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Herpes simplex virus type 1 ICP0 localizes in the stromal layer of infected rabbit corneas and resides predominantly in the cytoplasm and/or perinuclear region of rabbit keratocytes

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Abstract: Herpes stromal keratitis (HSK) results from the reactivation of herpes simplex virus type-1 (HSV-1) in the cornea. The subsequent corneal inflammation and neovascularization may lead to scarring and visual loss. The cellular and molecular mechanisms underlying HSK remain unknown. The presence of stromal HSV-1 viral proteins or antigens in the HSK cornea remains a subject of debate. It was recently reported that HSV-1 ICP0 rapidly diffuses out of infected rabbit corneas. To investigate further the presence of HSV-1 ICP0 in the infected cornea, particularly in the corneal stroma, *ex vivo* confocal microscopy was used to scan rabbit corneas infected with the virus ICP0–EYFP, an HSV-1 derivative (strain 17+) that expresses ICP0 fused to the enhanced yellow fluorescent protein (EYFP). These results demonstrate that ICP0 is expressed in the corneal epithelium and stromal cells (keratocytes) of infected rabbit corneas throughout acute infection. Furthermore, expression of ICP0–EYFP appears localized to punctate, granular deposits within stromal keratocytes, showing both a cytoplasmic and perinuclear localization. These findings provide new data demonstrating that anterior corneal keratocytes become infected and express ICP0 during acute HSV-1 infection.

Introduction

Herpes simplex virus type-1 (HSV-1) is an ancient virus infecting a large percentage of humans (Cumston, 1926; Wildy, 1973). A wide range of clinical manifestations occur as a result of HSV-1 infections, from mild uncomplicated mucocutaneous infections to life-threatening encephalitis (Whitley & Roizman, 2001). Over 80 % of the population is sero-positive for HSV-1 (Nahmias *et al.*, 1990). The route and time of viral transmission are not clear but they appear to be associated with close human contact at a very young age (Roizman & Whitley, 2001).

During the acute infection, HSV-1 travels by retrograde axonal transport to the sensory ganglia where brief virus replication occurs. Subsequently, one of two mutually exclusive scenarios can take place, either neuronal destruc-

tion results from virus replication or neuronal survival from which viral latency is established. The latent virus stays in the individual for life (Stevens & Cook, 1971) and the virus reactivates as the result of external stimuli, such as emotional stress, tissue damage, systemic and/or topical steroid therapy, and UV exposure (Whitley, 2001). Reactivated virus travels down the sensory nerve axon and spreads to and infects the peripheral mucosal epithelium and, in some cases, the eye. In some individuals reactivation causes a recurrent manifestation of infection and the development of herpes stromal keratitis (HSK). Generally, the pattern of clinical disease resulting from primary or recurrent HSV-1 infection is mild or asymptomatic. When clinical symptoms do occur, they range from the mild but common fever blister on the lip to more severe eye infections resulting in visual loss, and to devas-

tating but rare infections of the central nervous system (Roizman & Sears, 1996).

Ocular infections with HSV-1 can induce HSK that is clinically apparent in only 1–6 % of patients (Pavan-Langston, 2000) and most episodes of HSK are associated with reactivation of HSV-1 infection (Shimeld *et al.*, 2001). The recurrence is shown as the epithelial keratitis with typical dendritic or geographical ulcers. The other manifestation of recurrence of herpetic disease is immune stromal keratitis (Holland & Schwartz, 1999), which is believed to result from an immune response to the resident herpes virus antigens. Stromal keratitis is characterized by cellular infiltration and neovascularization; this rarely leads to necrotizing stromal keratitis, clinically showing necrosis, ulceration and severe cellular infiltration with epithelial defect. These clinical manifestations vary but affect the patient's vision. Indeed, herpetic keratitis is still the most common infectious cause of corneal opacity that leads to blindness in the developed world, in spite of the availability of anti-HSV drugs. The actual underlying mechanisms by which corneal scarring develops are unclear. It is assumed that viral antigens trigger an immunopathological response that leads to tissue damage in the corneal stroma (Deshpande *et al.*, 2004; Streilein *et al.*, 1997). Several mechanisms of immunopathology have been suggested. First, viral antigens and specific antibody complexes may be trapped in the stromal layer of the cornea and trigger inflammatory responses, in which complement and polymorphonuclear neutrophils (PMNs) play a pivotal role (Deshpande *et al.*, 2002, 2004; Streilein *et al.*, 1997). Second, molecular mimicry triggered by HSV-1, which induces antiviral immunity, may lead to immune responses against self-antigens or autoimmune responses (Avery *et al.*, 1995; Zhao *et al.*, 1998) leading to bystander effects (Deshpande *et al.*, 2001). Third, corneal latency in which viral DNA persists in corneas causing persistent infection has also been proposed (Cantin *et al.*, 1992; Polcicova *et al.*, 2005). Perhaps, it is difficult to detect viral antigens using antibodies in HSK corneas. Thus, it remains unclear which, if any, viral protein(s) is involved in HSK.

We recently demonstrated by Western blot analysis and immunocytochemistry that the HSV-1 immediate-early protein ICP0 rapidly diffuses out of infected rabbit corneas and that ICP0 could be diffusely localized to the anterior stroma of infected corneas (Naito *et al.*, 2005). To investigate further the presence of ICP0 in the anterior stroma of infected rabbit corneas, confocal microscopy images were taken from *ex vivo* rabbit corneas infected with an HSV-1 ICP0–EYFP, a previously described recombinant virus that expresses ICP0 as a fusion protein with enhanced yellow fluorescent protein (EYFP) (Everett *et al.*, 2003). Our data confirm that HSV-1 ICP0 is in the stromal layer of infected rabbit corneas during the course of acute infection. In addition, ICP0–EYFP is localized to punctate deposits within the cytoplasm and/or perinuclear region of rabbit corneal fibroblasts or keratocytes.

Methods

Cell culture. Normal rabbit eyes were purchased from Pel-Freez Biologicals. Primary rabbit corneal stromal cells (rabbit keratocytes, RKs) were isolated using collagenase digestion as reported previously (Jester *et al.*, 1996). Briefly, corneal epithelium was removed using a no. 10 surgical blade (Feather Safety Razor). After wiping with 70 % isopropyl alcohol, corneas were excised. The corneal endothelium was removed with a sterile cotton-tipped applicator. Corneas were digested in sterile 2.0 mg collagenase (Gibco Invitrogen) ml⁻¹ and 0.5 mg hyaluronidase (Worthington) ml⁻¹ in Dulbecco's modified Eagle medium (DMEM; Invitrogen) overnight at 37 °C. After digestion, RKs were plated in serum-free medium (RPMI vitamin mix and glutathione, non-essential amino acids, pyruvic acid, 1 % glutamine, 10 U penicillin, 10 µg streptomycin ml⁻¹ and 25 µg fungizone ml⁻¹ in DMEM) at 2.5×10⁴ cells per cm² onto 3 cm glass-bottomed dishes coated with 1.5 mg collagen (Cohesion; Invitrogen) ml⁻¹ for morphological observation or at 5.0×10⁴ cells per cm² onto 10 cm plates for Western blot analysis. RKs were also activated to corneal fibroblasts (RCF) by culturing with 10 % fetal bovine serum prior to evaluation. Rabbit skin (RS) cells were grown in Eagle's minimal essential medium supplemented with 5 % fetal bovine serum as described previously (Perng *et al.*, 1994).

Animals. Eight- to 10-week-old New Zealand white male rabbits (Irish Farms) were used. Rabbits were treated in accordance with Association for Research in Vision and Ophthalmology, American Association for Laboratory Animal Care and National Institute of Health guidelines.

HSV infection. HSV-1 ICP0–EYFP, a derivative of wild-type (wt) HSV-1 strain 17+, has been characterized previously (Everett *et al.*, 2003, 2004). wt HSV-1 McKrae and Glasgow strain 17+ were triple plaque purified, passaged only one or two times and titrated in RS cells prior to use (Perng *et al.*, 1994). RSs, RKs and RCFs in culture were infected with 0.1, 1.0 and 10.0 m.o.i. of ICP0–EYFP. Rabbits were bilaterally infected after scarification by placing, as eye drops, 2×10⁵ p.f.u. of virus into the conjunctival cul-de-sac, closing the eyes and rubbing the lid gently against the eye for 30 s as described previously (Perng *et al.*, 1994).

Tissue staining. Three rabbits were euthanized with pentobarbital (100 mg kg⁻¹) at indicated times and corneas fixed by anterior chamber perfusion with 2 % paraformaldehyde in PBS. Corneas were excised and observed under a fluorescent microscope to identify ICP0–EYFP-positive lesions. Corneas were then dissected into smaller corneal blocks (1–1.5 mm), washed in PBS and then further stained overnight at 4 °C with phalloidin (Alexa Flour 633 phalloidin; Molecular Probes) or 5 µm SYTO 59 (Molecular Probes) in 50 % TD buffer (0.5 % DMSO, 0.5 % Triton X, 2.5 % dextran 40 in PBS, pH 7.4) to identify actin cytoskeleton or nuclei, respectively. After staining, samples were mounted with 1 : 1 glycerol/PBS on coverslips.

ICP0, cytoskeleton and nuclei imaging. Samples were mounted on a microscope (Axiovert 100; Zeiss) and observed by laser confocal microscopy (LSM 510 META; Zeiss). In order to detect the localization of ICP0–EYFP in corneas, the entire tissue block was scanned from the endothelial side to the epithelial side using a x20 objective lens (Zeiss) at 2 µm steps. To localize better the ICP0–EYFP to corneal cells, the anterior, subepithelial region of the cornea was scanned using a x40 oil immersion objective lens (Zeiss) at 1 µm steps. The ICP0–EYFP fusion protein was excited by a 514 nm argon laser line and the emission was detected using a 535–590 nm band-pass filter. Actin or nuclei were imaged by excitation with the 633 nm helium–neon laser and the emission was collected with a 650 nm long-pass filter.

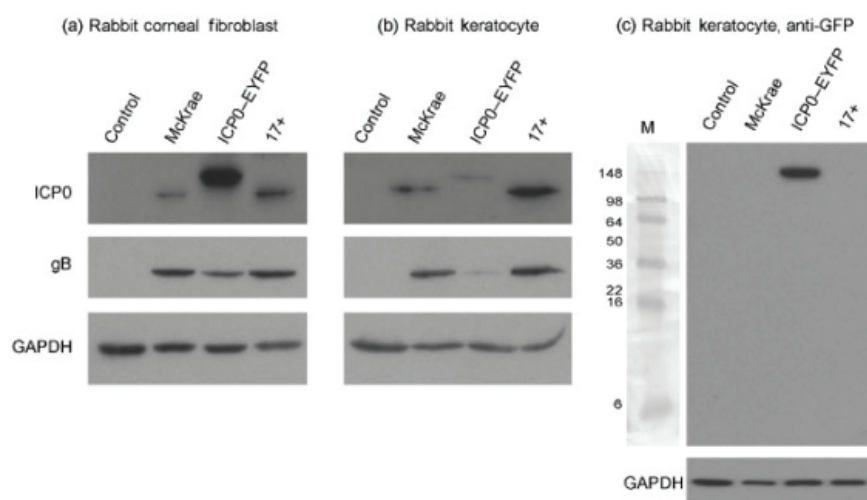


Figure 1. Expression of ICP0–EYFP in rabbit corneal fibroblast cells and primary RKs. RCF cells (a) and primary RKs (b) were isolated, infected with the indicated virus, and Western blot assays were performed as described in Methods. A parallel RK lysates and Western blot assay was probed with anti-GFP antibody (RK, anti-GFP) (c). ICP0 proteins were detected with mAb against ICP0; gB was detected with mAb against glycoprotein B; GAPDH was detected with mAb against glyceraldehyde-3-phosphate dehydrogenase. Control, parallel non-infected cell lysates; McKrae, McKrae-infected cell lysates; ICP0–EYFP, ICP0–EYFP-infected cell lysates; 17+, 17+–infected cell lysates.

Image analysis. Scanned images of ICP0–EYFP, actin or nuclei were three-dimensionally reconstructed using LSM Examiner software (Zeiss). From each three-dimensional dataset, four 10 μ m wide x – z slices were cut at equally spaced regions through the dataset. Cut regions were then rotated and maximum intensity projections generated along the z – y plane. For each projection, the corneal thickness and depth of ICP0–EYFP fluorescence was measured using MetaView image processing software (Universal Imaging).

Western blot analysis. Protein samples in gel sample buffer [2 % (v/v) SDS, 50 mM Tris, pH 6.8, 3 % (v/v) sucrose, 5 % 2-mercaptoethanol, 0.1 % bromophenol blue] were subjected to electrophoresis in 10 % bisacrylamide gels, transferred to PVDF membranes, blocked for 2 h with 5 % (v/v) non-fat dried milk in PBS and reacted with the appropriate primary antibody, which was diluted in PBS with 1 % BSA and 0.05 % Tween 20, overnight at 4 °C. The appropriate secondary antibody conjugated to horseradish peroxidase was diluted 1 : 5000 in PBS with 1 % BSA and 0.05 % Tween 20 and was reacted to the membrane for 1 h at room temperature. The blot was rinsed in PBS with 1 % BSA and 0.05 % Tween 20. Bound antibody was detected by SuperSignal West Pico Chemiluminescent substrate (Pierce) and visualized by autoradiography. For reprobing the membrane, blots were stripped by incubating with stripping buffer (PBS with 7 μ l 2-mercaptoethanol ml^{-1} and 2 % SDS) for 30 min at room temperature with constant agitation and then reprobed.

Results

Characterization of ICP0–EYFP virus in primary corneal cells

Virus ICP0–EYFP, which expresses ICP0 fused to an enhanced yellow fluorescent protein (EYFP) has been extensively characterized in established cell lines (Everett *et al.*, 2003, 2004). However, the biological properties of ICP0–EYFP have not been examined in primary cultures prepared from cornea. Primary rabbit corneal fibroblast and keratocyte cells were isolated and infected with 0.1 p.f.u. per cell of McKrae, 17+ or ICP0–EYFP for 24 h. Cell lysates were analysed by Western blot analysis using monoclonal antibodies (mAbs) directed against glycoprotein B (gB) or ICP0. The ICP0–EYFP fusion protein was expressed in both primary, serum-free cultured RK (primary RKs) and serum cultured RCF (primary rabbit corneal fibro-

blast) (Figure 1a and b). The increased molecular mass of ICP0–EYFP compared with that of normal ICP0 expressed by McKrae and 17+ was in accordance with a previous report (Everett *et al.*, 2003). The viral gB was expressed from both RCF and RK cells as well (Figure 1a, b). The specificity of ICP0–EYFP fusion protein was demonstrated in cell lysate from RK, probing with an anti-GFP antibody (Figure 1c). To ensure the integrity of ICP0–EYFP fusion protein, membranes were left for longer exposures and no smaller bands were observed (data not shown). Therefore, the ICP0–EYFP fusion protein does not undergo detectable processing, confirming that the fluorescence signal detected in later experiments must be derived from the intact fusion protein, rather than an EYFP fragment. To determine the loading quantities of the protein from individual lysates, mAb to GAPDH was used as a marker (Figure 1).

To check for ICP0–EYFP expression in primary corneal cells, RKs were seeded on coverslips, infected with ICP0–EYFP at 0.1 p.f.u. per cell, fixed and stained with an ICP0 mAb. A yellow fluorescent signal was detected in keratocytes 24 h after infection (Figure 2). The ICP0–EYFP signal was localized to granular deposits mainly within the cytoplasm, and it co-localized with the red fluorescent signal generated by staining for ICP0 with a mAb (Figure 2, upper panel). The granular deposits detected in the cytoplasm of infected keratocytes were also observed in strain 17+ and McKrae-infected keratocytes (Figure 2, middle and lower panels, respectively). Thus, the biological properties of the ICP0–EYFP in primary keratocytes are similar to those of the parental virus, 17+ or wt McKrae.

ICP0–EYFP localization in epithelial layer of infected rabbit corneas

Having established that ICP0–EYFP was expressed during HSV-1 infection in cultured primary RKs, we next set out to examine the expression and localization of ICP0–EYFP in infected rabbit corneas. The ICP0–EYFP signal was readily detectable in corneal cells 1 day after infection of

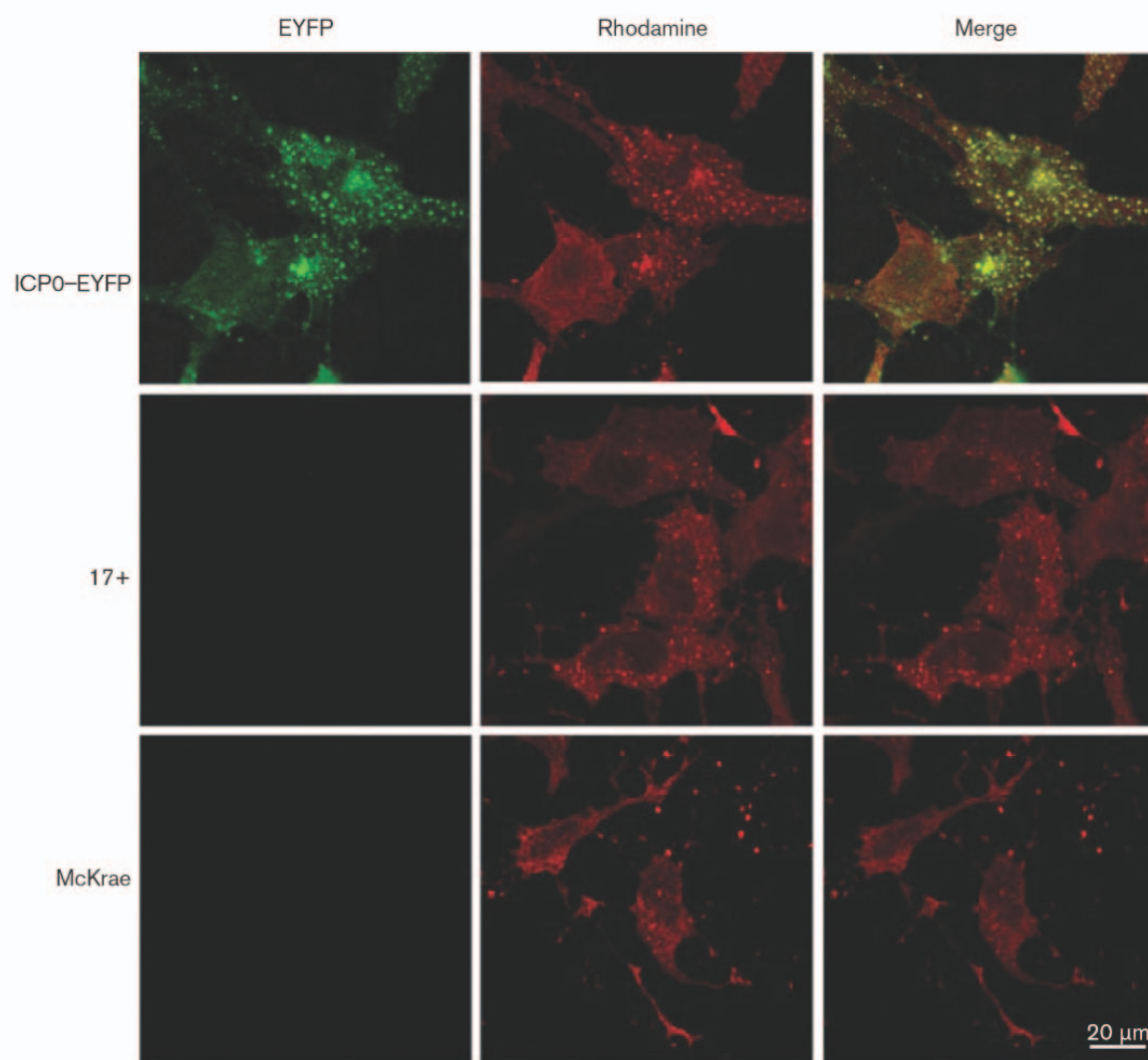


Figure 2. Granular distribution of ICP0 in cytoplasm of infected RK cells. Primary RKs were isolated and immunohistochemistry was performed as described in Methods. EYFP, Fluorescence signal from ICP0–EYFP; rhodamine (red), stained with ICP0 mAb conjugated with rhodamine; merge, both EYFP and rhodamine signals were combined. McKrae, ICP0–EYFP and 17+ indicate the viruses used. Bar, 20 μ m.

rabbit eyes (Figure 3). A typical cluster of fused epithelial cells expressing ICP0–EYFP (pseudo-coloured green) was detected (Figure 3a, b \star). An ICP0–EYFP signal was also observed along the scarified surface of the infected cornea (Figure 3c). It is common practice to scarify the surface of corneas with a fine needle to enhance the infectivity of the wt HSV-1 strain 17+, the parent of ICP0–EYFP. This scarification results in cutting of the corneal epithelium and, at times, incision of the anterior corneal stroma. This leads to migration of epithelium into the cut region of the stroma and direct contact with stromal keratocytes. In z - y projections, extension of infected epithelial cells that express ICP0–EYFP into the stroma were detected (Figure 3d).

ICP0–EYFP in the stromal layer of infected rabbit corneas

We previously reported that low levels of ICP0 were detected in the stroma of wt McKrae-infected rabbit corneas

during acute infection (Naito *et al.*, 2005). However, an independent report concluded that no viral antigens were detected in the stroma of acutely infected mouse corneas (Polcicova *et al.*, 2005). To further strengthen our findings, rabbits were ocularly infected with ICP0–EYFP and corneas were collected at different days after infection. The ICP0–EYFP signal was detected in infected corneas using confocal microscopy as described in Methods (Figure 4a). The ICP0–EYFP signal was hardly visible in the epithelial and stromal layers 1 day after infection (Figure 4a, day 1). A much more intense signal was detected in the anterior stromal layer of infected cornea 3 days after infection (Figure 4a, day 3) and the intensity of the ICP0–EYFP signal was reduced 7 days after infection (Figure 4a, day 7). By 14 days after infection, the ICP0–EYFP signal mainly localized underneath epithelial cells (Figure 4a, day 14). By 28 days after infection, the ICP0–EYFP signal was hardly detectable (data not shown). ICP0–EYFP was local-

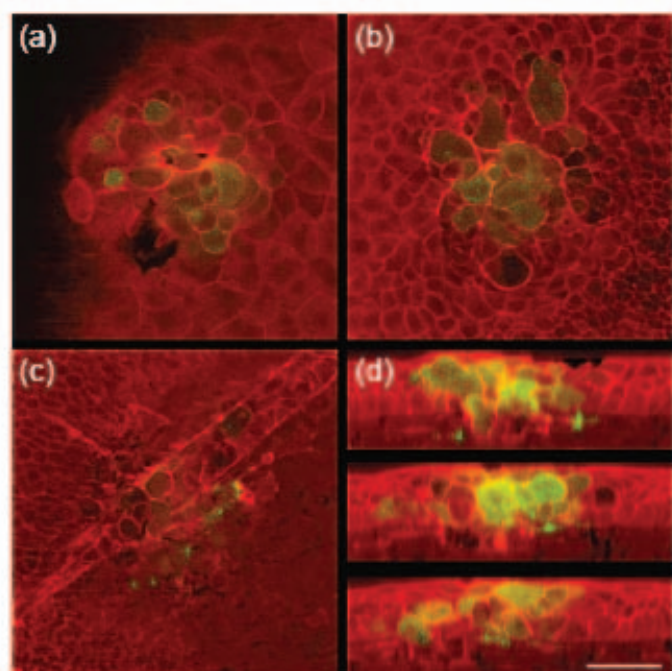


Figure 3. Expression of ICP0–EYFP in the epithelial layer of the rabbit cornea 1 day after infection. Rabbits were infected with ICP0–EYFP virus and *ex vivo* confocal microscopy was performed as described in Methods. (a, b) Green (pseudo colour) signal was observed in the epithelial layer of infected corneas; (c) green signal was also observed along the line of scarification; (d), z–y projections of infected corneas. Bar, 50 µm.

ized primarily to corneal keratocytes from day 3 to 14. A z–y projection from infected cornea 10 days after infection is shown in Figure 4(b).

Granular localization of ICP0–EYFP in keratocytes

To assess the distribution of ICP0–EYFP in the cornea, serial images were captured by confocal microscopy as described in Methods. Granular deposits were detected in infected keratocytes (Figure 5a), which were similar to those detected in cultured primary keratocytes infected with ICP0–EYFP (Figure 2). In this particular infected cornea, the ICP0–EYFP signal could be seen up to 78 µm below the basal epithelial cells (Figure 5a, 78/89). A cartoon drawing to demonstrate the depth of the scan section is shown in Figure 5(b). Very few keratocytes were observed right underneath the layer of epithelium. At the centre, most obviously from 12 to 43 µm, the majority of keratocytes were gone and a scratched lesion was seen in the stromal layer. With this approach, we were able to calculate the depth of the ICP0–EYFP signal distributed in the stromal layer of the infected cornea (Table 1). The overall thickness of the stroma increased gradually from days 3 to 14 after infection and decreased by 28 days after infection. The maximum

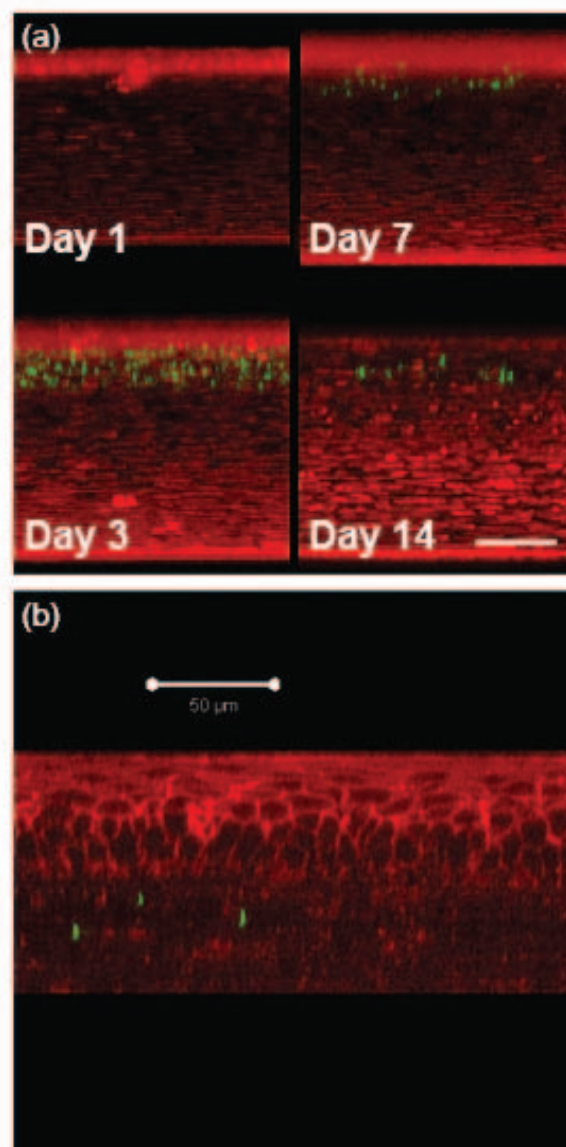


Figure 4. Expression of ICP0 in the stromal layer of infected rabbit cornea. Rabbits were infected with ICP0–EYFP virus, enucleation of corneas was at indicated days post-infection, and *ex vivo* confocal microscopy was performed as described in Methods. (a) Kinetic distribution of ICP0 (pseudo-coloured green signal) in infected rabbit corneas. Bar, 100 µm. (b) z–y projection of infected rabbit cornea, ICP0 (green signal) was predominantly seen in the stromal layer of infected cornea. Bar, 50 µm.

depth of the ICP0–EYFP signal remained constant from days 3 to 14, achieving 16–17 % depth. The depth of the ICP0–EYFP signal, 7 days after infection, was slightly less (10 %) than that detected at days 3 and 10, which may be due to either experimental error or a second wave of virus replication within the stroma. The finding that a maximum depth of infection was achieved as early as 3 days after exposure suggested that infection of keratocytes originates from epithelial virus rather than from lateral and axial spread of virus from adjacent keratocytes.

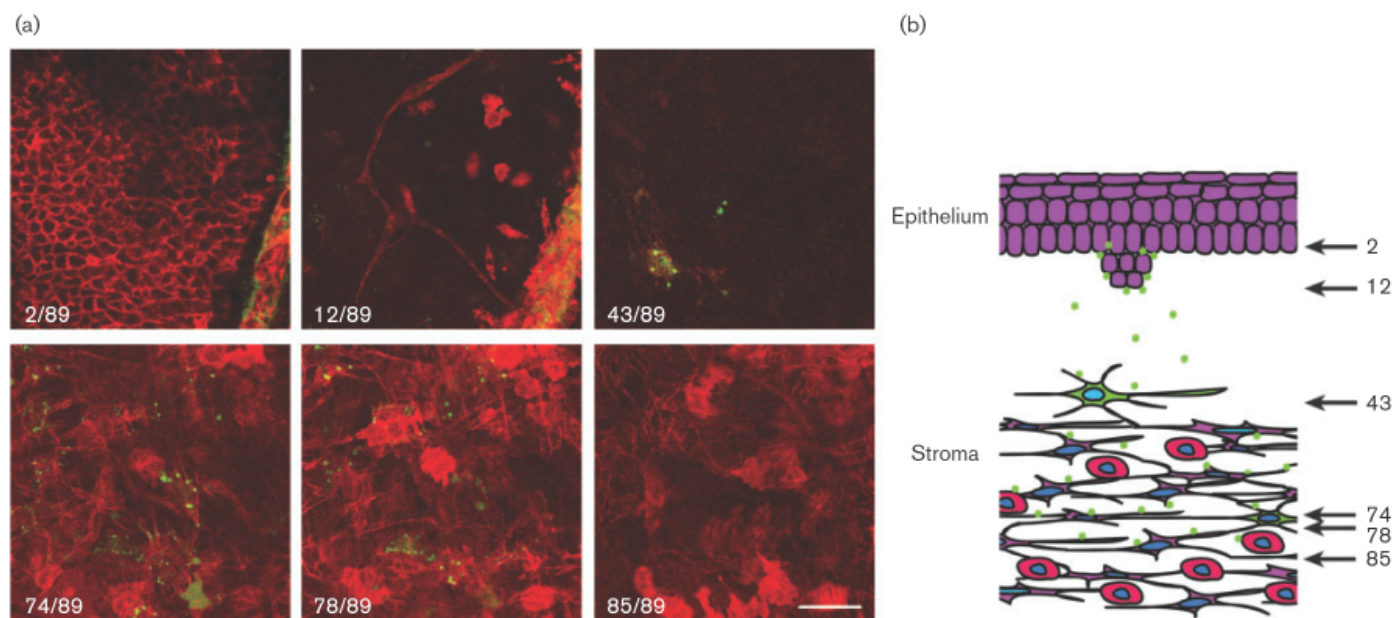


Figure 5. Depth distribution of ICP0 in stromal layer of infected cornea. Rabbits and corneas were processed as in Figure 4. Numbers indicate the distance in micrometres below the basal epithelial cells. (a) In this particular infected cornea, ICP0 can be seen up to 78 μm deep (78/89) and granular deposits were observed in infected keratocytes. Bar, 50 μm . (b) Cartoon drawing of the depth distribution. In the drawing, epithelium and stromal layers are shown. Green dots indicate ICP0-EYFP granules. Green-coloured keratocytes indicate ICP0-EYFP distribution in cytoplasm of infected keratocytes. Arrows and numbers corresponding to the sections in (a) in micrometres.

Discussion

HSK is the leading infectious cause of blindness in the developed world (Liesegang, 2001). The clinical manifestations range from mild blurry vision, photophobia and watery eyes to, in the worst cases, a devastatingly scarred cornea. These clinical symptoms are primarily due to recurrent keratitis within the stromal layer of the cornea. In addition, it is generally thought that HSK is the result of an immune response directed against one or more viral proteins. Although the presence of HSV-1 antigens can be sporadically found in stroma or keratocytes using immunohistochemical analysis and probing with anti-HSV-1 polyclonal antibodies in HSK corneas (Anaka & Kimura, 1967; Easty *et al.*, 1987; Holbach *et al.*, 1991; Kaye *et al.*, 2000; Koelle *et al.*, 2000; Meyers-Elliott *et al.*, 1980; Robertson *et al.*, 1984; Verjans *et al.*, 2000), detecting specific viral antigens may be more difficult using immunohistochemical analysis in HSK corneas. Thus, it remains unclear which, if any, viral protein(s) is expressed in the eyes of patients with HSK.

The mechanism underlying the pathogenesis of corneal scarring is still an enigma in spite of intense study into this disease. A role for both a T-helper (Th)1 and a Th2 response has been suggested (Deshpande *et al.*, 2002; Gangappa *et al.*, 2000), although this viral antigen hypothesis has been hard to prove (Banerjee *et al.*, 2002). In addition, cytotoxic CD8⁺ T cells involved in an autoimmune response against corneal autoantigens (Avery *et al.*, 1995;

Zhao *et al.*, 1998), CD4⁺ T-cell-mediated inflammatory reaction (Deshpande *et al.*, 2001) and CpG induced immune response have also been proposed to induce HSK (Cantin *et al.*, 1992; Zheng *et al.*, 2002). A recent report has concluded that the ability of HSV to persist in the cornea may lead to HSK in mice (Polcicova *et al.*, 2005). In this previous study, a mutant HSV-1 strain was used that exhibits diminished neuronal transport. Since these previous studies were performed in mice, in which *in vivo* reactivation from latency does not frequently occur (Feldman *et al.*, 2002), it is not clear how frequent reactivation from latency influences these results. We recently reported that soluble ICP0 is found in the tears of infected rabbit eyes, a model in which high levels of spontaneous reactivation from latency occur (Barsam *et al.*, 2005), and that a diffuse staining pattern for ICP0 can be detected by immunohistochemistry in the anterior stromal layer of acutely infected corneas (Naito *et al.*, 2005). To further confirm and extend our finding that ICP0 is present in the stromal layer of infected cornea, confocal microscopy was used to scan the *ex vivo* rabbit corneas infected with virus ICP0-EYFP, a well-characterized recombinant virus that expresses ICP0 fused to EYFP (Everett *et al.*, 2003).

The biological properties of ICP0-EYFP in primary corneal fibroblast cells and keratocytes were similar to those of the parental 17+ or wt McKrae HSV-1 strains. A striking finding in this study was that ICP0 appeared to be localized predominantly in the cytoplasmic and perinuclear region

Table 1. Depth of ICP0–EYFP expression in the stroma of infected rabbit corneas

Rabbit corneas were scarified prior to infection with 2×10^5 p.f.u. per eye of ICP0–EYFP virus. Corneas were fixed with 2 % paraformaldehyde in PBS prior to enucleation.

Day	No. blocks*	No. regions†	Stromal thickness‡ (µm)	ICP0 from stromal depth§ (µm)	Relative depth of ICP0 in stroma (%)
1	3	12	305.3 ± 15.7	0	0
3	5	20	335.8 ± 40.1	59.4 ± 24.72	17.4 ± 7.4
7	5	16	329.3 ± 78.5	30.3 ± 21.9	12.5 ± 5.5
10	6	24	342.5 ± 61.2	57.5 ± 27.7	16.6 ± 7.7
14	5	20	400.7 ± 34.0	70.9 ± 43.7	17.0 ± 11.0
28	3	12	362.0 ± 54.7	0	0

*Trephined corneas were quickly scanned under a confocal microscope and the ICP0–EYFP-positive corneas were cut into 1.5 mm square blocks and three-dimensional datasets were collected from the surface epithelium to the endothelium at 2 mm steps.

† Four regions from each block were sequentially reconstructed along the x–z projections.

‡ For each region, the thickness of the stroma from the basal epithelium to the corneal endothelium was measured and the mean±SD for each block recorded.

§ The depth (mm) of ICP0–EYFP signal extending from the basal epithelium into the anterior stroma was measured and the mean±SD for each block recorded.

|| The relative ICP0 distribution (%) to that of the thickness of the stroma was obtained by dividing the depth of ICP0 expression by the total stromal thickness and the mean ± for each block recorded.

rather than in the nucleus of infected keratocytes. This phenomenon may be related to the rate and efficiency of ICP0 translocation from the nucleus to the cytoplasm, a phenomenon that occurs at later stages of infection and which is cell-type specific (Advani *et al.*, 2001; Elliott *et al.*, 2005; Lopez *et al.*, 2001). Alternatively, it may be due to lower amounts of protein kinase G in keratocytes, which may lead to inefficient phosphorylation of ICP0. Interestingly, the degree of ICP0 phosphorylation affects the organelle localization of ICP0 in infected cells (Advani *et al.*, 2001; Chen *et al.*, 2000; Davido *et al.*, 2005). Furthermore, we observed that lower levels of protein kinase G were clearly detected in keratocytes compared to corneal fibroblast cultures. In contrast, there was no visible difference between p70S6K kinase and protein kinase C in the respective cells (data not shown). This may also be due to increased deposition of ICP0 in primary keratocytes and stromal fibroblasts within the cytoplasm and adjacent perinuclear envelope, which may mask detection of intranuclear ICP0. Additional studies will be necessary to determine the factors that regulate the subcellular localization of ICP0 in primary cells.

In infected animals, the ICP0–EYFP signal could be seen within epithelial cells of the infected cornea as early as 1 day after infection and it gradually penetrated into the stromal layer of the cornea by day 3. The depth distribution of the ICP0–EYFP signal ranged from 10 to 20 % of the total stromal depth (Table 1). Furthermore, ICP0 was mainly in the anterior stromal layer of infected corneas throughout the course of acute viral infection. Coincidentally, anterior corneal scarring caused by epithelial keratitis mainly occurs in the anterior third of the stroma (Pepose,

1991; Pepose *et al.*, 1996). Our data and these clinical manifestations indicate that infectious herpes virus invades the corneal stroma at the sites of epithelial keratitis. However, it is unclear whether the presence of ICP0 in the stromal layer of infected cornea associates with the development of corneal scarring. Taken together, these results suggest that ICP0 may be an important HSV-1 protein present in HSK cornea, although the presence of other viral antigens cannot be excluded. Thus, if HSK is due to an immune response to an HSV-1 antigen, ICP0 is a likely candidate. However, this does not exclude the possibility that small amounts of other viral proteins that are undetectable by Western blot assays can trigger an immune response that contributes to HSK.

It has been shown that reactivation can occur in latently infected rabbits (Perng *et al.*, 1994; Barsam *et al.*, 2005). Whether the ICP0–EYFP signal can be detected in the stromal layer of rabbit corneas during latent infection with ICP0–EYFP virus or during reactivation from latency is currently under investigation.

In conclusion, we demonstrated that using *ex vivo* confocal microscopy the ICP0 protein was expressed in the stromal layer of the cornea of acutely infected rabbits.

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