HHS Public Access

Author manuscript

Curr Eye Res. Author manuscript; available in PMC 2017 March 14.

Published in final edited form as:

Curr Eye Res. 2016 ; 41(3): 284–291. doi:10.3109/02713683.2015.1020172.

Large Amounts of Reactivated Virus in Tears Precedes Recurrent Herpes Stromal Keratitis in Stressed Rabbits Latently Infected with Herpes Simplex Virus

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Abstract

Aim—Recurrent herpetic stromal keratitis (rHSK), due to an immune response to reactivation of herpes simplex virus (HSV-1), can cause corneal blindness. The development of therapeutic interventions such as drugs and vaccines to decrease rHSK have been hampered by the lack of a small and reliable animal model in which rHSK occurs at a high frequency during HSV-1 latency. The aim of this study is to develop a rabbit model of rHSK in which stress from elevated temperatures increases the frequency of HSV-1 reactivations and rHSK.

Materials and methods—Rabbits latently infected with HSV-1 were subjected to elevated temperatures and the frequency of viral reactivations and rHSK were determined.

Results—In an experiment in which rabbits latently infected with HSV-1 were subjected to ill-defined stress as a result of failure of the vivarium air conditioning system, reactivation of HSV-1 occurred at over twice the normal frequency. In addition, 60% of eyes developed severe rHSK compared to <1% of eyes normally. All episodes of rHSK were preceded four to five days prior by

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DECLARATION OF INTEREST The authors report no conflicts of interest.

None of the authors have a commercial relationship in the form of financial support or personal financial interest with any company.

an unusually large amount of reactivated virus in the tears of that eye and whenever this unusually large amount of reactivated virus was detected in tears, rHSK always appeared 4–5 days later. In subsequent experiments using well defined heat stress the reactivation frequency was similarly increased, but no eyes developed rHSK.

Conclusions—The results reported here support the hypothesis that rHSK is associated not simply with elevated reactivation frequency, but rather with rare episodes of very high levels of reactivated virus in tears 4–5 days earlier.

Keywords

Herpes simplex virus; rabbit; reactivation; recurrent herpes stromal keratitis; stressed

INTRODUCTION

Estimates are that 50% to over 80% of adults in the United States are infected with herpes simplex virus type 1 (HSV-1).[1–3] Following primary infection of the eye, HSV-1 establishes lifelong latency in sensory neurons of the trigeminal ganglia (TG). Spontaneous reactivation of HSV-1 from latency results in virus returning to and replicating in the eye. Here it is shed in tears and can be detected by plating tears (eye swab) on indicator cells in tissue culture. In the majority of adults sporadic episodes of spontaneous reactivation occur frequently. By PCR analysis for viral DNA which is more sensitive that virus cultures, over 30% of tears in 50 healthy individuals contained reactivated HSV-1.[4] In contrast, recurrent HSV ocular disease (recurrent herpes stromal keratitis or rHSK) is rare with less than 1% of people having experienced such disease.[5] Nonetheless, rHSK is an important medical problem, with approximately 500,000 people in the US having a history of this potentially blinding disease.[3,5] rHSK, triggered by recurrent bouts of HSV-1 reactivation and shedding into the cornea,[6–8] includes stromal opacity, edema and neovascularization. It is the leading cause of corneal blindness due to an infectious agent in the developed world.[9]

rHSK is due to an inflammatory response. However the viral/host proteins involved, and the specific innate and adaptive immune effectors and their kinetics, remain to be elucidated. There is no therapeutic vaccine for rHSK, long term antiviral therapy (Acyclovir family drugs) only decreases rHSK by ~40%,[10] and viruses resistant to Acyclovir are often selected for in immunocompromised patients.[11–13] Approximately 5–10% of AIDS patients and 18% of bone marrow transplant recipients develop Acyclovir resistant HSV-1 isolates.[14–16]

Most animal studies of ocular HSV-1 latency, reactivation and recurrent eye disease have been done in rabbit and mouse models.[8] Rabbits support spontaneous reactivation of HSV-1 from latency.[17,18] The virus returns to the eye, is shed in tears, and can occasionally cause rHSK that appears similar to rHSK in humans.[19] However, as in humans, rHSK is rare in rabbits (<1% of eyes).[19] In mice latently infected with HSV-1, little or no spontaneous reactivation is detected *in vivo*[20,21] (i.e. reactivated virus is not detected in tears) and thus no recurrent HSK occurs during latency in mice.

As discussed above, studies of the mechanisms involved in the development of rHSK and studies to decrease recurrent herpetic ocular disease by therapeutic vaccines or drugs have

been severely hampered by the lack of a small animal model in which rHSK occurs at a frequency that is sufficiently high to allow detailed qualitative and quantitative studies. In this report, we present the results of a serendipitous rabbit experiment in which latently infected rabbits developed severe rHSK during latency at a very high frequency (60% of eyes during a 26 day period). Attempts to develop a model of high frequency rHSK in rabbits are discussed.

MATERIALS AND METHODS

Cell Lines

Rabbit skin (RS) cells were maintained in Eagle minimal essential medium (MEM) with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 10% fetal bovine serum (Promega Scientific, Promega, Madison, WI), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Sigma, St. Louis, MO).

Viruses

The McKrae strain of HSV-1 was used in all studies. Virus was plaque purified at least three times and then passaged only one or two times in RS cells prior to use. Wild-type McKrae has been previously described.[17]

Rabbits

Eight- to 10-week-old female New Zealand Rabbits were used. Infection of eyes was done using eye drops (2×10^5 pfu/eye in 25 μl of tissue culture media) without corneal scarification as we previously described.[22–24]

Eye Swabs and Titration of Reactivated Virus in Tears of Latently Infected Rabbits

Tear films were collected from both eyes of all rabbits once a day from day 31 to 56 post-infection (p.i.) at which time latency was well established, using a Dacron-tipped swab as we previously described.[17] Each swab was placed in 1 ml of tissue culture medium, squeezed and removed from the tube. Tubes were stored at −80 °C and later thawed and the amount of virus determined by plating either 10 or 100 μl of the 1 ml sample on RS cells for a standard plaque assay and counting viral plaques 3 days later.

RESULTS

A small latency-reactivation study in rabbits was performed to test out some newly renovated vivarium space. Ten rabbits were ocularly infected in both eyes with 2×10^5 pfu/eye of wild type HSV-1 strain McKrae. This was done as eye drops without corneal scarification as described in Materials and Methods. Between days 31 and 56 p.i., when latency is well established, tears were collected daily from each eye for analysis of the presence of reactivated virus as described in Materials and Methods. We later found that the air handling system had malfunctioned as had the light cycling system. The temperature in the room was estimated to have reached 90+ °F range (~33+ °C) each afternoon and the room lights were on 24 h/day.

Stressful Environmental Conditions Appear to have Significantly Increased *In Vivo* HSV-1 Reactivation as Judged by Shedding of Virus in Tears

The cumulative number of tear cultures/eye that was positive for reactivated virus (i.e. shedding of reactivated virus) was significantly elevated under the above stressful conditions. For comparison, the pooled results from four unrelated studies with the same wt strain of HSV-1 were compared to the results with the stressed rabbits. The cumulative number of positive tear cultures/eye over a 26 day period was less than three under normal conditions compared to almost six under the stressful conditions (Figure 1). This difference was highly significant ($p = 0.0001$). The percent of eye swabs that were positive for virus under normal conditions was approximately 11% (295 virus positive swabs from a total of 2704 swabs) while under stressful conditions it was approximately 23% (59/260 virus positive swabs) (Table 1). This was highly significant ($p<0.0001$). Another method we often use to compare *in vivo* reactivation in rabbit eyes is the number of reactivation episodes. A reactivation episode is defined as the number of reactivation events, assuming that virus positive tear cultures on successive days result from a single reactivation event. For example, if shedding of virus is detected in an eye on days 7 and 8 it is considered one event. In contrast, if virus is detected in an eye on days 7 and 9, but not on day 8, it is considered to be two events. By this analysis, the number of reactivation episodes/eye under normal conditions was approximately 1.6, while for the stressed rabbits it was 4.0 (Table 1). Again, this was highly significant ($p<0.0001$). Thus, it appeared that when the rabbits were housed in the stressful environment (high temperatures, lights on 24 h/day, and/or other unknown environmental factors) HSV-1 reactivation from latency, as judged by the frequency at which virus was detected in tears, was significantly increased.

Stressful Environmental Conditions Appeared to Significantly Increase the Frequency of Severe Recurrent Herpes Stromal Keratitis

Similar to **Recurrent Herpes Stromal Keratitis** (rHSK) in humans, rHSK in rabbits is rare (<1% of eyes of rabbits latently infected with wt McKrae eyes).[19] In the above stressed rabbits, obvious and severe rHSK that could be seen easily even by an untrained observer, occurred at a high rate (Table 1). Six of 10 eyes of the stressed rabbits developed severe rHSK during the 26 day latency study period compared to 0/104 eyes in the normal unstressed rabbits latently infected with the same virus. This was highly significant ($p<0.0001$). Examples of rHSK in two rabbit eyes on day 56 p.i. are shown in Figure 2. In panel A severe corneal opacity is visible in the lower left portion of the eye. In panel B similar disease is seen at the upper portion of the cornea. Panel A also shows obvious neovascularization above the cornea and in the upper portion of the cornea.

Recurrent HSK was Preceded by a Large Bolus of Reactivated Virus in Tears 4–5 Days Earlier

Analysis of the above results from stressed rabbits indicated that when an extraordinary amount of reactivated virus appeared in rabbit eyes (tears), severe rHSK developed in that eye 4–5 days later. No rHSK developed in the absence of this large bolus of virus. Figure 3 shows the relative amount of virus shedding (black squares) and the relationship to the time of first appearance of rHSK in the same eye (black bars). In all 6 of the eyes that developed

rHSK, the disease was preceded 4 or 5 days earlier by a large bolus of virus being detected in the tears (panels B, C, E, G, H and J). This is represented by an arrow. In contrast, in eyes that did not develop rHSK, no such large bolus of virus was detected (panels A, D, F and I). Thus, when a large amount of virus was detected, obvious and severe rHSK always developed 4–5 days later. In contrast, rHSK did not develop in eyes in which this large amount of virus was not detected. This was highly significant (Table 2; 6/6 versus 0/4; $p<0.0005$, Fisher's exact test).

Other comparisons between the eyes of stressed rabbits that developed rHSK (rHSK eyes) and those that did not develop rHSK (non-rHSK eyes) revealed additional interesting findings (Table 2). The overall reactivation frequency (shedding) was similar in the two groups. Reactivated virus was detected in 27/104 (26%) of the non-rHSK eye swabs compared to 32/156 (21%) of the swabs from rabbits that developed rHSK. This was not significantly different ($p = 0.36$). In eyes that developed rHSK, 31/86 (35%) of the eye swabs were positive prior to the first appearance of rHSK. The average time at which rHSK appeared in the rHSK eyes was day 15 of the study period (day 45 p.i.). Prior to this time (i.e. days 1–14 of tear swab collection) only 13/56 (23%) of the eye swabs from the non-rHSK eyes were virus positive. However, the trend towards more HSV-1 reactivations prior to the time of the first appearance of rHSK in the rHSK compared to the non-rHSK eyes did not reach significance ($p = 0.14$). After rHSK was first seen, only 1 of 62 (2%) of eye swabs had detectable virus, while in the non-rHSK group 12 of 44 (27%) of the eye swabs contained reactivated virus between day 16 and 26 of the study. This was highly significant ($p<0.0001$). Thus, either little or no additional reactivated virus returned to eyes with obvious rHSK (perhaps due to damage of the nerve endings in the cornea), or if virus did return to the eye, the rHSK interfered with virus detection (perhaps the damage to the corneal tissue resulted in little or no virus replication in the eye).

Attempts to Develop a Controlled Rabbit Eye Model with High Reactivation and rHSK

The above stressed rabbit model would be an excellent model: (a) to investigate the events leading to/causing rHSK; (b) for pre-clinical therapeutic studies of candidate drugs to reduce rHSK in individuals with a history of rHSK and (c) for preclinical studies of candidate therapeutic vaccines to reduce/prevent rHSK in individuals with a history of multiple episodes of rHSK. Unfortunately, the above results were serendipitous. When the air handling system and the light cycling system were functioning properly both reactivation (approximately 10%) and rHSK (none) returned to normal. Thus, it is likely that the high reactivation and high rHSK levels were due to the elevated temperatures and/or the continuous room lighting, or possibly other unrecognized stressors. However, we did not know what the vivarium temperatures were on each day, nor did we know the highest temperature reached on any given day. We therefore tried to reproduce the results under more controlled conditions.

Since we knew that both the temperature and the light cycling had been defective throughout, we reprogramed the temperature controls in a fully functional vivarium room such that the temperature cycled as follows: (1) ~70°F (21 °C) from 7 PM to 10 AM; (2) gradual increase from 10 AM to 11 AM to ~90°F (~32 °C) and (3) gradual decrease from 5

PM to 6 PM to ~70°F. We hoped this would simulate the daily afternoon elevated temperatures in the original uncontrolled vivarium. The room lights remained on throughout. The original study was repeated under these conditions using more eyes of latently infected rabbits than originally (16 versus 10). Reactivation frequency was examined for 26 days during latency as in the original experiment. Sixty out of 337 eye swabs (18%) were positive for reactivated virus. This was significantly higher than the typical frequency of ~11% (295/2704; Table 1) in eyes of rabbits housed under normal environmental conditions (p = 0.0004). However no rHSK was detected. We repeated the experiment using an elevated temperature of 99°F (~37 °C), the highest temperature that could be achieved in the room. Shedding was not examined, but again no rHSK was detected. We then decided to subject rabbits to even higher temperatures by placing them into an incubator for a short time. To determine the highest temperature that could be used to stress rabbits without causing undo harm, individual uninfected rabbits were subjected to various temperatures for 20–30 min. An incubator with a large glass door was used to allow continuous monitoring by a veterinarian. The temperatures used were 43 °C (109°F), 45 °C (113°F), 47 °C (116°F), 49 °C (120°F), 51 °C (124°F), 53 °C (127°F), 55 °C (131°F), 57 °C (135°F) and 60 °C (140°F), the highest temperature that could be achieved by the incubator. Internal body temperature rose to ~106–107°F (~41.1–41.7 °C) at the higher temperatures. Even at 60 °C, no acute or long term undue stress or health problems were encountered other than panting, increased salivating and licking of front limbs, and aural vasodilation which immediately stopped upon return to 70°F. We therefore did the following study. Rabbits housed under normal environmental conditions were ocularly infected as above. On day 30 p.i., each rabbit was placed in the 60 °C incubator which equilibrated back to 60 °C within one minute. The rabbits were removed after 25 min at 60 °C. Shedding was not examined, but again, no rHSK was detected.

DISCUSSION

Following primary HSV-1 infection of the eye, the virus replicates locally, infects sensory axons in the cornea, and is transported up the axon to the nerve body in the TG. Here it establishes life-long latency with sporadic episodes of reactivation. Most HSK follows reactivation of virus in the TG, the return of virus to the eye, and inflammatory immune responses that lead to disease, including corneal clouding which can compromise sight. Most animal studies of HSK have used mouse models and have studied corneal disease that is a sequela of the primary infection, rather than disease that follows reactivation of HSV-1. Rabbits, in which HSV-1 reactivation occurs at high frequency, nonetheless only have a low frequency of HSK that is typically insufficient for systematic study.

We report here an experiment in which rabbits latently infected with the wt HSV-1 strain McKrae had a much higher frequency of *in vivo* reactivation (virus shedding in tears) than normal and in which 60% of eyes developed severe rHSK (Figure 1 and Table 1). Since rHSK in rabbits, as in humans, is a rare event (<1% of eyes), these results were quite intriguing. Furthermore, each rHSK episode was preceded 4–5 days earlier by the detection of a large amount of reactivated virus (>50,000 pfu) in the tears of the eyes that developed rHSK (Figure 3). This is in comparison to an average of ~200 pfu (median ~50 pfu) in HSV-1 positive eyes that did not develop rHSK shortly after. Thus, when a large amount of

virus was detected, the development of obvious and severe rHSK always followed 4–5 days later. In contrast, rHSK did not develop without this large amount of virus. This was highly significant ($p<0.0005$).

It is well known that in humans HSV-1 reactivations and recurrent disease are increased by various forms of environmental, emotional, physical and chemical stress. Indeed, we discovered after completion of the experiment, that both the vivarium air handling system and the vivarium room light controls had malfunctioned from prior to the time the rabbits were infected through the end of the experiment. This resulted in abnormally elevated afternoon temperatures and continuous room lighting (24 h/7 d). It was estimated that on most summer afternoons the temperature in the rabbit housing room rose to between ~92°F and ~98°F, with occasional afternoons of over 100°F. During the evening, nights and early morning the room temperature likely returned to more normal rabbit housing standards of ~70°F.

After the cooling and lighting dysfunctions were fixed, in future experiments virus reactivation returned to lower normal rates and rabbit eyes did not develop rHSK during latency. To try to develop the above exciting serendipitous findings into a useful working model of high frequency rHSK, we obtained IACUC approval to try to reproduce the stressful housing environment and arranged for Facilities to automate a rabbit room to simulate the stressful conditions. Unfortunately, as explained in the Results section, multiple attempts using increasingly elevated temperatures even with continuous room lighting failed to produce any rHSK during latency. In one experiment we even subjected latently infected rabbits to extremely high temperatures of 140°F or 60°C, for 25 min. The idea for this was based on a heat stress induced reactivation model in mice.[25] Placing latently infected mice into a 43 °C water bath for 10 min does not induce HSV-1 reactivations that can be detected *in vivo*, nor is rHSK induced. However, reactivated virus can be detected in TG harvested one day after the stress. Unfortunately, our one-time heat stress of latently infected rabbits failed to induce rHSK. Although we were unable to reproduce the original serendipitous results, several interesting pieces of information were gleaned as summarized in the next paragraph.

Environmentally stressful conditions such as excessive heat can significantly increase the *in vivo* reactivation frequency in latently infected rabbits from approximately 10–11% to over 18–23% of tear swabs. In an unpublished study in which latently infected rabbits were stressed by a different set of environmental factors resulting from an earthquake, the reactivation rate for wt McKrae was >20%, but no eyes developed rHSK. Altogether these results suggest that increased reactivation frequency alone does not result in more eyes developing rHSK. Rather, rHSK appeared linked to an unusually high amount of reactivated virus in the eye 4 to 5 days earlier. Thus, it appears that a large bolus of reactivated virus in the eye, rather than a higher than normal frequency of smaller amounts of reactivated virus, triggers the development of rHSK. We hypothesize that the large bolus of virus we detected in rabbit eyes was the result of much higher than normal replication of reactivated virus that had returned to the eye. If this is the case, treatment modalities that prevent large amounts of reactivated virus from replicating in the eye should be successful at decreasing rHSK in individuals with a history of multiple episodes of rHSK.

ACKNOWLEDGMENTS

We thank Dr. Nigel Fraser for reading this manuscript and providing helpful comments.

The authors alone are responsible for the content and writing of the paper. This study was supported by Public Health Service NIH grants 1R56AI098985, 1R56AI093133, R01EY013191, RO1EY019896, RO1EY14900 and EY024618, and The Discovery Center for Eye Research.

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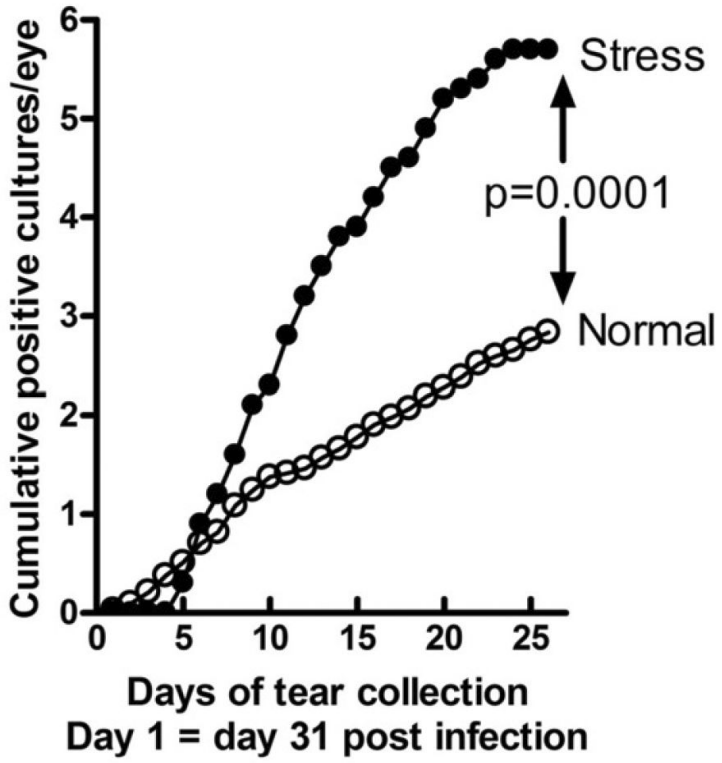

FIGURE 1.

Cumulative reactivation in eyes of stressed and unstressed (normal) rabbits latently infected with wild type HSV-1 strain McKrae. Rabbits were bilaterally ocularly infected with 2×10^5 PFU of McKrae per eye as described in Materials and Methods. Tear films were collected by swabbing eyes daily for 26 days beginning on day 31 post-infection (day 1) and plated on indicator cells for the presence of reactivated virus as described in Materials and Methods. The results are plotted as the cumulative number of virus-positive cultures divided by the total number of cultures divided by the number of eyes. This gives the cumulative number of the average virus positive tear film cultures per eye on each day. Stress: Solid circles represent the results from 10 eyes from rabbits latently infected with wt HSV-1 that underwent environmental stress in the dysfunctional vivarium room. Eyes averaged almost six virus positive cultures each. Normal: Open circles represent the results from 104 eyes from rabbits latently infected with wt HSV-1 that were housed in normal environmental conditions. These results are pooled from four experiments and averaged just under three virus positive cultures per eye.

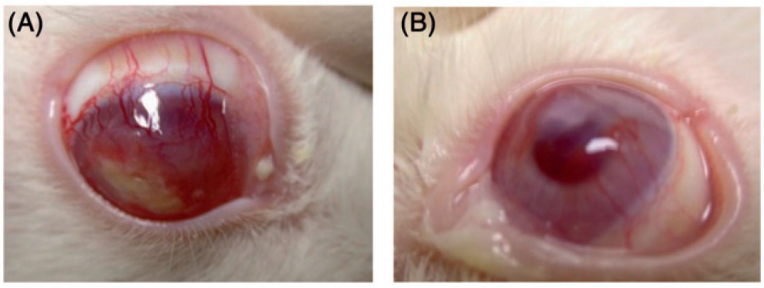

FIGURE 2.

rHSK in stressed rabbits. Photographs of eyes from stressed rabbits 56 days p.i. Areas of opaque clouding can be seen in both eyes. Neovascularization is also present in both eyes, but is more easily seen in the eye in panel A. Panels A and B are from different rabbits.

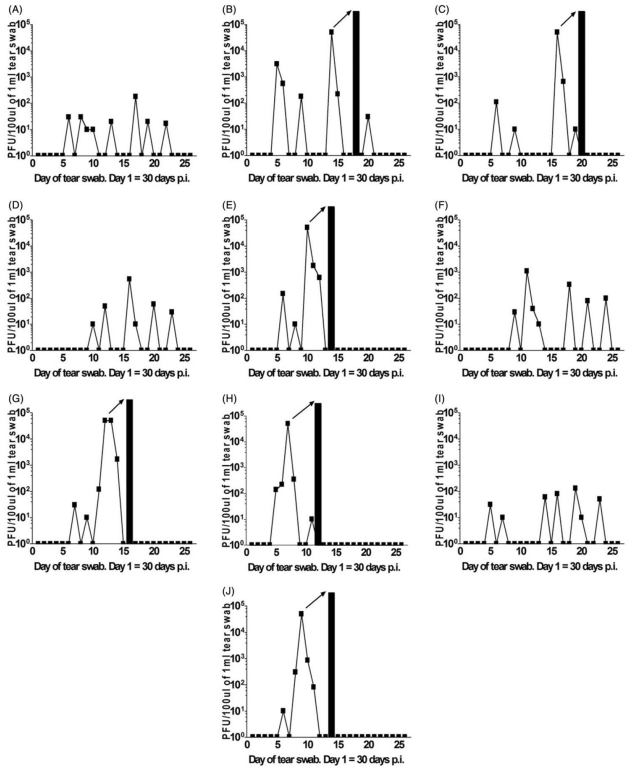

FIGURE 3.

Abnormally high amounts of reactivated virus detected in tears led to obvious rHSK 4–5 days later. The amount of virus detected in tears on each day is plotted as solid squares. The solid black bars indicate the time at which obvious rHSK was first seen. Panels A–J each show the results from a different rabbit eye. The arrows in panels B, C, E, G, H and J connect the abnormally high amount of virus in tears to the development of obvious rHSK 4–5 days later. The amount of virus in each eye was estimated by plating 10 and 100 μl of a 1 ml eye swab on RS cells and counting virus plaques 3–4 days later. The numbers for the highest amounts (which are the origins of the arrows) are shown here as 5×10^4, but this is a lower estimate, as there were too many plaques to count at the highest dilution plated from the eye swabs.

TABLE 1

Additional analyses of the experiments shown in Figures 1 and 2[a].

Groups	Number of reactivated virus positive tears/total number (%)[a]	Average number of reactivation episodes/eye[b]	Number of eyes developing severe rHSK during latency/total eyes[c]
Normal	295/2704 (10.9%)	1.59	0/104 (0%)
Stressed	59/260 (22.9%)	4.0	6/10 (60%)
p value[d]	$p<0.0001$ (Fisher's exact)	$p<0.0001$ (Student's *t*-test)	$p<0.0001$ (Fisher's)

[a]Tears were collected daily from day 31 to 56 p.i. and the presence of reactivated virus was determined as described in Materials & Methods and the legend to Figure 1.

[b]The definition of “reactivation episode” is defined in the text.

[c]All eyes were disease free on day 30 p.i. The presence of rHSK was determined daily from days 31 to 56 p.i. The rHSK detected was severe and obvious by casual observation.

[d]*p* Values were determined using the indicated test (two sided test). The groups are considered significantly different if $p<0.05$.

TABLE 2

Comparisons of eyes from stressed rabbits that did or did not develop rHSK[a].

	Eyes w/no disease	Eyes w/severe rHSK	Fisher's exact test[e]
Large bolus of reactivated virus leading to rHSK 4–5 days later	0/4	6/6	$p = 0.0005$
Pos. cultures/total cultures (%)[b]	27/104 (26%)	32/156 (21%)	$p = 0.36$
Pos. cultures prior to rHSK/total pre-rHSK cultures (%)[c]	13/56 (23%)	31/86 (35%)	$p = 0.14$
Pos. culture post rHSK/total post-rHSK cultures (%)[d]	12/44 (27%)	1/62 (2%)	$p<0.0001$

[a]All eyes are from the original serendipitously stressed rabbits in the “Stress” group of Figure 1.

[b]Aliquots of daily eye swabs (tear films) were collect from each eye over a 26 day period and plated on indicator cells to determine the presence or absence of reactivated virus.

[c]For eyes that developed rHSK, the total cultures are for the days prior to the appearance of rHSK in that eye. The average time was 14.3 days. For eyes that never developed rHSK, the first 14 days of eye swabs were used.

[d]For eyes that developed rHSK only the eye swabs after rHSK was seen in that eye are included. For eyes that never developed rHSK, only days 15–26 were used.

[e]A two sided analysis was done. A $p<0.05$ is considered significant.