Herpes simplex virus type 1 immediate-early protein ICP0 diffuses out of infected rabbit corneas

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INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a prevalent virus pathogen infecting 60–90 % of the adult world population. Following primary HSV-1 infection of the peripheral epithelium, the virus spreads to local sensory neurons and travels via axonal transport to the cell nucleus. Here it either replicates or establishes latency (Bastian et al., 1972; Kristensson et al., 1971, 1982; Stevens, 1989; Stevens et al., 1972). Reactivation of latent virus can occur spontaneously or in response to various stimuli such as ultraviolet radiation, stress and trauma. The reactivated virus travels via the axon back to peripheral sites. This often results in virus shedding and the recurrence of clinical disease.

Replication of HSV-1 in tissue culture is temporally orchestrated and tightly regulated (Roizman & Sears, 1996). Initiation of replication requires several immediate-early (IE) genes to be expressed sequentially and simultaneously. The HSV-1 IE gene ICP0 is a promiscuous activator of viral gene expression and is required for efficient initiation of lytic infection in tissue culture (Everett, 2000; Hagglund & Roizman, 2004), particularly at low m.o.i. values (Everett et al., 2004; Sacks & Schaffer, 1987; Stow & Stow, 1986). In addition, ICP0 may be important in reactivation from latency (Halford & Schaffer, 2001; Leib et al., 1989). It has been suggested that ICP0 regulates the balance between lytic and latent HSV-1 infection (Loiacono et al., 2003). The function of the protein itself has been extensively studied in vitro, and an increasingly detailed picture of the interactions of ICP0 with cellular proteins and its biochemical functions is emerging (Boutell & Everett, 2003; Boutell et al., 2002; Everett, 2000; Everett et al., 1998; Gu & Roizman, 2003; Hagglund & Roizman, 2004; Jackson & DeLuca, 2003; Kawaguchi et al., 1997, 2001; Parkinson et al., 1999). ICP0 can counteract the host antiviral response by disarming the interferon regulatory factor IRF3- and IRF7-mediated activation of interferon-stimulated genes. The RING finger domains of ICP0 are essential for this activity (Lin et al., 2004; Mossman et al., 2000).

Ocular infections of the cornea with HSV-1 can induce herpetic stromal keratitis (HSK). HSK is the most common infectious cause of corneal opacity leading to blindness. HSK is characterized by tissue destruction, oedema, opacification, corneal scarring and neovascularization (Pavan-Langston, 2000; Shimeld et al., 2001). Interactions between the recurrent virus and the pre-existing immune response are believed to be the main cause of corneal scarring (Deshpande et al., 2004; Streilein et al., 1997). The current knowledge of HSK immunopathology is mainly derived from murine models, although spontaneous reactivation is a rare event in mice (Feldman et al., 2002). In the HSK mouse model, CD4+ T cells play a pivotal role in the HSK immunopathological response (Gangappa et al., 2000). It has been hypothesized that HSK may be due either to HSV-1 inducing auto-reactive T cells (Avery et al., 1995; Zhao et al., 1998) or to bystander damage by infiltrating CD4+ T cells
(Deshpande et al., 2001). However, it is generally thought that HSK is the result of an immune response to one or more viral proteins. Surprisingly, no specific HSV antigens have been recovered from HSK corneas. Thus, it remains unclear which, if any, viral protein(s) is involved in HSK.

We report here that when HSV-1-infected rabbit corneal buttons were soaked in fixative or buffer, ICP0 could be consistently detected in the soaking solution. ICP0 was also consistently detected in virus-free tears from infected rabbits. In contrast, no other viral antigens were detected. This suggests that ICP0 is water soluble and either rapidly diffuses out of, or is actively secreted from, cells in the cornea.

**METHODS**

**Cells and virus.** Rabbit skin (RS) cells were grown in Eagle’s minimal essential medium supplemented with 5% fetal calf serum. Wild-type HSV-1 McKrae was triple plaque purified, passed only one or two times and titrated in RS cells prior to use (Perng et al., 1994).

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

**Rabbit model of HSV infection.** Rabbits were bilaterally infected without scarification or anaesthesia by placing, as eye drops, 105 p.f.u. virus into the conjunctiva cul-de-sac, closing the eyes, and rubbing the lid gently against the eye for 30 s as previously described (Perng et al., 1994). At this dose of HSV-1 McKrae, virtually all of the surviving rabbits harbour a latent HSV infection in both trigeminal ganglia. Latency is assumed to have been completely established by 28 days post-infection (p.i.).

Beginning on day 31 p.i., tear film specimens were collected daily from each eye for 26 days as previously described (Perng et al., 1994), using a nylon-tipped swab. The swab was then placed in 0.5 ml tissue culture medium and squeezed, and the inoculated medium was used for plaque assays as previously described (Perng et al., 1994).

**Collection of tears for the detection of viral proteins.** Tears were collected on indicated days using Whatman no. 41 Schirmer strips placed in the inferior lid margin of the eye for 2 min. The strips were then placed in microcentrifuge tubes containing 100 μl PBS with protease inhibitor and strongly vortexed for 5 min. The liquid in the tube was spun through a 0.22 μm filter for 10 min at 14,000 r.p.m. (Beckman Microfuge 22R centrifuge) to remove cell debris and virus particles. The supernatant was lyophilized and stored at −80°C until all the samples were collected. The dried samples were each resuspended in 20 μl SDS-PAGE sample buffer, boiled for 5 min and briefly spun. They were then subjected to SDS-PAGE, transferred to PVDF membrane and probed with the indicated antibody.

**Monitoring corneal scarring.** Recurrent ocular disease was monitored from day 30 to day 58 p.i. Briefly, corneal scarring was scored as being present or absent and was visualized by the unaided eye. A drop of 1% fluorescein was topically applied to the eye, photographed and confirmed by slit lamp biomicroscopy at the time of sacrifice.

**Collection of corneal buttons.** An 8 mm trephine (similar to a cork borer) was used to cut out and collect corneal buttons from the corneas of euthanized rabbits.

**Soluble corneal extract preparation.** Corneas were trephined and immediately immersed in 100 μl cold PBS buffer with a cocktail of protease inhibitors (Roche Diagnostics). The sample was kept on ice for 2 h and the supernatant was transferred to a new tube. The protein concentration was measured, adjusted and subjected to SDS-PAGE and Western blot analysis.

**Antibodies.** All HSV-1 monoclonal antibodies (mAbs) were purchased from Virusys Corporation except for the ICP27 mAb, which was a gift from Dr Sandri-Goldin (Department of Microbiology and Molecular Genetics, University of California at Irvine, USA). Goat anti-HSV-1 polyclonal antibody was from Biodesign. Goat anti-human albumin antibody was from BiosPacific. GAPDH mAb was from Research Diagnostics. Secondary antibodies conjugated to horseradish peroxidase were from Chemicon International.

**Immunoblotting.** 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 dry milk in PBS and reacted overnight at 4°C with the appropriate primary antibody diluted in PBS with 1% BSA and 0.05% Tween 20 (PBS/BSA/Tween). The appropriate secondary antibody conjugated to horseradish peroxidase was diluted 1:5000 in PBS/BSA/Tween and reacted with the membrane for 1 h at room temperature. The blot was rinsed in PBS/BSA/Tween and antibody bound to the blots was detected using SuperSignal West Pico Chemiluminescent substrate (Pierce) and visualized by autoradiography. For reprobing the membrane, blots were stripped by incubating with stripping buffer (PBS containing 7 μl 2-mercaptoethanol ml⁻¹ and 2% SDS) for 30 min at room temperature with constant agitation and then reprobed.

**Histology and immunohistochemistry.** Trephined corneas were fixed in 4% paraformaldehyde in PBS for a minimum of 24 h, embedded in paraffin and sectioned as previously described (Barsam et al., 2005). Sections (5 μm) were stained with haematoxylin and eosin (H&E) and morphology was examined by light microscopy. Serial adjacent sections were stained with HSV-1 polyclonal antibody or specific HSV-1 mAb using the Vectastain ABC kit (Vector Laboratories). Antigens were retrieved by antigen unmasking solution (Vector Laboratories) according to the manufacturer’s protocol. The mAb-stained sections were not counterstained in order to increase the clarity of the stain.

**RESULTS**

Recently, we established a viral model for studying corneal scarring following ocular infection of rabbits with HSV-1 (Barsam et al., 2005). To look for HSV-1 proteins in these HSK corneas, we examined over 30 fixed, sectioned, corneal buttons by indirect immunofluorescence using both polyclonal antibodies produced against total HSV-1 proteins and mAbs specific for gB, gC, gD, ICP4, VP5, ICP0 or ICP27. Surprisingly, these initial attempts to detect HSV-1 antigens in histological sections of HSK rabbit corneas were inconclusive (data not shown). This was consistent with previous immunohistological analyses of fixed HSK corneas from mice and humans using polyclonal antibody against total HSV-1 proteins (Banerjee et al., 2002; Hendricks, 1999; Holbach et al., 1991; Metcalf & Helmsen, 1977; Miyamoto...
et al., 1971; Pepose, 1991; Pettit & Meyers, 1976). Since HSK is thought to represent an immune response to one or more HSV-1 proteins (Deshpande et al., 2004; Hendricks, 1999; Metcalf & Kaufman, 1976; Meyers-Elliott et al., 1980; Pepose, 1991; Pettit & Meyers, 1976), we were surprised at the inability to readily detect any viral proteins in these severe HSK corneas.

Since some water-soluble corneal proteins can diffuse out of the cornea (Piatigorsky, 2001), we decided to look for potential viral proteins in the fixative. The fixative was dialysed against a large volume of PBS buffer overnight at 4°C. The solution inside the dialysis bag was collected and precipitated with acetone, and the precipitate was collected by centrifugation, vacuum dried and suspended in SDS-PAGE sample buffer. Western blotting was performed as described in Methods using the above well-characterized commercial HSV-1 mAbs. The HSV-1 ICP0 mAb consistently detected a specific band (Fig. 1), whereas no bands were observed with the other mAbs (not shown). All of the mAbs detected the appropriate HSV-1 protein by Western blotting of acutely infected RS cell lysates and by immunohistochemistry of acutely infected RS cells (Fig. 2a and b). These results suggest that ICP0 might have rapidly diffused into the fixative.

ICP0 detection in corneal soaking buffer

To determine whether ICP0 readily diffused out of acutely infected rabbit corneas, the following study was performed. Ten rabbits were infected with $2 \times 10^5$ p.f.u. HSV-1 McKrae per eye, as described in Methods. Replication of virus during acute infection was consistent with previous studies (Perng et al., 1994, 2000a) (Fig. 3). The infected corneas showed a progressive pattern of disease consistent with previous studies (Fig. 4a). On day 3 p.i., a hazy appearance was apparent. On day 5 p.i., ulceration of the cornea and corneal opaqueness were observed. By day 14 p.i., when infectious virus was not detectable, the cornea was similar to controls (Fig. 4a).

Rabbits were euthanized and corneal buttons collected on the indicated days p.i. (Fig. 4) and immediately incubated in 100 μl cold PBS containing protease inhibitors for 2 h on ice. The buffer was transferred to a new tube and centrifuged to remove debris. The supernatant was collected and the amount of protein was standardized and adjusted to a concentration of 6× running buffer by the addition of 6× running buffer. Equal amounts of protein were loaded onto the gel and Western blotting was performed. ICP0 was readily detected by Western blot analysis of the soaking buffer on days 5, 7 and 10 p.i. (Fig. 4a). Similar results were seen with the contra-lateral eyes of these rabbits (Fig. 4b). Note that the corneal photos in Fig. 4(a) were taken just prior to euthanasia and were the same corneas used for Western blot analysis in Fig. 4(a). Interestingly, no viral proteins were detected in the soaking buffer.

![Fig. 1. Detection of ICP0 in fixative. Briefly, the fixative was dialysed against a large volume of PBS. The solution inside the dialysis bag was collected, precipitated with acetone, vacuum dried, resolved by SDS-PAGE, transferred to membrane and probed with ICP0 mAb. Marker, protein molecular markers (kDa); HSK, fixative from HSV-1-induced keratitis cornea; Normal, fixative buffer from a cornea with a normal appearance; Lysate, HSV-1-infected cell lysates.](http://vir.sgmjournals.org)

![Fig. 2. Specificity of HSV-1 mAb. (a) RS cells were infected with HSV-1 McKrae at an m.o.i. of 1. Cell lysates were prepared at the indicated times as described in Methods and probed with mAb against the indicated protein. (b) RS cells were seeded in chamber slides and infected with HSV-1 McKrae at an m.o.i. of 1. Cells were fixed at 4 h p.i. and probed with mAb against the indicated protein.](http://vir.sgmjournals.org)
buffer by any of the other HSV-1 mAbs (anti-gB, -gC, -gD, -ICP4, -VP5 and -ICP27) (data not shown). To confirm the lack of these proteins, the proteins in the soaking buffer were concentrated by lyophilization, resuspended in one-sixth of the original volume and analysed by Western blotting as above. Again, no bands were detected. GAPDH can be detected in the soaking buffer of corneal buttons (Piatigorsky, 2001) and was used as a loading control. These results suggest that ICP0, but none of the other viral proteins examined, rapidly diffused out of corneal buttons harvested from rabbits on days 5, 7 and 10 p.i.

**ICP0 detection in rabbit tears**

To determine whether ICP0 could also be readily detected in the tears of rabbits infected with HSV-1, tears were collected daily as described in Methods between days 3 and 10 p.i. from each of the 10 rabbits described above. The tear films were centrifuged through a 0.22 μm membrane spin column to remove cellular debris and virus particles, and processed for Western blotting. ICP0 was readily detected in the tears from all 20 eyes (tears collected from four eyes are shown in panels a–d).

![Fig. 3.](image1) Replication of HSV-1 in infected rabbit eyes. Rabbits were ocularly infected with $2 \times 10^5$ p.f.u. HSV-1 McKrae per eye. Eye swabs were taken at the indicated days after infection and viral titres were assayed as described in Methods.

![Fig. 4.](image2) Detection of ICP0 in corneal soaking buffer from acutely infected rabbit eyes. (a) The physical appearance of corneas on the indicated days after infection was photographed. The corneas were trephined and treated as described in Methods. Western blotting was carried out using mAbs against the indicated proteins. (b) Contra-lateral eyes from (a). ICP0 was detected in corneal soaking buffer on days 5, 7 and 10 p.i. GAPDH were used as a reference protein marker. C, Control cornea; D#, days p.i.

![Fig. 5.](image3) Detection of ICP0 in tears collected from acutely infected rabbits. Tear fluid was collected daily from 3 to 10 days p.i. Collected tears were processed as described in Methods. Western blotting was performed by probing the membrane with HSV-1 ICP0-specific mAb. Membranes were reprobed with a polyclonal antibody against albumin as a loading control. (a–d) Results of representative collected tears from infected rabbits. C, Control tears collected from uninfected rabbit eyes; McKrae, tears collected from McKrae-infected rabbit eyes; D#, days p.i.; M, protein molecular mass marker.
shown in Fig. 5). In 17 eyes, ICP0 was detected on at least one day between days 7 and 9 p.i. In one eye, ICP0 was detected from day 3 to day 9 p.i. In two eyes, ICP0 was detected only on day 5 p.i. We were unable to detect any other HSV-1 proteins in any of the tear samples using the other HSV-1-specific mAbs. This suggests that detection of ICP0 was not the result of virus particles in the samples and that other viral proteins did not diffuse out of the cornea as efficiently as ICP0. Albumin is a major protein in tears and was used as a loading standard. Membranes were reprobed with anti-albumin antibody. All tear samples contained albumin (Fig. 5).

**Histological staining**

Corneal buttons from rabbits infected as in the above experiment were trephined at various times and fixed. Paraffin sections (5 µm) were processed for H&E staining.

**Fig. 6. Distribution of ICP0 in the stromal layer of acutely infected rabbit corneas.** Corneas were removed and fixed in 4% paraformaldehyde as described in Methods. Sections (5 µm) were cut, and histology and immunohistochemical staining were performed. The histology and patterns of antibody staining are described in detail in the text. Control, uninfected cornea; D#, days p.i.; H&E, haematoxylin and eosin staining; HSV-1, polyclonal antibody to HSV-1-infected cell lysates; gB, mAb to HSV-1 glycoprotein B; ICP0, mAb to HSV-1 ICP0; ICP4, mAb to ICP4.
Erosion of the epithelial layer was seen as early as 3 days p.i. By day 7 p.i., the epithelial layer was not readily detectable, but appeared to begin regenerating as early as day 10 p.i. By day 14 p.i., the corneal morphology appeared to be normal. The anterior stromal layer of rabbit corneas was progressively swollen and contained infiltrating immune cells during the acute infection. By day 14 p.i., infiltrating lymphocytes were not readily detected. The observed pathology in the infected rabbit eyes was similar to lesions in patients with herpetic eye disease (Meyers-Elliott et al., 1980).

ICP0 detection in the stromal layer by immunohistochemical staining

Serial corneal sections were stained with different antibodies to examine the distribution of HSV-1 antigens in acutely infected corneas. Using polyclonal antibody against total HSV-1 proteins, HSV-1 antigens were readily detected at the epithelial layer on days 3 and 5 p.i. (Fig. 6, HSV-1). By days 7 and 10 p.i., cells in the stromal layer were only lightly stained. A diffuse spatial distribution of staining in the anterior stromal lamellae was observed on day 10 p.i. The counterstain appeared to make it difficult to differentiate the light spatial distribution staining. Therefore, we did not perform counterstaining when specific mAbs were used.

The staining pattern seen with anti-gB and -ICP4 mAbs was similar to that seen with HSV-1 polyclonal antibody, with the exception that gB-positive staining appeared to be distributed closer to the epithelial layer, while ICP4-positive staining appeared to be deeper in the stromal layer. In contrast, sections stained with ICP0 mAb showed a different pattern (Fig. 6). ICP0-positive staining on the epithelial layer was not readily observed until day 5 p.i. On days 7 and 10 p.i., spatial distribution of ICP0 staining was visible in the stromal lamellae. By day 14 p.i., the staining was very weak and diffuse in the stromal layer.

ICP0 detection in the soaking buffer of scarred corneas

To readdress the issue of HSV-1 proteins in HSK corneas, we performed a more detailed study than that described in the first paragraph of Results above. Ten rabbits were ocularly infected in both eyes with wild-type HSV-1 McKrae. Beginning on day 31 p.i., eye swabs were collected daily from both eyes of all five surviving rabbits and the amount of recurrent virus shedding was determined by plaque assay (Table 1). A high spontaneous reactivation rate (23%) was observed and six of ten eyes developed corneal scarring (Table 1, CS). This is consistent with a report that eyes with a high HSV reactivation rate are more likely to develop HSK (Liesegang, 2001). Interestingly, a high burst of virus shedding (>500 p.f.u.) occurred prior to the appearance of corneal scarring (Table 1) and virus shedding was limited after the appearance of corneal scarring. Corneal scarring is known to interfere with the detection of spontaneous reactivation in rabbit tears (Perng et al., 2000b).

Four scarred corneal buttons (rabbits 4L, 5L, 5R and 10R; Table 1) and one normal corneal button (rabbit 2L; Table 1) were trephined and processed as described above. Eyes were photographed just prior to trephination (Fig. 7a). ICP0 was the only HSV-1 antigen detected in the corneal soaking buffer from scarred corneal buttons (Fig. 7a). ICP0 was not detected in the buffer from a normal eye (Fig. 7a). As expected, similar levels of GAPDH were detected in all samples (Fig. 7a). When the other HSV-1 mAbs were used in Western blot assays, no viral proteins or peptides were detected in the buffer (not shown).

**Table 1. History of the latently infected rabbit eyes**

Rabbits were ocularly infected with $2 \times 10^6$ p.f.u. HSV-1 McKrae per eye as described in Methods. Rabbits surviving past 28 days p.i. were assumed to have establish latency. Beginning at day 31 p.i., eye swabs were collected daily for 26 days. Plaque assays were done as described in Methods. Results are given as the observed number of viral plaques from an eye swab. No plaques were observed on days 31–34. CS, Appearance of corneal scarring.

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Two corneal buttons prepared from scarred eyes (rabbits 4R and 10L; Table 1) and one from a normal eye (rabbit 9L; Table 1) were fixed in 4% paraformaldehyde, embedded and sectioned for histological and immunohistochemistry staining (Fig. 7b). The H&E staining (Fig. 7b) of HSK paraffin sections was similar to our previous report (Barsam et al., 2005). One of 10 sections from one of the scarred corneal buttons appeared to be positive for ICP0 in the anterior stromal lamellae. All sections were negative for gB (Fig. 7b). Neither ICP0 nor gB was detected in the other scarred cornea. Combined with the lack of detection of ICP0 and other HSV-1 proteins in over 30 scarred corneas in our initial experiment in paragraph one of Results, this suggests that detection of ICP0 in cornea scarred by recurrent HSV-1 infection is very rare. This is consistent with only a very low percentage of human HSK corneas being positive for HSV-1 antigens (Garweg et al., 2003).

DISCUSSION

HSK produces severe scarring of the cornea that can lead to loss of vision. HSK is associated with recurrent herpes infections and is generally thought to be the result of an immune response to one or more HSV-1 proteins. Thus, the inability to detect HSV-1 antigens routinely in histological sections of human HSK corneas (Garweg et al., 2003; Kaye et al., 2000; Pramod et al., 1998) has been both disappointing and confusing. One possibility for this lack of detection is that human HSK corneal buttons are difficult to acquire and the quality of the tissue is hard to control. However, HSV-1 antigens have also not been readily detected in mouse HSK corneas (Banerjee et al., 2002). This might be due to differences in the mouse model, our primary source of knowledge on the development of HSK (Deshpande et al., 2004), compared with humans. In particular, spontaneous reactivation of HSV-1 in mice is very rare (Feldman et al., 2002) and most mouse studies therefore employ acutely infected mice. Here we have reported that HSV-1 proteins were also not readily detected in histological sections of corneas from our newly developed rabbit model of recurrent HSV-1 corneal disease. Since both the quality and quantity of these corneal buttons can be controlled, this initially suggested that HSK corneas did not contain any significant amount of viral protein. However, we subsequently found that ICP0 could be detected in the fixative in which the corneal button was soaked prior to processing of sections. Since fixation of corneas is a routine step in preparing corneal sections, the loss of ICP0 from the cornea during this procedure may explain why ICP0 has not been detected in HSK corneal sections.

We found here that ICP0 also diffused into the cornea soaking buffer from acutely infected corneas. In addition, ICP0 was also readily detectable in fixed corneal sections during acute infection. This suggests that (i) there is much more ICP0 present during acute infection than during HSK; (ii) some or all of the ICP0 is located in different compartments of the cornea during acute infection compared with HSK; (iii) the structure of ICP0 differs during HSK, resulting in increased diffusion out of the cornea; and/or (iv) the HSK cornea allows ICP0 to diffuse out more rapidly. These possibilities are not mutually exclusive.

In contrast to the diffusion of ICP0, none of the other HSV-1 proteins examined during acute infection was readily detected in the soaking buffer, although they were readily detected in corneal sections. The lack of these other HSV-1 proteins in the fixative and/or soaking buffer from HSK corneal buttons and in sections of the HSK corneal buttons therefore suggests that they are significantly less abundant than ICP0 in HSK cornea. Taken together, these results suggest that ICP0 may be the most abundant, if not the only, HSV-1 protein present in HSK corneas. Thus, if HSK is due to an immune response to an HSV-1 antigen, ICP0 is a likely candidate. However, this does not rule out the possibility that small amounts of other viral proteins below levels detectable by Western blotting may be able to trigger an immune-based aetiology of HSK.

Consistent with ICP0 diffusing into the soaking buffer, ICP0 was also found in cell-free and virus-free tears of acutely infected rabbits. Interestingly, the peak of ICP0 in tears (days 7–10 p.i.) was delayed compared with the peak of infectious virus in tears (day 5 p.i.) and the peak of ICP0 expression in corneal sections (days 5–7 p.i.). This suggests that in vivo it...
takes a few days for ICP0 to diffuse into the tears. The temporal disconnection between peak virus replication and the peak of ICP0 in tears also suggests that ICP0 is stable and can be retained for a significant time after virus can no longer be detected. This may explain how ICP0 is present in HSK cornea in the absence of detectable virus or other viral proteins.

The McKrae strain of HSV-1 does not usually cause high rates of recurrent corneal scarring in the rabbit model. Our experience is that typically <2% of eyes develop corneal scarring. However, in this study, six of ten eyes developed corneal scarring. We believe that this may have been the result of an unusually high spontaneous reactivation rate in this batch of animals (23% compared with a typical rate of 10%). This may have been due to one or more unusual environmental factors in our new vivarium facility, including elevated temperatures and 24 h lighting (rather than 12 h on, 12 h off).

Multiple forms of ICP0 occur because of extensive post-translational processing, and this may contribute to the biological properties of ICP0 (Ackermann et al., 1984). In addition, different phosphorylation patterns of ICP0 occur in infected cells (Davido et al., 2005). Whether one or several forms of phosphorylated ICP0 occur in the corneal soaking buffer is not known. How ICP0 might contribute to HSK immune-related disease is also unclear. However, if ICP0 remains stable in the cornea for long periods of time after acute and recurrent infection, it may be a key antigen recognized by the immune response leading to HSK. In addition, ICP0 plays an important role in blocking the antiviral effects of interferon (Leib et al., 1999). Thus, ICP0 may act as an antagonistic factor against the innate immune response. The extended presence of ICP0 might therefore facilitate virus replication during reactivation from latency, resulting in increased HSK.

In summary, our findings suggest that ICP0 is present in HSK corneas, but has not previously been detected because it rapidly diffuses out of corneal buttons into the fixative. In contrast, other viral proteins were not detected in either HSK corneas or fixative. This suggests that following acute infection and/or reactivation of HSV-1 from latency, ICP0 has a long-term presence in the cornea. ICP0 is therefore a candidate for a viral antigen involved in HSK. In addition, ICP0 is particularly important for virus replication following low m.o.i. values (Everett et al., 2004; Hagglund & Roizman, 2004) and this may be particularly relevant for acute or recurrent corneal infections (Kaye et al., 2000; Ling et al., 2003). Thus, the presence of ICP0 in tears may play a role in stimulating replication of small amounts of HSV-1 that return to the eye following reactivation.

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REFERENCES


**Miyamoto, K., Morgan, C., Hsu, K. C. & Hampar, B. (1971).** Differentiation by immunoferretin of herpes simplex antigens with the use of rabbit 75 and 195 antibodies from early (7-day) and late (7-week) immune sera. *J Natl Cancer Inst* 46, 629–646.


