Reactivation of latent infection and induction of recurrent herpetic eye disease in mice

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During primary ocular infection of mice with herpes simplex virus type 1 (HSV-1) strain McKrae, dendritic corneal ulcers developed and many eyes became permanently damaged. When primary infection had subsided, latent infection was detected in the three parts of the trigeminal ganglion and in the superior cervical ganglion. Such latently infected mice were treated with cyclophosphamide, dexamethasone and u.v. irradiation, or cyclophosphamide and dexamethasone alone. After treatment with immunosuppressive drugs and u.v. irradiation infectious virus was isolated from the ophthalmic part of the trigeminal ganglion, and in eyelids and eyewashings; recurrent herpetic eye disease was seen but only in eyes undamaged by primary infection. After treatment with cyclophosphamide and dexamethasone alone there was a lower incidence of virus isolated from eyewashings and no recurrent disease was seen. There was a good correlation between the pattern and distribution of recurrent lesions and the distribution of cells stained due to the presence of virus antigens.

Introduction

Detailed studies of animal models for recurrent eye disease caused by herpes simplex virus (HSV) provide valuable information on the pathogenesis of this condition and on the events surrounding the reactivation of latent infection. In rabbits, after inoculation of the cornea with HSV, latently infected animals show a high rate of spontaneous shedding of virus in tears and on some occasions this is associated with recurrent corneal disease (Nesburn et al., 1967; Se Kwon et al., 1981; Hill et al., 1987a; Haruta et al., 1987). Such frequent shedding (which is probably preceded by reactivation of latent virus in the sensory or sympathetic ganglia supplying the eye) complicates attempts to induce reactivation by specific stimuli as it is difficult to dissociate experimentally induced events from those occurring spontaneously. However in latently infected mice the incidence of spontaneous shedding of virus in eye secretions is very low (Tullo et al., 1982; Wille et al., 1984).

Using iontophoresis of epinephrine into the cornea Willey et al. (1984) were able to induce reactivation of virus in the trigeminal ganglion (TG) and shedding of virus from the eyes of latently infected mice. Gordon et al. (1986) were unable to repeat this work but after testing a variety of regimes, they found that iontophoresis of 6-hydroxydopamine, combined with epinephrine and prednisolone phosphate eye drops, caused shedding of virus in the tears. The incidence of such shedding was further increased by the addition of timolol eye drops to this arduous and time-consuming schedule of treatment (Harwick et al., 1987). However none of these workers commented on recurrent eye disease.

We now report on the development of a model of reactivation, virus shedding and recurrent herpetic eye disease in mice.

Methods

Mice. NIH/OLA inbred mice were obtained originally from Olac; they were maintained as a breeding colony in the Department of Microbiology. All were used at 8 weeks old; any with abnormal eyes were rejected (Tullo et al., 1983).

Inoculation. Mice were anaesthetized by intraperitoneal injection of sodium pentobarbitone and inoculated by scarification of the left cornea with a 26-gauge needle (Tullo et al., 1983) through 5 μl drop of medium containing 10⁴ p.f.u. HSV type 1 (HSV-1) strain McKrae. Control mice were inoculated in the same way with a preparation of uninfected Vero cells made in a similar manner to the virus inoculum (mock inoculum).

Examination of eyes and isolation of virus from eyewashings. Mice were anaesthetized and the cornea, iris and lids were examined for signs of disease using a slit lamp microscope. Eyewashings were put onto Vero cells for the isolation of virus (Tullo et al., 1983).

Detection of latent infection. Mice were killed with an overdose of sodium pentobarbitone and the following tissues were removed from the left side: the three parts of the TG and the superior cervical ganglion (SCG). The TG was divided in situ so that part 1 (TGI)
probably contained all the ophthalmic and some of the maxillary neurons, part 2 (TG2) contained maxillary neurons and part 3 (TG3), mandibular neurons (Gregg & Dixon, 1973; Arvidson, 1979; Tullo et al., 1982). The SCG and the parts of TG were each placed in 0·5 ml of medium and incubated at 37 °C in 5% CO₂ for 5 days (Tullo et al., 1982). The tissues were then ground and 50 μl of the suspension was put onto Vero cell monolayers. These were incubated at 37 °C for 2 days before being fixed and stained so that plaques could be identified.

**Reactivation of latent infection.** At least 40 days after corneal inoculation mice were given 5 mg of cyclophosphamide (Koch-Light Laboratories) intravenously in 0·2 ml of phosphate-buffered saline (PBS). One day later, 0·2 mg of dexamethasone in 0·2 ml (Dexamethasone, Intervet Laboratories) was injected via the same route. In all experiments except one, mice were anaesthetized immediately after this injection and held for 90 s with the left eye proptosed, below a Hanovia lamp so that the cornea and lids could be irradiated. The lamp emitted a peak of 4.03 mJ/cm².s at 320 nm. This irradiation produced mild erythema in the skin of the pinna of these mice (T. J. Hill & W. A. Blyth, unpublished results).

Detection of virus reactivated in vivo.

(i) **Isolation of infectious virus from tissues.** The three parts of the left TG and the left SCG were each ground in 0·5 ml of medium, then frozen and thawed three times to disrupt all the cells. The resulting cell-free suspensions were put onto a monolayer of Vero cells in 25 cm² flasks and incubated at 37 °C in 5% CO₂. Cultures were examined daily for c.p.e. for 5 days. The upper and lower left lids from each mouse were ground together in 0·5 ml of medium, frozen and thawed three times and the resulting cell-free suspensions were put onto a monolayer of Vero cells as above. These cultures were examined for c.p.e. for up to 21 days.

(ii) **Detection of virus antigens by peroxidase-antiperoxidase (PAP) staining.** Mice were killed with an overdose of sodium pentobarbitone, followed by the removal of the left eye and the lids (or part of the lids). Flat mounts of the corneal epithelium and of the remainder of the eye (the corneal stroma and endothelium, the uvea and sclera, referred to as 'globe') were prepared and stained by the PAP method for the detection of HSV-1 antigens, as described by Dyson et al. (1987). Upper and lower lids were treated separately or selected diseased areas of lids were fixed in Bouin's fluid for 30 min to 1 h and then immersed in 70% ethanol for at least 24 h. They were then embedded in paraffin wax and serial 5 μm sections were cut for PAP staining.

**Results**

**Primary disease and incidence of latent infection**

Nineteen female mice and 20 male mice were inoculated with virus on the cornea. Eyewashings were taken for the isolation of virus on days 3 to 7 after inoculation and mice were examined for signs of eye disease on days 3, 5, 7 and 10. Seventeen female mice and 12 males survived. On day 25 the eyes of female mice were examined, the mice were killed and tissues were taken to determine the incidence of latent infection in the three parts of the TG and in the SCG; on day 43 after inoculation male mice were treated similarly.

Eye disease followed a similar pattern in both sexes. Three days after inoculation 50% of corneas had multiple dendritic ulcers, accompanied in some mice by localized corneal haze. Lid disease (swelling, ulcers and scabs) was seen in the majority of mice on day 7. By day 10, 50% of surviving mice had opaque corneas with peripheral vascularization and swollen lids devoid of lashes.

Virus was isolated from eyewashings of nearly all mice on days 3 to 6; on day 7 the incidence of isolation was 80% in males and 65% in females.

Latent infection was detected in TG1 from 82% of animals. It was also detected, in TG2 (58% in males, 71% in females), in TG3 (33% in males, 41% in females) and in SCG (50% in males, 35% in females). In two male and three female mice latent infection was not detected in any of the tissues tested.

**Reactivation of latent infection**

Fifty-seven days after virus inoculation on the cornea, 47 male mice (the survivors from a group of 75) were treated with cyclophosphamide, dexamethasone and u.v. irradiation; 31 female mice (the survivors from a group of 75) were treated similarly on day 54 after inoculation. The day of treatment with dexamethasone was defined as day 0. Eyes were examined immediately before the mice were treated with cyclophosphamide and eyewashings were taken for virus isolation at this time and daily for 5 days. The eyes of groups of 10 to 14 mice were examined for signs of recurrent disease on days 2, 3 and 4. They were then killed and their tissues removed for the isolation of virus or for PAP staining to detect HSV-1 antigens.

Infectious virus was first isolated from one of 14 TG1 samples from male mice on day 2 and four of 14 on day 3; it could not be isolated from this tissue in female mice. Only a small amount of virus was isolated, an average of 3 p.f.u. and a maximum of 6 p.f.u. per sample. Infectious virus was isolated from a small proportion of lids from male and female mice at all times tested; the maximum was from five of 10 female mice on day 4. Where infectious virus was isolated c.p.e. appeared within 5 days of culture, except on two occasions when c.p.e. was first seen after 16 days. Infectious virus was not isolated from SCG at any time (Table 1a).

Recurrent corneal disease (dendritic ulceration with or without associated corneal haze) was seen in one male mouse on day 3 and one female mouse on day 4. Virus antigen was seen in cells bordering branched ulcers in the corneal epithelial sheets from these mice (Fig. 1). It was also seen in this tissue on day 3 in small groups of cells although these two corneas were not ulcerated. Virus antigen was never seen in globes (Table 1a). In addition to those mice with recurrent corneal disease, six mice had recurrent lid disease (ulcers or scabs). Such disease was seen in two male mice on day 2 and four female mice, one on day 2, one on day 3 and two on day 4 (Table 1b). Infectious virus was isolated from lids of all eight mice
Table 1. **Eye disease, isolation of virus and staining of virus antigens following treatment of latently infected mice** with cyclophosphamide, dexamethasone and u.v.† irradiation

(a) Incidence

<table>
<thead>
<tr>
<th>Day after treatment‡</th>
<th>Isolation of infectious virus</th>
<th>HSV-1 antigen stained by PAP</th>
</tr>
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<tr>
<td></td>
<td>TS1§</td>
<td>SCG</td>
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<tr>
<td>Male mice</td>
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<tr>
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<td>4/14 (29)</td>
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<tr>
<td>4</td>
<td>0/14</td>
<td>0/14</td>
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<tr>
<td>Female mice</td>
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<tr>
<td>-1</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>0</td>
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<td>0/10</td>
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<tr>
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<td>0/11</td>
<td>0/11</td>
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<tr>
<td>4</td>
<td>0/10</td>
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</tr>
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</table>

(b) Details of mice with evidence of recurrent ocular infection

<table>
<thead>
<tr>
<th>Mouse number</th>
<th>State of corneas†† immediately before treatment</th>
<th>Day killed</th>
<th>Virus from eyewashings on day</th>
<th>Infectious virus from lids</th>
<th>Antigen in corneal epithelium</th>
<th>Recurrent disease</th>
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<tr>
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<td>-0-1-2-3-4</td>
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<td>V</td>
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<td>84</td>
<td>&gt;100</td>
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<tr>
<td>9</td>
<td>V</td>
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<td>10</td>
<td>V</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>&gt;100</td>
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</tr>
</tbody>
</table>

*: Inoculated 57 days (male) or 54 days (female) previously with 1 x 10⁴ p.f.u. on left cornea.

†: Treatment: 5 mg of cyclophosphamide intravenously (i.v.) followed 24 h later by 0-2 mg of dexamethasone i.v. and 90 s of u.v. irradiation to the left eye.

§: Dexamethasone was given on day 0.

‡: TG1, ophthalmic part of trigeminal ganglion.

¶: ND, Not determined.

**: Number with virus or antigen/number tested. Percentage value is given in parentheses.

**: Six mice died from anaesthetic.

††: V, Vascularization of the cornea; N, normal. All lids were normal.

‡‡: Dendritic ulceration with or without associated corneal haze or infiltrate.

§§: Ulcers or scabs.

||: Number of p.f.u.

with recurrent disease and from the lids of a further six mice without such disease. Virus was also isolated from the eyewashings of three of the mice with recurrent lesions and three other mice. However, virus antigens were seen in corneal epithelia and/or virus was isolated from the lids of these latter three animals. It is noteworthy that the lids of mice which yielded virus and the corneas of mice with virus antigens in corneal epithelial sheets were all normal when examined on day -1 (Table 1b).
In a further experiment, 104 male mice that had survived primary inoculation were examined. Twenty-four of them were from a group of 75 inoculated 40 days previously, 80 from a group of 124 inoculated 92 days previously. The 52 with normal corneas and lids were selected; 29 were treated with cyclophosphamide, dexamethasone and u.v. irradiation, and 23 with cyclophosphamide and dexamethasone only. Eyewashings were taken before treatment and daily for 7 days. From day 3, mice were examined each day for signs of recurrent eye disease. If virus was isolated from eyewashings and/or recurrent disease was seen, the mouse was killed, and the corneal epithelium, globe and selected lid tissue were taken for PAP staining.

In the group of mice treated with only cyclophosphamide and dexamethasone, none showed signs of recur-
Recurrent herpetic eye disease in mice

Table 2. Recurrent eye disease, isolation of virus and staining of virus antigens in male mice*

<table>
<thead>
<tr>
<th>Mouse number</th>
<th>Virus from eyewashings on day</th>
<th>HSV-1 antigen stained by PAP</th>
<th>Day recurrent disease first seen</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Corneal epithelium</td>
<td>Globe</td>
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<tr>
<td>1</td>
<td>- - - - - - &gt;100‡ 1005 - - K§</td>
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<td>- - - - - -</td>
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<tr>
<td>2</td>
<td>- - - - - - &gt;100 &gt;100 53 K</td>
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<td>3</td>
<td>- - - - - - &gt;100 &gt;100 53 K</td>
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<td>4</td>
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<td>7</td>
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<td>- - &gt;100 80 - - - -</td>
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* Inoculated on the cornea at least 40 days previously with 1 x 10⁴ p.f.u. All had normal eyes immediately before treatment. Mice were treated as previously except numbers 10 and 11 which received no u.v. irradiation.
† From mice 1 and 3, the suspected recurrent lid lesions were taken for staining and from mice 4, 5 and 9, whole lids (upper and lower) were taken.
‡ Number of p.f.u.
§ K, Killed.
| ND, Not determined.
¶ D, Died from anaesthetic.

Discussion

As reported by others (Willey et al., 1984) dendritic lesions were seen during primary infection following present in a single focus in the conjunctival epithelium; in the other lid it was more extensive with foci in the epidermis of the eyelid, in hair follicles and in glandular tissue beneath the conjunctiva (Fig. 1).

Drug treatment and u.v. irradiation of uninfected mice

Ten mice were inoculated on the cornea with the mock inoculum and then treated 77 days later with cyclophosphamide, dexamethasone and u.v. irradiation. Eyewashings were taken but discarded and eyes were examined immediately before injection of cyclophosphamide and then daily for 5 days. On days -1 and 0 all eyes were normal. On day 1, four mice showed haze, suggesting oedema, in the central cornea; five had corneal epithelial deficits. These were ovoid, extended from the medial to lateral canthus and were underlaid by haze. Nine mice had such deficits by day 2 and in addition two had iris hyperaemia. On day 3, five corneas were normal; the others had very small patches of slight haze in the centre and all corneas were normal by day 4. Lid disease was not seen at any time.

Over the period of observation no signs of systemic toxicity were seen. However, in other experiments seven of 16 (44%) died within 22 days of similar treatment.
inoculation of the cornea with HSV-1 strain McKrae. In contrast this type of ulcer was rarely seen after a similar inoculation with strain SC16 (Tullo et al., 1983). However, with both strains the incidence and timing of lid disease, and the proportions of surviving mice that had severely and irreversibly damaged corneas, were similar (Tullo et al., 1983). In addition the distribution of latent infection was similar to that found when SC16 was inoculated at this site (Tullo et al., 1982). The incidence of spontaneous shedding was low; in the present work only one of 130 latently infected mice was found to have virus in eyewashings before treatment to induce reactivation. This confirms previous reports for the McKrae strain (Willey et al., 1984) and SC16 (Tullo et al., 1982). However a disadvantage of the McKrae strain is its greater neurovirulence when compared with strain SC16.

The most commonly recognized, diagnosed and reported sign of recurrent herpetic eye disease in humans is the corneal dendritic ulcer (Duke Elder, 1965). After treatment with immunosuppressant drugs and u.v. irradiation this characteristic lesion was seen on the corneas of latently infected mice. Confirmation that these lesions were caused by virus was provided by the close similarity between the pattern of the lesion and that of cells containing the virus antigen. Recurrent lid disease, also confirmed by the presence of virus antigen, was seen more frequently than recurrent corneal disease. In humans, recurrent lid disease due to HSV (Jacobiec et al., 1979; Egerer & Stry, 1980) appears to occur less frequently than corneal disease although the difference may be artificial because corneal disease is more likely to be recognized and reported than localized lid disease. Recurrent herpetic conjunctival disease has also been reported in man (Brown et al., 1968; Nauheim, 1969; Colin et al., 1980). In this present study no such disease was seen, but only the bulbar conjunctiva of mice can be examined clearly. However subclinical disease was present in this tissue as virus antigens were seen in epithelial cells at this site.

Isolation of virus from eyewashings provides further evidence of infection although this does not help in identifying the precise site of infected cells. When corneal disease was present (usually with lid disease) large amounts of virus were isolated for at least 2 consecutive days. However when clinical disease was not seen virus was isolated only sporadically and in small amounts. Four mice shed only small amounts of virus in tears on one occasion only; in three of these no recurrent disease was seen. Shedding of virus in the absence of clinical disease has been reported also in humans and rabbits (Kaufman et al., 1967; Haruta et al., 1987). By chance the likely source of such virus was identified in one mouse when virus antigens were demonstrated in corneal epithelium.

This study and others (Metcalf & Michaelis, 1984; Stulting et al., 1985) show that primary infection with HSV in the mouse cornea is often followed by severe and irreversible eye damage. It is noteworthy that recurrent eye disease and/or shedding of virus was seen only in those mice with undamaged eyes. In mice with damaged eyes recognition of recurrent lesions might be difficult but the absence of such lesions did not appear to be due to a failure of reactivation in the ganglia since, on some occasions, virus was isolated from TG1 of such mice following treatment with cyclophosphamide, dexamethasone and u.v. irradiation. In eyes damaged from the primary disease the sub-epithelial plexus of the cornea is severely depleted whereas in undamaged corneas the plexus appears normal (C. Shimeld, unpublished results). Similar observations have been reported in rabbits after inoculation of the cornea with HSV-1 (Asbell & Beuerman, 1985). Since an intact nerve supply to the cornea or to the skin is necessary for induced shedding of virus in tears (Rootman et al., 1988) or the production of recurrent herpetic skin lesions (Hill et al., 1983) respectively, the absence of the nerve plexus in damaged eyes may provide a further explanation for the absence of recurrent disease in such eyes. Other factors may influence the production of recurrent eye disease. For example in rabbits, the strain of virus is known to affect its incidence (Hill et al., 1987b) and the strain of mouse was shown to play a role in influencing the incidence of recurrent skin disease in mice (Harbour et al., 1981).

Following treatment to induce reactivation, only small amounts of virus were isolated from TG1. This observation, the times at which virus was isolated and the number of animals yielding virus were similar to previous results involving different reactivation stimuli in latently infected mice (Openshaw et al., 1979; Harbour et al., 1983). A nerve supply to the cornea is required to produce shedding of virus in the tear film (Rootman et al., 1988). Therefore the reactivated virus in TG1 was the most likely source of virus to produce recurrent ocular disease and shedding in the tears. However in humans (Shimeld et al., 1981; Tullo et al., 1985; Cook et al., 1986), in mice (Abghari & Stulting, 1988) and in rabbits (O’Brien & Taylor, 1989) there is evidence that the cornea may be persistently or latently infected with virus. Therefore reactivation may also occur in this tissue.

The small amounts of reactivated virus isolated from TG1 may account for the absence of virus antigens within ocular nerves. During primary infection, when much larger amounts of virus are present in ganglia (Blyth et al., 1984) such antigens are frequently seen in Schwann cells of ocular nerves on days 2 to 6 after corneal inoculation of mice with HSV-1 strain SC16 (Dyson et al., 1987).

Ultraviolet irradiation appeared to be the major factor in inducing recurrent lesions since treatment with
cyclophosphamide and dexamethasone alone produced a very low incidence of shedding of virus in tears, and no recurrent disease. In contrast treatment with these immunosuppressive drugs plus u.v. irradiation produced a much higher incidence of both of these signs of recurrent infection. u.v. irradiation induces recurrent skin lesions in man (Wheeler, 1975; Spruance, 1985), reactivation of latent virus in mice (Blyth et al., 1976) and, in combination with systemic and topical corticosteroids, recurrent corneal lesions in rabbits (Spurney & Rosenthal, 1972). The dose of irradiation used in the present study produced temporary and slight damage to the corneal epithelium and no detectable damage to the lids and bulbar conjunctiva. U.v. irradiation is known to produce many changes in the skin including damage to epithelial cells and their replication (Epstein et al., 1971), the release of prostaglandins (Harbour et al., 1983) and the reduction of ATPase staining on Langerhans' cells (Bergstresser et al., 1980). Its effect on the mouse cornea has not been well documented but in the rabbit cornea, swelling (Riley et al., 1987), epithelial loss and killing of keratocytes (Ringvold & Davanger, 1985) have been recorded. It is not clear how such observations relate to reactivation of latent HSV. However, reactivation may arise from the effects of u.v. irradiation on corneal nerves because in humans, exposure to a low dose of u.v. irradiation produced a loss of corneal sensitivity lasting for 4 h (Millodot & Earlam, 1984). In addition, u.v. irradiation of the corneal epithelium may result in the release of mediators of inflammation that could also affect the neuronal cell body.

In latently infected rabbits the combination of cyclophosphamide and dexamethasone used here was successful in inducing the shedding of virus in tears (Stroop & Schaefer, 1986; Rootman et al., 1988) and recurrent ocular disease (Stroop & Schaefer, 1987). The disease varied from very severe conjunctivitis and keratitis to mild conjunctivitis; some rabbits died from the toxic effects of the drugs. Despite the potent actions of these drugs on the immune system and the higher dose of cyclophosphamide than that used previously in rabbits, drug treatment without u.v. irradiation produced little shedding of virus and no recurrent disease in mice. However once reactivation has occurred, either spontaneously or induced by u.v. irradiation, these immunosuppressive drugs might increase the duration of shedding of virus from the eye. Evidence for such activity was provided by one mouse which shed virus spontaneously before treatment. Previously in spontaneous shedding we found virus only in small amounts on 1 or 2 days (Tullo et al., 1982). In the present study one mouse was found to be shedding virus spontaneously before treatment with the drugs. After treatment, the virus was shed for 6 consecutive days and for 2 of these days, large amounts were isolated. Even so, and despite the generalized immunosuppression, no disease was seen. This supports the concept that conditions in the peripheral ocular tissue have an important role to play in the development of recurrent disease (Hill & Blyth, 1976).

Besides closely mimicking the clinical features of recurrent herpetic ocular disease in humans this model has the following advantages. Firstly, the method of inducing such disease is far less demanding than iontophoresis and results in less damage to the cornea. Gordon et al. (1986) commented on the damage caused to the mouse cornea after 3 consecutive days of iontophoresis; indeed such damage may decrease the chance of inducing or recognizing recurrent disease. Secondly the staining of HSV-1 antigens in corneal epithelial sheets allows the specific identification of virus-infected cells. This can confirm clinical observations and identify subclinical foci of infection. Moreover lesions caused by the virus can be distinguished from damage caused by the reactivation stimulus, a distinction which might be difficult to make on clinical examination alone. The excellent correlation between recurrent corneal disease, the isolation of virus from eyewashings and the presence of virus antigens in corneal epithelial sheets provides complete definition of recurrent disease in other systems such definition has not been achieved. For example in latently infected rabbits, after iontophoresis, corneal lesions have been seen in the absence of virus in tears (Haruta et al., 1987; Hill et al., 1987a). Lastly, compared with models of recurrent herpetic eye disease in the rabbit (Hill et al., 1987a; Haruta et al., 1987) use of mice has the advantages of economy (thereby allowing larger experimental groups) and the availability of well defined inbred strains for immunological studies.

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References


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