration during HSVK, suggesting that the different cell types play different roles in VACVK. Further, studies are needed to define the roles of the different cell types in the pathologic course and resolution of VACVK.

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