Non-traumatic acquisition of herpes simplex virus infection through the eye

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Abstract
Primary ocular herpes is usually seen as a follicular conjunctivitis and blepharitis, with or without involvement of the cornea. It is unknown, however, to what extent asymptomatic and/or subclinical primary disease occurs, and whether primary ocular herpes follows direct droplet spread to the eye. Previous models of murine ocular herpes have used trauma (scarification) to introduce virus into the cornea, producing disease which results in significant corneal scarring. To mimic a likely route of infection in humans, a droplet containing virus was placed on the mouse eye and clinical disease recorded. At least 1 month after inoculation, serum was assayed for neutralising antibodies and the cornea, iris, and trigeminal ganglion were investigated for evidence of herpes simplex virus type 1, by cocultivation and the polymerase chain reaction. Some animals showed a severe ulcerative blepharitis with little to no involvement of the cornea, while disease was undetectable in others. The development of disease depended on the dose and strain of virus and age of the animal, with older mice appearing more resistant. Virus was isolated from the trigeminal ganglion of younger animals inoculated with higher doses of virus, after 21 days in culture, suggesting that latency had been established. Neutralising antibodies were present in most mice irrespective of the presence of recognisable clinical disease. Using primers for the thymidine kinase and glycoprotein C regions of the viral genome, herpes simplex virus type 1 DNA was found in the cornea, iris, and trigeminal ganglion of most animals and showed a good correlation with the presence of neutralising antibodies. It would thus appear that herpes simplex virus type 1 is able to accede into the cornea, iris, and trigeminal ganglion following non-traumatic application of virus onto the mouse eye. This model mimics primary ocular disease in humans and may be useful for studies on recurrent disease and the spread of ocular herpes.

Herpes simplex virus type 1 (HSV-1) is an important cause of ocular disease in both developed and underdeveloped countries. At present, despite advances in elucidating the molecular biology of HSV-1, we are still unable to prevent infection and the subsequent recurrences which may occur in the eye. The mechanism by which virus gains access to the cornea and trigeminal ganglion (TG) to infect and to establish a latent infection is central to the pathogenesis of herpes keratitis (HSK), its prevention, treatment, and eventual eradication.

The frequent occurrence of asymptomatic salivary shedding of HSV-1 provides indirect evidence of asymptomatic primary oral disease and for implicating the mouth as the main site for the acquisition and spread of HSV-1 within the community. The proportion of cases of primary herpetic eye disease that are asymptomatic however, is not known and they may play an important role in the development of recurrent corneal disease. The sensitive polymerase chain reaction (PCR), has been used to detect HSV-1 DNA in the corneas of patients with previous HSK in the absence of an isolation of cocultivation. HSV-1 DNA has also been detected in non-herpetic corneal diseases such as aphakic bullous keratopathy, keratoconus, and interstitial keratitis, a finding consistent with the occurrence of asymptomatic HSV-1 ocular infection.

Primary ocular infection in the mouse has been well studied using corneal trauma for the inoculation of virus. Although non-invasive inoculation has been used in rabbits there has been only limited study and no murine study in which disease was reported. The clinical findings, however, were not described nor were the ocular tissues and TG tested for the isolation of virus.

After scarification the disease which occurs is predominantly corneal, and usually results in extensive scarring. In humans, however, there is no evidence that such corneal trauma is necessary for ocular infection or the establishment of latency. In addition, cases of primary ocular herpes in humans with follicular conjunctivitis show varying degrees of blepharitis with or without corneal involvement and generally heals with no residual damage.

In this study, virus was applied to the mouse eye in droplet form, in the absence of trauma, to mimic a possible route of human primary ocular infection. Following inoculation mice were examined for clinical disease and the humoral immune response assessed for serum neutralising antibodies. At least 1 month after infection, the cornea, iris, and trigeminal ganglion were investigated for evidence of HSV-1 by the PCR or for a latent infection by cocultivation.

Methods

INOCULATION
NIH/OLA inbred female mice were anaesthetised with 0.1 ml of Hypnovel (Roche) and 0.1 ml of Hypnorn (Janssen). The left eye was inoculated either by application of virus onto the cornea in a 5 μl or 10 μl droplet (topical inoculation) of medium (medium E-199, 0-15% sodium bicarbonate, streptomycin 0-1 mg/ml,
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polymyxin B sulphate 50 U/ml penicillin 10U/ml (SPA) and 20% fetal calf serum) or by scarification through the 5 μl or 10 μl droplet of medium containing virus. The lids were not massaged and the animals were placed on shredded paper with their left eye uppermost. Control mice were inoculated with uninfected vero cells prepared in the same manner as the virus pool (mock inoculum).

EXPERIMENT 1
Fifty-six 12-5-week-old mice were divided into four groups and inoculated with 5 μl as follows: group A (n=30), totally with 5x10^6 pfu of HSV-1 strain McKrae,^64 group B (n=5) topically with mock inoculum, group C (n=14) by scarification with 5x10^7 pfu of HSV-1 strain McKrae and group D (n=5), by scarification with mock inoculum.

Two mice were left uninfected for additional PCR controls.

EXPERIMENT 2
Fifty, 5-5-week-old mice were divided into four groups and inoculated with 10 μl (groups E to G) or 5 μl (group H) as follows: group E (n=15) and group G (n=15) topically with 10^5 pfu of HSV-1 strain McKrae or strain SC16 respectively, and group F (n=10) and group H (n=10), by scarification with 10^6 pfu of HSV-1 strain McKrae or SC16 respectively.

EXPERIMENT 3
Twenty-four 4-week-old mice were topically inoculated with 5 μl of medium containing 3x10^5 pfu of HSV-1 strain SC16 and four animals were left uninfected.

CLINICAL EXAMINATION
Both eyes were examined for signs of disease using slit-lamp biomicroscopy, daily for the first week, alternate days for the second week, and thrice weekly for the remainder of the experiments.

DISSECTION AND REMOVAL OF TISSUES
Mice were killed at 8 weeks (expt 1) and 4 weeks (expt 2) post infection, by an overdose with pentobarbitone (Sagatal, May & Baker). A 2 mm diameter corneal disc was trephined from the left cornea, following which a substantial piece of iris and ciliary body was removed by microdissection. The left trigeminal ganglion was then removed. Tissues from animals in expt 1 were either cocultivated with Vero cells (with and without 15 μM 5 azacytidine) for the isolation of virus, or immediately frozen and kept at -70°C for subsequent DNA extraction. Tissues from all animals in expt 2 were cocultivated.

TITRATION OF SERA FOR ANTIBODIES TO HSV-1
Serum collected before inoculation, at 8 weeks (expt 1) and 4 weeks (expt 2) after infection was titrated for neutralising antibodies using the plaque reduction method. The ED_{50} was calculated by probit analysis. Neutralising antibodies were not measured in experiment 3.

POLYMERASE CHAIN REACTION
The cornea, iris, and TC (expt 1) were digested with proteinase K (Sigma) and the DNA extracted as previously described. Dilutions were made so that 5 μl of nucleic acid solution contained 150–250 ng of DNA. Negative control DNAs from the corneas, irides, and TGs of mock infected and uninfected mice were prepared in the same manner. Positive control DNA was obtained from Vero cells acutely infected with HSV-1.

Three sets of primers were used in the PCR: two for overlapping regions of the thymidine kinase gene (TK), and one pair for the glycoprotein C (gC) region. The two TK primers, TK1 and TK2 amplify a 110 and a 278 bp fragment respectively, and the gC a 115 bp fragment. The TK1 primers were used as they have been previously shown to be informative in mouse tissue and were thus used as a control. The TK2 primers have the sequences 5'-TTATTGCGCTATAGGCCGG 5'-GGCCAGCTGATAACCTGTT, and were selected for optimisation using Primer Designer program (Scientific and Educational Software, Pennsylvania, USA). A GeneAmp DNA amplification kit (AmpliTaq, Perkin Elmer) was used for the PCR, the 50 μl reaction mixture containing 5 μl of total nucleic acid mixture. Following 60 cycles of denaturation (94°C), annealing (55°C), and extension (72°C), on an automated thermal cycler (Perkin Elmer), products were separated by polyacrylamide or agarose gel electrophoresis and visualised by staining with ethidium bromide. A pBR322 DNA-Hae III digest (Sigma) was used as a molecular size standard. In all experiments a (no-DNA) blank control was included. A titration of HSV-1 infected Vero cell DNA from 1 μg to 10^4 pg, showed the TK2 and gC primers to give better signals than the TK1 primers (confirming the TK2 optimisation).

PRODUCT DIGESTION AND HYBRIDISATION
The identity of the product was confirmed by restriction endonuclease digestion using Sma I (Boehringer Mannheim) for TK1 (to produce 73 and 37 bp fragments) and TK2 (210 and 68 bp fragments), and Ksp I (Gibco BDH for the gC products (to produce 59 to 56 bp fragments) as previously described. Additional confirmation of the TK products was by Southern blot hybridisation. A 5'end labelled probe from a 26 base oligonucleotide corresponding to a sequence between the TK1 and TK2 primers was prepared using [γ-32P]ATP (Amersham) and 20 U of polynucleotide kinase (Pharmacia). Hybridisation was in 5 ml/200 cm^2 of rapid prehybridisation buffer (Amersham) at 65°C for 1.5 hours, followed by two 10 minute washes at 20°C in 2xSSC, and one 15 minute wash at 65°C in 1xSSC and 0.1% SDS.

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Table 1  Disease, antibody response, cocultivation, and PCR results

<table>
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<tr>
<th>Group</th>
<th>Inoculation</th>
<th>Isolations*</th>
<th>ED50</th>
<th>PCR†</th>
<th>Disease</th>
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<tr>
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<td>14/16</td>
<td>1/16C</td>
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</tr>
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<td>B (5)</td>
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<td>0/5</td>
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<td>0/3</td>
<td>0/3</td>
<td>13/14 severe lid and corneal 3 encephalitis</td>
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<td>D (5)</td>
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<td>Exp 2 (5½-week-old mice)</td>
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<td>G (15)</td>
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<td>H (10)</td>
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<td>2/8/2/22</td>
<td>2/8</td>
<td>2/8</td>
<td>2 encephalitis</td>
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*Number of isolations/number tested (average no of days in culture to isolate virus following removal of tissue)
†Mean neutralising antibody response
‡Number of specimens positive with three primers/number tested
McK=HSV-1 strain McKrae; SC16=HSV-1 strain SC16; C=cornea; I=iris; TG=trigeminal ganglion

REACTIVATION OF LATENT INFECTION

On day 30 after inoculation, mice were irradiated with UV light (Hanovia lamp 420 nm, 4·02 J/cm²) for 2 minutes with the left eye protected. Before irradiation (days -4, -3, 0) and after irradiation (days 1, 2, 3, 4) eye washings were taken for the isolation of HSV-1.

Results

CLINICAL RESPONSE (TABLE 1)

Experiment 1

No evidence of detectable disease could be found in group A (topical virus inoculation) or in group B (topical mock inoculum).

All scarification lines in groups C and D had healed by day 2. From day 3, 13/14 mice from group C developed disease. This began as blepharospasm, followed by the development of lid ulceration, central corneal geographic ulceration, and varying degrees of mydriasis. By the end of the third week there was lid scarring with hair loss and the corneas were vascularised and scarred.

The right eyes remained normal throughout the study.

Experiment 2

Topically infected mice (groups E with strain McKrae and G with strain SC16) developed disease in the inoculated eye in the following manner: mild lid oedema and a squamous blepharitis was present by day 3 (10/15 in group E and 13/15 in group G), with two mice in Group G showed lid vesicles. By the end of the first week mice had developed varying degrees of the upper and lower lid ulcerative blepharitis (6/15 in group E and 9/15 in group G). In addition, four of these mice (one from group E and three from group G) had a mild punctate epithelial keratoconjunctivitis. One mouse (group G) developed an encephalitis by day 10 and was killed. At 2 weeks there were varying degrees of lid scarring and hair loss (Fig 1) and two mice (group G) had developed an ipsilateral mydriasis.

Mice in groups F (scarified with strain McKrae) and H (scarified with strain SC16) developed disease as in group C. Two mice from group F and one from group H developed an encephalitis and were killed. No detectable difference in severity of disease was found between groups F and H.

Experiment 3

Mice developed varying degrees of blepharitis in a similar time course to the topically inoculated animals in experiment 2, although the disease was milder.

NEUTRALISING ANTIBODY RESPONSE

Sera from pre-infection and control mice (groups B and D) showed no detectable levels of antibody.

Experiment 1

Twenty-five of the 30 (83%) topically infected mice (group A) had detectable levels of antibody (Table 2) with a mean ED50 of 14-0 (range: 2 to 121) which was significantly lower than those scarified (group C, mean ED50=157-0, range: 78 to 240 t test, p<0·001) (Fig 2a).

Experiment 2

Mice topically inoculated with strain SC16 (group G, mean ED50=237, range: 26 to 575) had significantly higher levels of neutralising antibody than those topically infected with strain McKrae (group E, mean ED50=116, range: 2 to 401) (t test p<0·05). Conversely, mice infected by scarification with strain McKrae (group F, mean ED50=880, range:234 to 2205) had a significantly higher mean ED50 than with strain SC16 (group H, mean ED50=330, range:170 to 464 (t test, p<0·05), (Fig 2b.) There was no significant difference between the mean ED50 of
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Table 2. Polymerase chain reaction and ED50

<table>
<thead>
<tr>
<th>Mouse</th>
<th>TK1</th>
<th>TK2</th>
<th>gC</th>
<th>ED50</th>
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<td>C</td>
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TK1, TK2=primers for thymidine kinase; gC=primers for glycoprotein C; ED50=neutralising antibody response; + = presence of PCR amplification product; - = absence of PCR amplification product; C=cornea; I=iris; TG=trigeminal ganglion; A1-A16=topical inoculation with 5 x 10^6 pfu HSV-1 strain McKrae; B1-B3=topical inoculation with mock inoculum; C1-C5=scarified with 5 x 10^6 pfu HSV-1 strain McKrae; D1-D3=scarified with mock inoculum; U1 and U2=uninfected mice.

POLYMERASE CHAIN REACTION (EXPT 1)

Topical infection with virus

Thirteen of 16 mice had an antibody response to topical HSV-1 and evidence of HSV-1 DNA in their cornea and/or iris and/or TG (Tables 1 and 2). Of these 13 mice, viral DNA was detected using all three primers as follows: (a) in the cornea, iris, and TG of five mice (A1, A2, A4, A5, A13), (b) in two of the tissues in four mice (A9, A14, A15, and A16), and (c) in one of the tissues in three mice (A3, A6, and A12).

Both the TK1 and TK2, but not the gC amplification products were present in the iris and TG of A3, and only in the iris of A9. Conversely, the gC but not the TK amplification products were present in the TG of two animals (A6 and A15). These may represent genomic retention of false positives. Furthermore, there was evidence of HSV-1 DNA in the TG of A6 and in the cornea of A11 using the TK2 and gC primers, but not the TK1 primers. The absence of TK1 in the presence of TK2 and gC probably reflects the stronger signals obtained with TK2 and gC than with TK1, rather than incomplete HSV-1 DNA, as the TK2 product overlaps the TK1 product.

There was no significant difference in the number of positives that is, presence of all amplification products, in any of the tissues tested (Fisher exact test, p>0.1 for all).

Three mice with no detectable antibody response showed no evidence of HSV-1 DNA in the cornea, iris, or TG.

Scarcification with virus. HSV-1 DNA was detected (using all three primers) in all tissues of each mouse in group C, with the exception of the iris and cornea of one animal (C2), where it could be detected using the TK2 and gC primers but not with the TK1 primers (Table 2).

Controls. There were no detectable amplification products using the three primers in the cornea, iris, or TG of eight control mice, apart from the presence of the TK amplification products in the TG of B1 (Table 1).

PRODUCT DIGESTION AND HYBRIDISATION

The amplification products (Fig 3a) were cleaved as predicted by the relevant restriction enzymes, confirming their identities (Fig 3b and c). Similarly, the TK1 and TK2 amplification products from positive samples hybridised to the internal oligonucleotide probe, whereas the negative control samples did not (Fig 3d).

ISOLATION OF VIRUS BY COCULTIVATION

Experiment 1

No virus was detected in cornea, iris, or TG (either cultured with or without 5'-azacytidine), from topically infected or control animals.

Virus was isolated from the TG of 5/6 scarified mice (group C) between days 10 to 14 (average day 12), but not from the iris or cornea.

Experiment 2

Virus was isolated from the TG of 5/14 mice topically infected with strain SC16 (average day 15) (group G), and from the TG of 3/15 mice infected with strain McKrae (average day 21) (group E), are of which also had virus cultured from its iris on day 23. Virus was isolated from the TG of 7/9 mice which had been scarified with strain McKrae (average day 14) and from the TG of 6/9 mice which had been scarified with strain SC16 (group H) (average day 15). In addition, virus was also isolated from the iris of two mice from the latter group on days 22 and 23 of culture.

No virus was isolated from the cornea in any of the above groups.

REACTIVATION

No virus was detected in eye washings taken from the tear film, either before irradiation or in the week following irradiation.

All mice (including the controls) developed UV induced diffuse punctate epithelial keratitis with epithelial oedema and swollen eyelids 1 day after UV irradiation. By day 2 significant areas of epithelial loss had developed.
Kaye, Shimeld, Grinfeld, Mainland, Hill, Easty

Figure 2 Neutralising antibody response expressed as the ED_{50}. (A) (Expt 1); group A, topically inoculated with 5 \times 10^4 pfu HSV-1 strain McKrae; group G, scarified with 5 \times 10^4 pfu HSV-1 strain McKrae. (B) (Expt 2); groups E and G, topical inoculation with 10^6 pfu HSV-1 strain SC16 or McKrae respectively; groups F and H, scarified with 10^6 pfu HSV-1 strain SC16 or McKrae respectively.

spreading across the cornea which was associated with mild iris hyperaemia. By day 3, the corneas of all control and 19/24 infected mice had re-epithelialised. Five of the 24 infected mice had persistent central oval epithelial defects and focal areas of lid excoriation. At 1 week, all the central epithelial defects had healed, two mice being left with scarred vascularised corneas.

**Discussion**

HSV-1 infection is usually acquired by direct or indirect contact spread from an infected individual. The non-traumatic acquisition of HSV-1 by direct droplet spread to the eye, however, has not been fully investigated. Asymptomatic ocular infection\textsuperscript{17-18} has been postulated to occur based on the finding of anti-HSV-1 antibodies in the tear film of seronegative individuals\textsuperscript{17} and although reports are contradictory, the isolation of HSV-1 in the tear film of persons with no prior history of herpes.\textsuperscript{6,24}

Lower doses of HSV-1 strain McKrae did not produce disease after topical inoculation of older mice. Primary ocular disease did occur, however, following topical inoculation with either strain of HSV-1 (SC16 or McKrae), when higher doses were used in younger mice. This probably reflects the combination of increasing the dose of virus and the greater susceptibility of younger mice.\textsuperscript{19} The incidence and severity of disease was slightly greater when SC16 was used for inoculation. For both strains of virus the disease was predominantly confined to the lids and resembled that which occurs in human primary ocular herpetic blepharitis.\textsuperscript{27-28}

The pattern of disease (lid and corneal ulceration) in mice scarified with HSV-1 was similar to that reported previously.\textsuperscript{12,24-26} Topical application of HSV-1, induced the production of neutralising antibody in 83\% of animals in expt 1 and in all animals in expt 2, irrespective of recognisable clinical disease. Mice which developed clinical disease, however, had significantly higher levels of antibody than those which did not. The level of antibody produced was highest in younger mice which had been scarified with HSV-1 strain McKrae. Interestingly, there was no significant difference in the level of antibody between mice which had been topically infected with 10^4 pfu of SC16 or scarified with 10^4 pfu of SC16, while there was a significant difference between mice topically infected with 10^6 pfu of McKrae and those scarified with 10^4 pfu of McKrae. These findings suggest that production of antibody following ocular infection is not only dependent on the dose of virus used, but also on the strain of virus and the method of inoculation.

A positive result for the likely presence of large stretches of HSV-1 DNA in a tissue was defined as the presence of both TK and \( \delta \)C amplification products. The presence of only one amplification product was taken to represent incomplete fragments of HSV-1 DNA (genomic retention or false positives), and a negative result defined as the absence of both the TK and \( \delta \)C amplification products. Using this definition HSV-1 DNA was present in the majority of mice that had been topically infected with HSV-1. There was a good correlation between the presence of HSV-1 DNA and the presence of detectable antibody in that only mice that had evidence of antibody were PCR positive. HSV-1 DNA has previously been detected in the TG and eyes of mice inoculated by scarification,\textsuperscript{29} so that finding viral DNA in the TG, cornea, and iris or scarified mice was not unexpected. In the majority of tissues tested from mice topically infected or scarified with virus, all three amplification products were present, which indicates the likelihood that intact HSV genomes, rather than fragments of HSV-1 DNA, were present.

The isolation of HSV-1 between days 17 and 23, from the TG in 27\% (8/29) of mice following topical inoculation (expt 2), suggests that a virus had been able to establish a latent infection. Although there were isolated infections from the TG of mice inoculated with HSV-1 strain SC16 than with McKrae, the difference was not signi-
Non-traumatic acquisition of herpes simplex virus infection through the eye

Significant. The isolation of virus from approximately 80% of the TG of scarified mice is similar to previous reports, and probably reflects the greater virus load reaching the TG.

The inability to isolate HSV-1 from the central cornea of mice (where it was detected by PCR in 11/16 cases), is somewhat at odds with previous work. The amount of virus in the 2 mm cornea may have been below the sensitivity of cocultivation, (particularly following topical inoculation) but well within the sensitivity of the PCR. In previous reports (using scarification), although virus was isolated from the anterior segment and cornea, the amount of tissue taken was not specified. It is not clear therefore, whether virus may have been present in higher amounts in the peripheral cornea and limbus, conjunctiva, or iris, or in all three tissues. This is supported in the present study by the isolation of virus from the iris of three mice (one topical and two scarified) in the absence of corneal isolation.

Whereas virus was detected by cocultivation in the iris of 3/50 (6%) mice in expt 2, viral DNA was detected in the iris in 12/21 (56%) animals by PCR in expt 1. Although this may have been due to an absence of reactivatable virus, it probably reflects the greater sensitivity of the PCR, particularly in view of the higher doses of virus used for inoculation in expt 2.

UV irradiation induces shedding of virus in the tear film and recurrent eye disease in mice previously inoculated by scarification with HSV-1 strain McKrae. In this study, after similar stimulation, we were unable to detect virus in the tear film of mice previously topically inoculated with 3 × 10^3 pfu of HSV-1 strain SC16. To increase the likelihood of induced reactivation in topically infected animals, further work is underway, varying the dose and strain of HSV-1 used for inoculation.

HSV-1 virus can establish a latent infection in the trigeminal and superior cervical ganglion in animals and humans. In addition, recent work suggests that HSV-1 may also be able to establish a latent infection in the anterior segment of mice, and the corneas of rabbits and humans.

Clearly, either in the presence or absence of overt ocular disease, ocular inoculation is the most direct way in which HSV-1 may reach these sites to establish a latent infection. In experimentally infected mice however, virus may also reach these sites, and establish latency in the ophthalmic division of the TG, by the ‘back door route’ following a primary infection in non-ophthalmic parts of the trigeminal dermatome, for example the lower lid.

Following ocular inoculation neural spread of virus from tissues such as the cornea or eyelids seems the most likely route of the virus to the TG. Ocular infection, however, may involve a more complex pattern of spread. Thus drainage of virus from the eye via the nasolacrimal duct can lead to infection via the olfactory route. Hence involvement of the TG might occur from the infected nasal mucosa via the nasociliary branches of the ophthalmic division.

The results of the present study show that, following non-traumatic topical application of HSV-1 to the mouse eye, virus accedes to the cornea, iris, and TG. By analogy in humans this might represent a symptomatic or asymptomatic primary infection, which leads to a latent infection of the TG and possibly of the iris and cornea. Ocular disease may thus potentially result from reactivation of virus at any of these sites of latency.

The clinical features and events following topical application of virus to the mouse cornea mimic primary ocular disease in humans and may prove useful for studying the pathogenesis of HSV-1 ocular disease.

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