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Confocal Microscopic Analysis of a Rabbit Eye Model of High-Incidence Recurrent Herpes Stromal Keratitis

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Purpose: Using CJLAT, a chimeric herpes simplex virus (HSV-1) that produces a high incidence of herpes stromal keratitis (HSK) in latently infected rabbits, and in vivo confocal microscopy (CM), we characterized the cellular events that precede the development of HSK.

Methods: Thirty days after infection, in vivo CM was performed daily for 10 days and then weekly for up to 80 days after infection.

Results: We detected 3 types of subclinical corneal lesions before HSK was clinically apparent: (1) small epithelial erosions; (2) regenerating epithelium overlying small cell infiltrates within the basal epithelial cell layer; and (3) dendritic-like cells within the basal epithelial layer overlying stromal foci containing infiltrating cells. Sequential in vivo CM observations suggested that subclinical foci resolved over time but were larger and more abundant with CJLAT than with wild-type HSV-1 McKrae. Active HSK was observed only with CJLAT and was initially associated with a large epithelial lesion overlying stromal immune cell infiltrates.

Conclusions: These results suggest that replication in the cornea of reactivated virus from the trigeminal ganglia produces epithelial lesions, which recruit immune cell infiltrates into the basal epithelial layer and anterior stroma. The virus is usually cleared rapidly eliminating viral antigens before the arrival of the immune cells, which disperse. However, if the virus is not cleared rapidly, or if an additional reactivation results in an additional round of virus at the same site before the immune cells disperse, then the immune cells are stimulated and may induce an immunopathological response leading to the development of HSK.

Key Words: herpes simplex virus, herpes stromal keratitis, rabbit, confocal microscopy

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In developed countries, recurrent HSV-1–induced eye disease, called herpes stromal keratitis (HSK) or more properly recurrent HSK, is a major cause of infectious corneal blindness,[1] with ~500,000 afflicted individuals in the United States alone.[2,3] Primary ocular HSV-1 infection is typically very mild or asymptomatic. By its conclusion, virus has ascended through axons and established lifelong latent infection in neurons of the trigeminal ganglia (TG). As a result of poorly understood cellular and molecular mechanisms, the virus periodically reactivates and returns through axons to the cornea where it replicates, is shed in tears, and can produce HSK.

Most episodes of HSK begin as superficial herpes epithelial keratitis (HEK) that progresses to stromal disease.[4–6] Although long-term antiviral therapy can reduce HSK by ~40%[7] (presumably by decreasing viral replication after reactivation), antiviral treatment has no significant impact on preventing progression from HEK to HSK.[7,8] This suggests that reactivated virus is involved in recurrent HEK, but additional virus replication is not needed to progress to stromal disease. In contrast, immunosuppressants such as corticosteroids and cyclosporine A, which are not suitable for long-term therapeutic use, are useful during recurrent HSK.[9] This suggests that progression from recurrent HEK to HSK involves a pathologic inflammatory response. For these and other reasons, it is generally believed that HSK is an immunopathologic disorder in response to virus that returns to the cornea after reactivation from latency in the TG. Although the specific underlying immunopathologic mechanism(s) and the specific viral antigens (Ags) remain to be determined, the viral Ags seem to trigger an already highly primed local immune response, producing exacerbated immunopathological and

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inflammatory responses with accompanying tissue damage and stromal scarring.[10] HSV-1–specific cellular immune responses are implicated because immunosuppressed individuals have decreased HSK despite often having increased viral reactivation (ie, shedding of virus).[11] It has also been proposed that HSK is triggered by small amounts of viral Ags remaining in the cornea after clearance of the primary infection or that the cornea may be an alternate site of latent or persistent viral infection.[12,13]

Because long-term oral acyclovir only reduces ocular HSV-1 recurrences by ~40%[7] and no prophylactic or therapeutic clinical vaccines against ocular herpes are currently available, there is an urgent need for a better understanding of the cellular and molecular mechanisms leading to HSK for the eventual development of efficacious interventions. To address this problem, we have developed a unique ocular rabbit model in which clinically relevant recurrent HSK occurs in approximately 80% of the eyes. CJLAT is an HSV-1 (strain McKrae) chimera in which the first 1.5 kb of the 8.3 kb HSV-1 latency-associated transcript (LAT) gene was replaced by the latency-related (LR) gene of bovine herpes virus 1.[14,15] We have previously reported that acute eye disease after CJLAT ocular infection in rabbits is similar to that produced by wild-type (WT) McKrae,[16] in that clinical corneal disease resolves completely by postinfection day 21 and on clinical examination the eyes are unremarkable. However, in CJLAT-infected eyes starting between approximately postinfection day 33 and 80, up to 80% of the corneas develop clinical HSK,[16] whereas less than 2% of corneas from rabbits latently infected with WT HSV-1 McKrae (or other HSV-1 strains) develop any overt recurrent disease. Although the virological, immunological, and molecular mechanisms that lead to the high level of HSK produced by CJLAT have not yet been determined, the rabbit–CJLAT model has provided us with the first clinically relevant small animal model with which to systematically study the development of recurrent HSK. In this study, we report our initial in vivo confocal microscopic studies of HSK in the rabbit–CJLAT model.

MATERIALS AND METHODS

Viruses and Tissue Culture Cells

All viruses were triple plaque-purified and passaged only 2 or 3 times in rabbit skin cells before use. CJLAT has been previously described.[15,16] Rabbit skin cells were grown in Eagle minimal essential media supplemented with 5% fetal calf serum.

Rabbits

Female New Zealand white rabbits aged 8 to 10 weeks 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 Institutes of Health guidelines for the care and use of animals in research. Rabbits were bilaterally infected without killing or anesthesia by placing 2×10^5 pfu of virus, as eye drops, into the conjunctival cul-de-sac, closing the eye and rubbing the lid gently against the eye for 30 seconds as we previously described.[15,17] Both eyes were used because in our experience there is no significant correlation between the left and right eyes of a rabbit for virus shedding or ocular disease.[17] Thus, the eyes can be treated as independent variables.

Cohorts of Latently Infected Rabbits

Five rabbits were infected with WT HSV-1 McKrae (WT) without corneal scarification and 18 were identically infected with CJLAT. One rabbit was maintained as a control. Seven CJLAT-infected rabbits, 3 WT infected rabbits, and the uninfected rabbit survived up to postinfection day 31 (p.i.). At this time, eyes were clinically evaluated by a hand-held slit lamp to assess acute disease, and only eyes that appeared clear (11 of 14 CJLAT-infected eyes; 6 of 6 WT infected eyes; and both uninfected eyes) were subsequently studied. At day 31 p.i., 2 CJLAT-infected rabbits with clinically normal corneas were removed from this study for use in a different study (data not shown). Six of the 11 eyes (3 eyes were previously excluded) in the 7 CJLAT-infected rabbits that were initially clear developed HSK at 39, 59, 60, and 79 days p.i. None of the 6 eyes from WT infected rabbits developed HSK. The experiment was terminated at day 80 p.i., and the remaining animals were killed.

In Vivo Confocal Microscopy

Starting at day 31, clinically normal eyes from CJLAT, WT, or control latently infected rabbits were evaluated daily up to day 40 and then weekly up to day 80 by in vivo confocal microscopy (CM) for the development of recurrent HSK using methods previously described.[18] Briefly, rabbits were anesthetized with intramuscular ketamine HCl (50 mg/kg body weight, Phoenix Pharmaceutical Inc, St Joseph, MO) and xylazine (5 mg/kg body weight, Phoenix Pharmaceutical Inc) and topical 0.5% proparacaine HCl (Bausch & Lomb Inc, Tampa, FL). Rabbit eyes were then scanned using a Tandem Scanning Confocal Microscope (TSCM, Tandem Scanning Corporation, Reston, VA) with a ×24 surface contact objective (numerical aperture = 0.6 and working distance = 1.5 mm) and 2.5% hydroxymethylcellulose coupling solution (Gonak, Akorn Inc, Buffalo Grove, IL). The axial focal plane position of the objective was controlled by an Oriel 18011 encoder Mike Controller (Oriel Corp, Stratford, CT). Images were captured using a Dage MTI VE-1000 camera (Dage MTI, Michigan City, IN) and digitized using a digitizing board (Data Translation, Marlboro, MA) controlled by specially designed software.[19] For each examination, images were collected of the corneal epithelium, basal lamina, and stroma from the central, nasal, temporal, and inferior corneal regions as well as selected areas showing epithelial damage. Within each region, a through-focus series of images (a 3D data set) extending from the corneal epithelium to the corneal endothelium was also collected, and projections along the *xz* and *yz* plane were generated.

RESULTS

Corneas From Uninfected Rabbits Showed Light Scattering From Superficial Corneal Epithelium, Stromal Keratocyte Nuclei, and Stromal Nerves by In Vivo CM

In vivo CM of healthy uninfected rabbit corneas (Fig. 1) was consistent with our previous studies[20,21] showing light scattering from only the superficial corneal epithelium (Fig. 1A), stromal keratocyte nuclei (Fig. 1B, arrows), and stromal nerves (Fig. 1B, arrowhead). Major light-scattering structures in the normal cornea are better identified in *xz* projections through the 3D data set (Fig. 1C) in which the corneal surface epithelium, basement membrane, and endothelium are detected.

Small Numbers of Subclinical Foci in Corneas of Rabbits Latently Infected With WT HSV-1

In contrast to uninfected corneas, a few corneas from the WT latently infected group of rabbits showed small numbers of isolated regions containing brightly reflecting small cells at the level of the basement membrane (Fig. 2A), which overlaid regions in the stroma that contained granular deposits (Fig. 2B). These granular deposits appeared to contain cellular infiltrates (arrows). We designated these regions (ie, basement membrane + stroma) subclinical foci because these corneas were clear by slit-lamp examination without detectable acute disease. *xz* projections showed that these subclinical foci are small, measuring approximately 100 to 150 μm in diameter, and are limited to the basement membrane/anterior stroma region (Fig. 2C, arrow). These isolated subclinical foci were identified at all time points evaluated (Figs. 2D–F) and showed differing degrees of cell infiltration both at the basement membrane (Fig. 2D) and within the stroma (Fig. 2E), but in the stroma, these foci always appeared limited to the anterior stroma (Fig. 2F, arrow). Subclinical foci could not be identified in every WT infected cornea at every time point because of both difficulty in locating these small lesions and their rapid resolution/disappearance over time.

Numerous Subclinical Foci Revealed by In Vivo CM of Corneas From Rabbits Latently Infected With CJLAT

In CJLAT rabbit corneas, subclinical foci were much more abundant. They were easily detected by in vivo CM in every CJLAT-infected eye at all times examined after day 31 p.i. They appeared similar to the subclinical foci in WT infected corneas (Fig. 3A) except that they extended deeper within the corneal stroma (Fig. 3A, upper left panel, asterisk). These larger subclinical foci in the CJLAT-infected rabbit corneas appeared to contain cellular infiltrates similar to those identified in WT infected corneas (Fig. 3A, lower panel). Using a mid-stromal nerve identified in the 3D data set at day 35 (3A, upper right panel, arrow), the same region was identified at day 40 (Fig. 3B, upper right panel, arrow). At this time, the stromal foci appeared less localized and scattered considerably less light (Fig. 3B, left upper panel, asterisk). They also appeared to

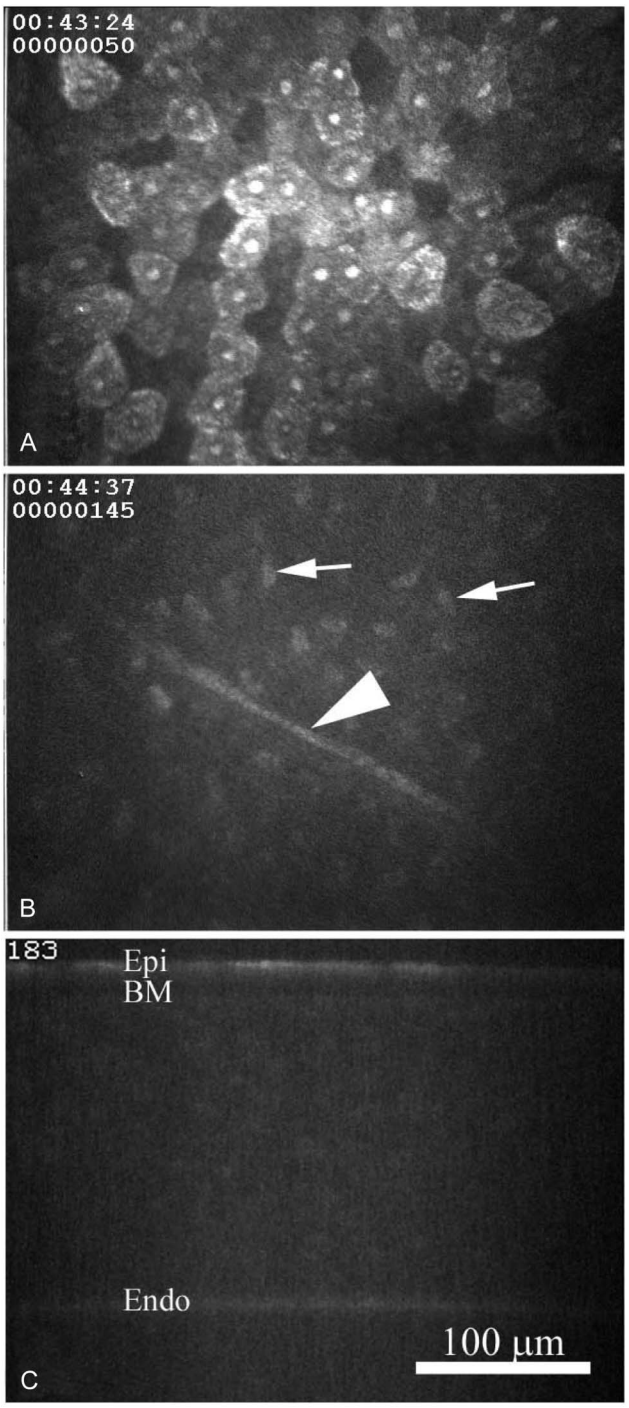

FIGURE 1. In vivo confocal microscopic images of the rabbit cornea showing (A) the normal superficial epithelial cell surface layer, (B) the corneal stroma containing keratocyte nuclei (arrows) and a stromal nerve (arrowhead), and (C) an *xz* cross-sectional view of the 3D data set showing the epithelium (Epi), epithelial basement membrane (BM), and the corneal endothelium (Endo).

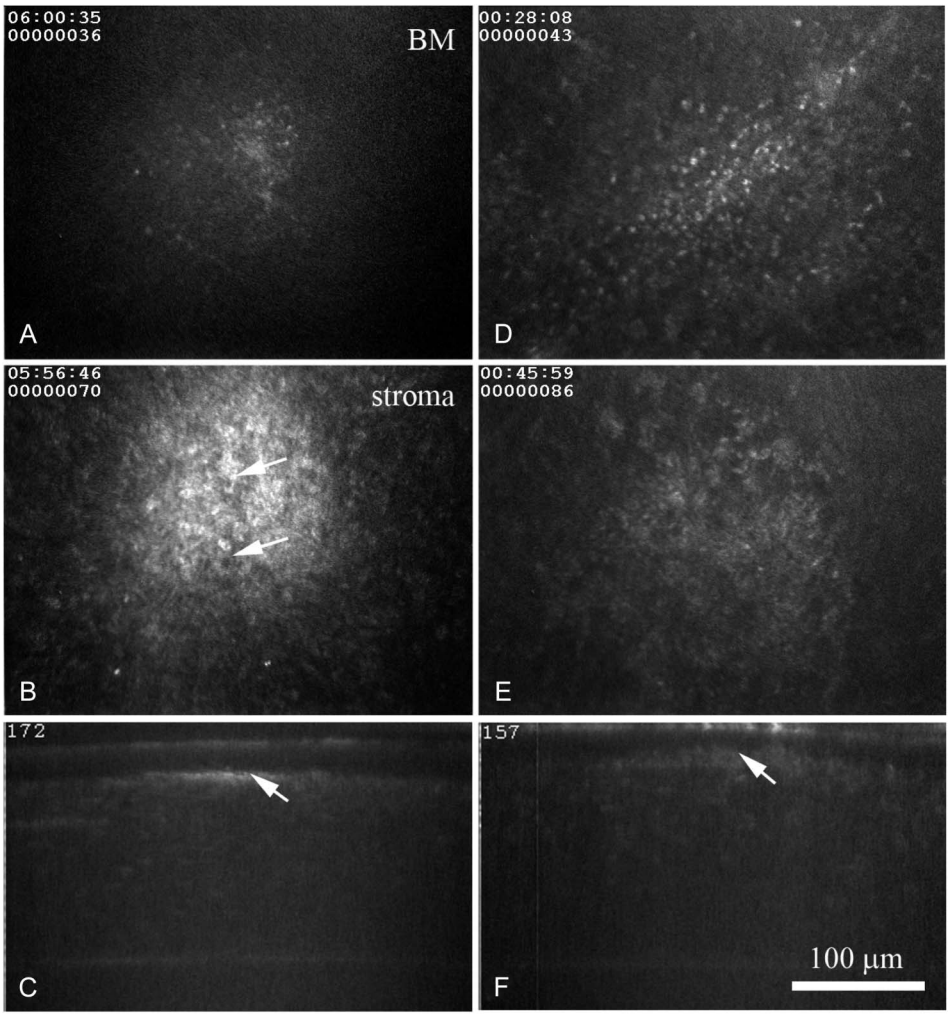

FIGURE 2. In vivo confocal microscopic images from a WT latently infected rabbit eye at 35 days (A–C) and 60 days (D–F) after infection. Images through the basement membrane region (A and D) detected brightly reflecting small cell infiltrates of variable sizes that overlaid granular deposits (B and E) that appeared to be composed of immune cell infiltrates (B, arrows). In the *xz* projections (C and F) note the small region of light scattering (arrow) located at the basement membrane anterior stroma.

contain fewer infiltrating cells (Fig. 3B, lower panel arrows) suggesting resolution of the foci over time.

No epithelial damage was noted in normal corneas or any of the corneas from rabbits latently infected with WT HSV-1. In contrast, epithelial damage was fairly common in corneas of the CJLAT latently infected rabbits and ranged from small epithelial defects (Fig. 4A), to elongated surface epithelial cells suggesting migration (Fig. 4C), to disorganized surface epithelial cells (Fig. 4E). Epithelial defects or epithelial lesions appeared to extend deep within the epithelial sheet, showing necrotic basal epithelial cells adjacent to the basement membrane (Fig. 4B, arrow). Interestingly, regions of epithelial migration, suggesting epithelial lesion healing, were associated with infiltrates of small round cells at the level of the basement membrane (Fig. 4D, arrow). Regions of disorganized surface epithelial cells (late epithelial lesion healing) were also associated with small cell infiltrates within the basement membrane region intermingled with cells that seemed to have dendritic processes (Fig. 4F, arrows). Taken together, these data suggest that reactivated CJLAT virus from the TG produced self-limiting regions of damage that healed by epithelial migration and restratification, and that epithelial lesions recruited small migrating cells that seem to be the start of a subclinical focus.

In all 6 of the corneas from the CJLAT rabbit group that went on to develop active recurrent HSK, clinically recognizable HSK was preceded by the development of an epithelial lesion (Fig. 5A, asterisk, day 34) with an underlying subclinical focus (Fig. 5B) similar to the subclinical foci noted in pre-HSK above (Fig. 3). Interestingly, these overlapping epithelial lesions and subclinical foci persisted and could be detected the next day as regions of bare basement membrane (Fig. 5C, day 35) and the beginning of acute inflammatory cell infiltration into the underlying stroma (Fig. 5D, arrows), respectively. Over the next 5 days, there was a persistent epithelial lesion with bare basement membrane (Fig. 5E, asterisk, day 40) with continued acute inflammatory cell infiltration and massive polymorphonuclear cell infiltration of the stroma that completely obscured normal stromal details (Fig. 5F). Such lesions persisted over the next days and weeks, later developing corneal neovascularization and stromal scarring (not shown).

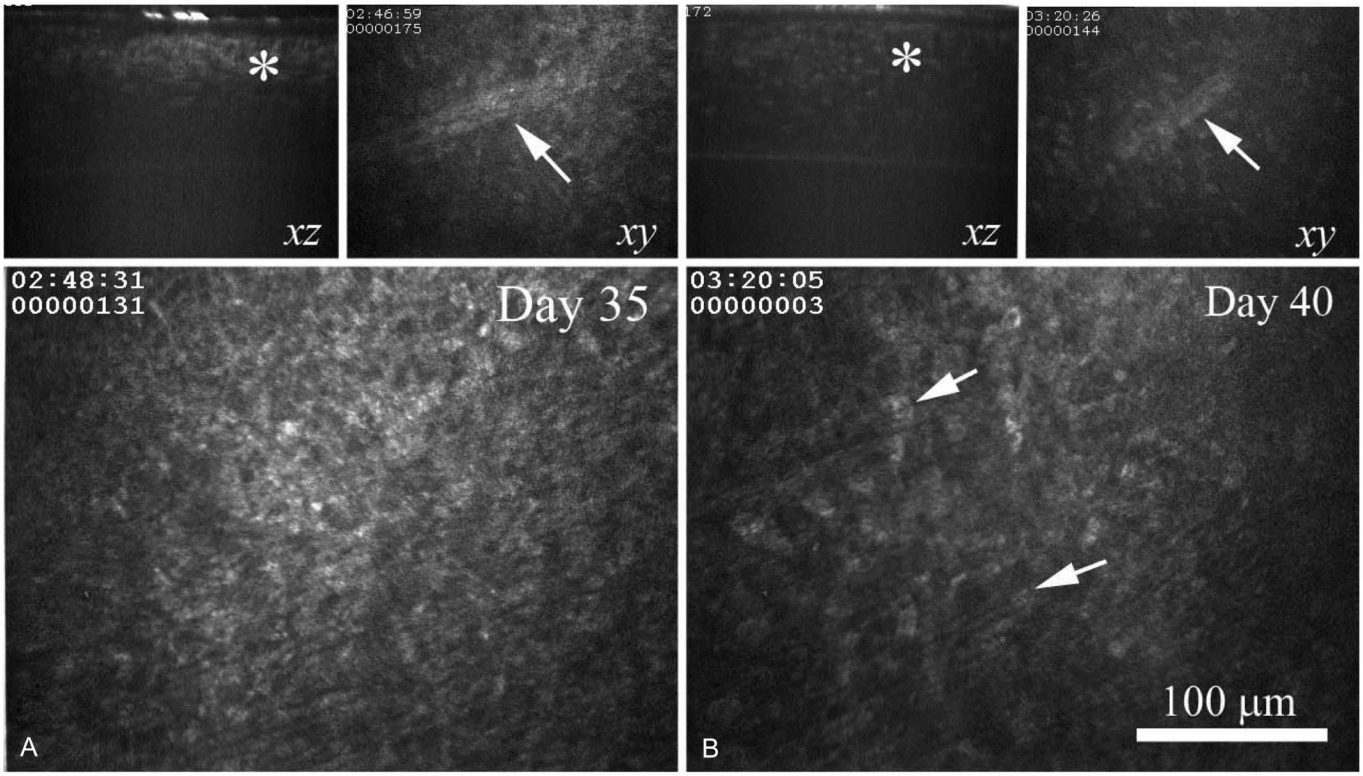

FIGURE 3. In vivo CM of the same eye from a CJLAT latently infected rabbit at day 35 (A) and day 40 (B). Upper left panels in (A) and (B) show *xz* projection through the same subclinical foci (asterisk), the upper right panel shows a nerve (arrow) that was located immediately below the subclinical foci in the same region. The lower panel shows the anterior stroma in the region of the subclinical foci, immediately below the basement membrane. Note that the subclinical foci appear less infiltrated (arrows) at day 40 compared with day 35 suggesting resolution of the lesion.

DISCUSSION

Because of the lack of a reliable animal model, there is a paucity of information regarding the early events in the cornea that lead to recurrent HSK. Human studies on the development of recurrent HSK are generally limited to ex vivo studies of HSK corneal buttons obtained during corneal replacement surgery or in vivo studies of full-blown or resolved HSK in individuals with a history of repeated recurrent HSK. In either situation, it is difficult to extrapolate from the full-blown or resolved disease state back to the important early steps of the disease process. In addition, even in studies of individuals with repeated episodes of recurrent HSK, the early stages of the first recurrent episode cannot be studied.

In the mouse model, severe corneal disease can occur after acute HSV-1 infection, and this has been studied as a model for recurrent HSK (reviewed in Ref. 22), although it is not a recurrent infection and the gross features of the corneal disease do not closely mimic clinical HSK. Spontaneous recurrent HSK does occasionally occur in the rabbit ocular model of HSV-1 latency and reactivation, and the clinical corneal disease is very similar to that seen in humans.[16] However, as in humans, only a very small fraction of rabbit eyes develop recurrent HSK (<2%), making the model refractory to detailed analytical study.[16] Our model of recurrent HSK in rabbits latently infected with the McKrae chimera, CJLAT,[15,16] has allowed us for the first time to investigate early stages of recurrent HSK by in vivo CM.

Acute HSV-1 infection in rabbits (and humans) initially induces epithelial keratitis that is self-limiting and usually resolves within 7 to 10 days. It involves viral replication, recruitment of acute inflammatory cells and macrophages, which scavenge infected cells, and release of cytokines attracting circulating lymphocytes. Although it is generally believed that the presence of lymphoid cells after acute inflection is a transient phenomenon, recent clinical in vivo CM studies have identified clusters of dendritic-like cells in the basal corneal epithelium that overlie regions of "granular fibrosis" in approximately 60% of cases with previous recurrent HSK.[23] The authors speculated that the dendritic-like cells, structures, or particles are clusters of Langerhan cells (LCs) or dendritic, antigen-presenting cells (APCs) recruited to the cornea in response to viral antigens. Although LCs are not specific to viral infection and have been seen occasionally in the normal peripheral cornea and after keratitis of various pathogenic mechanisms, the authors suggest that identification of LCs by in vivo CM may be a valuable tool for the diagnosis of HSK.[23] Interestingly, our in vivo CM data evaluating the progression of recurrent HSV-1 corneal disease in eyes of rabbits latently infected with

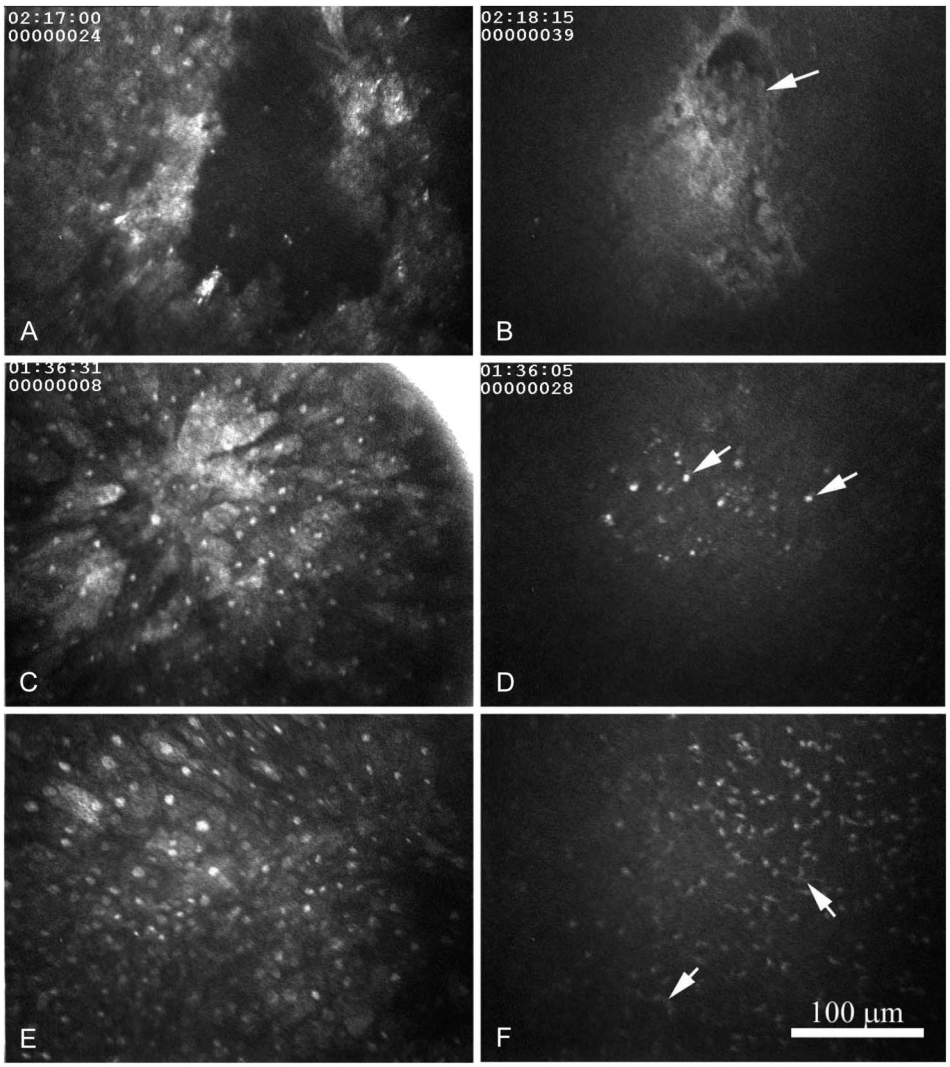

FIGURE 4. In vivo confocal microscopic images of the surface epithelium (A, C, and E) and basement membrane (B, D, and F) showing epithelial erosion (A and B), migrating epithelium (C and D), and disorganized surface epithelium (E and F). At the level of the basement membrane, epithelial erosions appear to contain necrotic basal epithelial cells (B, arrow), whereas regions of regenerating epithelium (C and E) were associated with varying degrees of small cell infiltrates (D and F, arrows).

CJLAT show cellular and structural changes in the cornea that appear identical to those observed by in vivo CM of human cases of resolved HSK.[23]

Thus, in these studies, corneas of rabbits latently infected with CJLAT contained numerous small subclinical foci containing dendritic-like cells within the basal epithelium and stromal cell infiltrates or "granular fibrosis" immediately underneath. These subclinical foci and our in vivo CM images of the pre-HSK CJLAT cornea are consistent with the description and in vivo CM images reported in over half of human corneas with previous recurrent HSK.[23]

We also performed some ex vivo CM studies on the cornea from these studies. Unfortunately, although several different mAbs can be used to detect a variety of rabbit immune cells by FACS, the only mAb we found useful for detection of rabbit immune cells by ex vivo CM was a pan-leukocyte CD45 antibody. Because CD45 is a marker for bone marrow–derived immune cells, we were able to confirm that the infiltrating cells seen in the cornea by in vivo CM were in fact immune cells (not shown), but the type of immune cells present could only be extrapolated based on the relative size and morphology. Based on the in vivo CM studies and the knowledge that the infiltrating cells seen were CD45 positive, it seems that subclinical foci develop after transient focal epithelial injury (epithelial lesion; perhaps because of return of reactivated virus to the corneal epithelium), which in turn recruits epithelial dendritic cells/Langerhans cells (DCs/LCs) and stromal immune cell infiltrates. Subclinical foci also seemed to resolve over time. In eyes with no clinical disease, active epithelial lesions and active subclinical foci were never seen to overlap. In contrast and importantly, in all 6 CJLAT eyes that developed HSK, the recurrent HSK seemed to develop around subclinical foci that had an overlying epithelial lesion. These combined foci (epithelial lesion + subclinical foci) appeared to persist for days, later involving infiltration of what appeared to be acute inflammatory cells and developing into HSK. Thus, there seems to be an important relationship between subclinical foci, and particularly subclinical foci combined with an epithelial lesion (epithelial lesion + subclinical foci), and

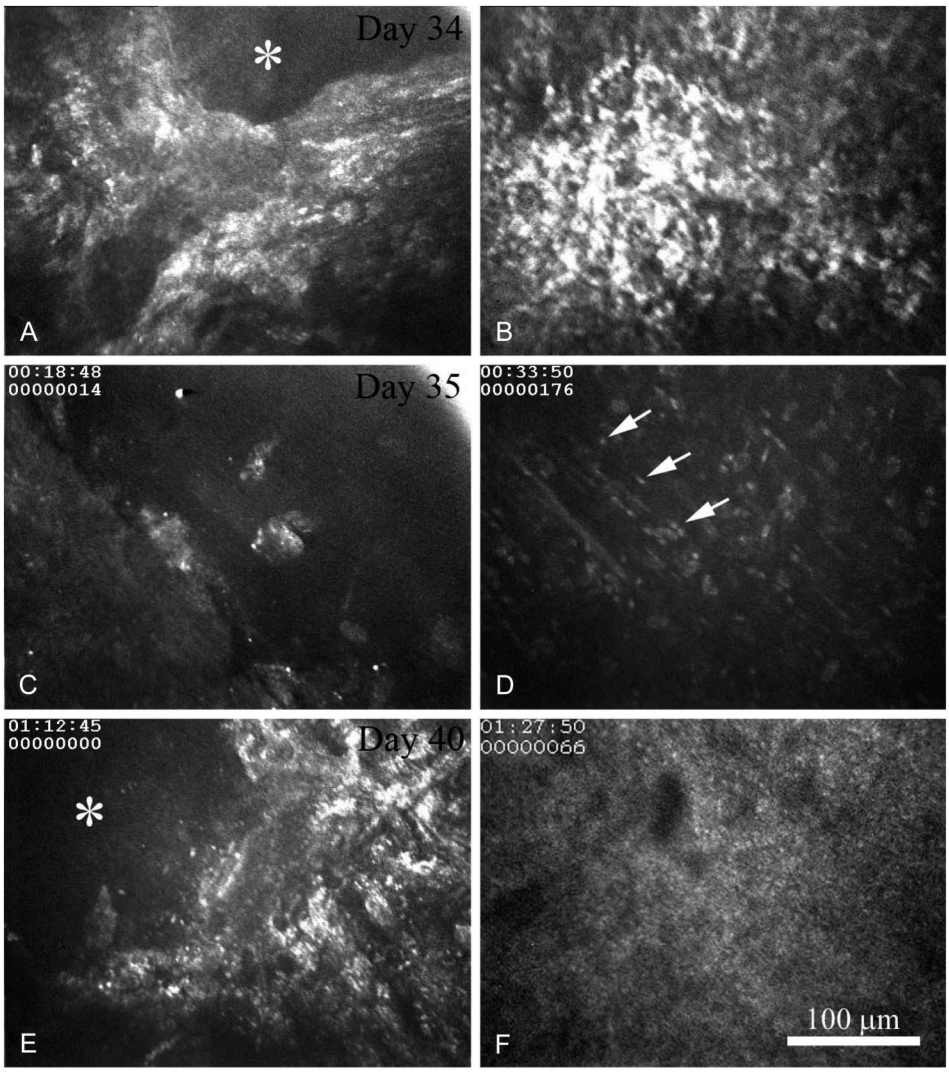

FIGURE 5. In vivo confocal microscopic images of active recurrent HSK in the same eye beginning at day 34 (A and B) and extending to day 35 (C and D) and day 40 (E and F). Images are taken at the level of the corneal surface (A, C, and E) and anterior corneal stroma (B, D, and F) in the same 3D data set. The first initiation of recurrent HSK at day 35 was associated with the appearance of an epithelial lesions and exposure of the basement membrane (A, asterisk) that immediately overlaid a subclinical focus (B). Progression of recurrent HSK at day 36 was associated with persistent epithelial erosion (C) and the infiltration of the anterior stroma with acute inflammatory cells (D, arrows). Four days later, at day 40, persistent epithelial erosion (E, asterisk) was associated with massive polymorphonuclear cell infiltration (F).

HSK. Interestingly, subclinical foci in the CJLAT-infected corneas were larger and much more frequent than those seen in eyes of rabbits infected with WT McKrae. If these subclinical foci are important for the ensuing development of HSK, the increased size and number in CJLAT compared with WT infected eyes are consistent with the much greater frequency of HSK development in rabbits latently infected with CJLAT compared with WT McKrae. Unfortunately, because of the relatively small number of eyes in the above studies, a rigorous statistical analysis to quantify the frequency at which subclinical foci resulted in HSK was not possible.

We propose a model of the relationship between reactivated virus from the TG returning to the cornea, epithelial lesions, subclinical foci, and the development of HSK. Primary ocular infection, usually clinically unnoticed, results in priming of T cells in draining lymph nodes, lifelong viral latency in the TG, clearing of corneal disease, and a normal-appearing cornea. Spontaneously reactivated HSV-1 from the TG returns to the cornea and infects and kills epithelial cells producing small transient punctate epithelial lesions lasting less than 24 hours. Antigens produced by viral replication are taken up by APCs (LCs/DCs and macrophages) and cause signaling to the cornea, which recruits immune cells to the site in 2 to 4 days, by which time the epithelial lesion has resolved. In the absence of target viral Ags, the infiltrating immune cells remain as subclinical foci composed of DCs/LCs in the basal epithelium and leukocytes in the underlying anterior stroma. These subclinical foci are detectable by in vivo CM but not by clinical examination. In the absence of new viral Ag, the immune cells slowly leave (~3–7 days) and the subclinical foci are resolved. This cycle can occur repeatedly at the same and/or different locations in the cornea, resulting in new subclinical foci comprising increasingly more specific and more stimulated functional leukocytes. In the rare event that a new epithelial lesion forms over the subclinical foci before it has had time to resolve or the original epithelial lesion persists until the subclinical foci fully form, a fast and robust immunopathologic response is induced in the subclinical foci because of viral Ags, APCs stimulation, and cross-priming of local memory T cells.

Because the memory T cells are highly responsive because of numerous previous rounds of subclinical foci, even small amounts of remaining viral Ags are likely sufficient to cause multiple rounds of immunopathological responses resulting in persistent and more extensive epithelial lesions similar to HEK in humans and the development of early HSK, immunopathologic damage to both stroma and epithelium, and ongoing HSK.

In summary, these studies provide new insights into the cellular mechanisms that lead to the development of HSV-1–induced immunopathological recurrent HSK and will be useful for helping to direct the development of effective antiviral immunotherapeutic and antiviral drug strategies.

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