Spread of Herpes Simplex Virus within Ocular Nerves of the Mouse: Demonstration of Viral Antigen in Whole Mounts of Eye Tissue

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(Accepted 6 August 1987)

SUMMARY
Spread of herpes simplex virus to and within the mouse eye after inoculation of the cornea or the skin of the snout was examined by peroxidase–antiperoxidase (PAP) staining of viral antigen in flat mounts of the eye and by isolation of virus from nervous tissue. Following inoculation of virus at either site, viral antigen was found in ocular nerves. One to three days later antigen was also found in the iris, ciliary body and choroid/sclera suggesting that virus spread to these tissues occurred via their nerve supply. Viral antigen was also found in the retina of the uninoculated eye after corneal inoculation. After inoculation of the snout, virus was isolated from ophthalmic and maxillary parts of the trigeminal ganglion and the superior cervical ganglion and then from the brainstem, eye and mandibular part of the trigeminal ganglion. This sequence also suggested that virus reached the eye via the nerves and that this may occur indirectly via the brainstem. The PAP method allows rapid determination of the distribution of antigen in various tissues. Our observations suggest that widespread involvement of ocular tissue may occur by spread of virus in nerves within the eye.

INTRODUCTION
There has been little study of specific sites of infection in the eye in either human or experimental herpes simplex virus (HSV) keratitis. A fluorescence method of detecting antigen in corneal epithelial sheets has been used (Peeler et al., 1985) and we have recently described peroxidase–antiperoxidase (PAP) staining of HSV in similar tissue sheets (Shimeld et al., 1986). These methods have the advantage that the overall distribution of antigen can easily be determined since the whole area of tissue can be examined rather than the relatively small sample available from selected histological sections. We have now developed them for detecting antigen in similar preparations of retina and the remainder of the eye (i.e. the corneal stroma and endothelium, the uvea and sclera) after removal of the corneal epithelium, lens and retina. This technique, together with assay of infectious virus, has allowed detailed study of the spread of HSV to and within the mouse eye following inoculation of virus on the cornea or snout. The PAP method has proved particularly useful for demonstrating viral antigen along the length of peripheral nerves in the eye.

METHODS
Mice. Male outbred Swiss white Bristol/2 mice (Blyth et al., 1984) were 7 to 8 weeks old at the time of inoculation; 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 (Tullo et al., 1983) or shaven skin of the left snout (Shimeld et al., 1985a) through a 5 μl drop of medium containing 105 p.f.u. HSV type 1 (HSV-1) strain SC16 (Hill et al., 1975) using a 26-gauge

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needle. Control mice (mock-inoculated) were inoculated in the same way with a preparation of uninfected Vero cells made in a similar manner to the virus inoculum.

**Examination of the eyes and isolation of virus.** Mice were anaesthetized and examined for signs of eye disease using a slit lamp. Any mice showing signs of infection of the central nervous system were excluded. The eye was washed with 20 µl of medium which was then placed on Vero cells for isolation of virus (Tullo et al., 1983). The mice were then killed with an overdose of sodium pentobarbitone, the eye was removed for staining by the PAP method and the following tissues were removed from the left side of some mice: the three parts of the trigeminal ganglion (TG), superior cervical ganglion (SCG) and 2 mm³ of brain stem (BS) at the root entry of the trigeminal nerve. The trigeminal ganglion was divided so that part 1 (TG1) probably contained all the ophthalmic and some of the maxillary neurons, part 2 (TG2) maxillary neurons and part 3 (TG3) mandibular neurons (Gregg & Dixon, 1973; Arvidson, 1979; Tullo et al., 1982a). Each tissue was ground in 0.5 ml of medium, frozen and thawed three times and 0.1 ml samples of the suspensions were put on Vero cells in multiwell plates for assay of infectious virus (Tullo et al., 1982a).

**PAP staining.** After 2 h in a solution of EDTA the eyes were transferred to phosphate-buffered saline (PBS), the corneal epithelium was removed (Shimeld et al., 1986) and the lens and retina were taken out through a slit close to the optic nerve. The remainder of the eye and the corneal epithelium were fixed for 30 min in Bouin’s solution followed by at least 20 min in 70% ethanol. The tissues were sometimes stored in 70% ethanol for up to 2 weeks before PAP staining by the method previously described (Shimeld et al., 1986) except that blocking of non-specific protein binding by incubation in swine serum was omitted, dilution of the primary and secondary antibodies was increased to 1:500 and 1:50 respectively and the incubation time for secondary and tertiary antibodies was increased to 1:500 and 1:50 respectively and the incubation time for secondary and tertiary antibodies was increased to 1:500 and 1:50 respectively and the incubation time for secondary and tertiary antibodies was 30 min. A polyclonal rabbit antiserum to HSV-1, swine antiserum to rabbit immunoglobulins and rabbit antiperoxidase complex were all obtained from Dakopatts (High Wycombe, U.K.). Tissues were mounted in half-strength polyvinylpyrrolidone (Mr 40000; Burstone, 1957). Controls for each day were eyes from two mock-inoculated mice, eyes from two inoculated mice that were incubated in PBS instead of primary antibody and HSV plaques in Vero cells on coverslips. Coded slides were examined independently by the first two authors.

The method can be applied to stain antigen in the retina either by fixing the retina after removing it from the eye or, if only the retina is required, by removing it after fixing the whole eye; the latter may give better preservation of retinal structure.

**Silver staining.** Parts of the eye, as described above, from uninoculated mice were stained for nerve fibres by the method of Palmgren (1948).

## RESULTS

### Corneal inoculation

**Eye disease**

For the first 2 days after inoculation corneal disease (including corneal ulceration and stromal opacity) was seen in all mice. The proportion of animals showing signs of disease decreased by 12 of 24 (50%) by day 6 but then increased again (Fig. 1b). Mydriasis was first seen on day 3 and disease of the lids and skin of the snout on day 4. Corneal ulceration and ground glass cornea were seen in mock-inoculated mice for up to 3 days after inoculation but after this their eyes were normal.

**PAP staining and isolation of virus**

All epithelial sheets showed viral antigen on days 1 and 2 after inoculation but by day 8 the antigen had disappeared (Fig. 1a). The proportion of sheets showing antigen was similar to that of eye washings from which virus was isolated (Fig. 1b). Stained cells were seen in the corneal stroma of two of nine mice (22%) on day 1 and two of 10 mice (20%) on day 2 after inoculation (Fig. 2a). Viral antigen was first detected in structures morphologically identified as nerves 2 days after inoculation (Fig. 2b, d, f). Stain was seen in single fibres or bundles of up to eight in one or more of four tracts passing from the centre at the back of the eye to the ciliary body. Sometimes the main nerves were seen to branch around the eye at the ciliary body and into the iris (Fig. 2d). Branches were also seen in the choroid/sclera running towards the ciliary body but no stained fibres were seen in the cornea. Staining occurred intermittently or along the whole length of the nerves, and nuclei (presumed to be those of Schwann cells) contained viral antigen (Fig. 2b). The incidence of staining in nerves rose to a peak of 18 of 19 (95%) on day 4, disappearing by day 7. Staining was seen in the iris on day 1 and days 3 to 7 after inoculation, the
HSV antigen in mouse eye

Fig. 1. (a) Incidence of viral antigen in the corneal epithelium (■), nerves (○), stroma (▲), iris (□) and choroid/sclera (○) and (b) incidence of eye disease (excluding lid disease) (○) and isolation of virus from eye washings (□) after inoculation of $1 \times 10^5$ p.f.u. HSV-1 strain SC16 on the cornea.

maximum incidence being 10 of 19 (53%) on day 5. At first the stain appeared as a network of single cells which sometimes seemed to be linked to stained nerves (Fig. 2d). On days 4 to 7 patches of stained cells were also seen (Fig. 2e); by day 7 such staining was the only type seen in the iris. The patches of stain occasionally joined together forming a ring around the pupillary margin. On the 5th and 6th days after inoculation iris staining was present in all eyes with mydriasis, it was not seen in such eyes at other times. Staining of nerves and patches of cells in the ciliary body were often associated with iris staining. Patchy stain was seen in the choroid/sclera on days 4 to 6 (Fig. 2f).

PAP staining of the retina from both eyes of six mice on day 7 after inoculation showed extensive foci of antigen in the uninoculated eye of one mouse (Fig. 2g), no antigen was seen in retinas from inoculated eyes.

Inoculation on the snout

Eye disease

Eye disease (mydriasis, iris hyperaemia, corneal ulceration, ground glass cornea and stromal opacity) was first seen on day 5 and affected half the mice by day 8 (Fig. 3b). Lid disease was first seen on day 4.

PAP staining and isolation of virus

Staining was first seen in the corneal epithelium and in nerves in the choroid/sclera 3 days after inoculation rising to a peak of 10 of 20 (50%) on day 4 (Fig. 3a). The proportion of epithelial sheets with antigen was again similar to that of eye washings from which virus was isolated (Fig. 3b). Staining was seen in the iris on days 4 to 6, the maximum incidence being seven of 20 (35%) on day 6. Throughout the experiment antigen was seen in the choroid/sclera of only one mouse, on day 5.

Isolation of virus from nervous tissue

One day after inoculation, virus was isolated from the TG2 of one of 12 mice (8%) (Fig. 4). On day 2 virus was isolated from the TG1 of 20 of 24 mice (83%) and from the TG2 of three of 12 mice (25%); thereafter the incidence of isolation from these two parts was similar with a maximum of 24 of 24 and 12 of 12 respectively on days 3 and 4. Virus was first found in the TG3 on day 4 with a maximum incidence of eight of 12 (67%) on day 5. In the SCG virus was present in one of 24 mice (4%) 2 days after inoculation rising to a peak of 20 of 24 (83%) on day 4. The
Fig. 2. Viral antigen (dark staining) in the inoculated eye (a, b, d, e, f) and uninoculated eye (g) of mice given $1 \times 10^5$ p.f.u. HSV-1 strain SC16 on the cornea and silver staining (c) in the eye of an uninoculated mouse. Bar markers represent 10 μm. (a) Stained cells in the stroma on day 1, (b) antigen in Schwann cell nuclei (arrowed) in the choroid/sclera on day 5, (c) silver staining of nerve fibres in the choroid/sclera, (d) branching nerve and network of stained cells in the iris on day 5, (e) patches of stained cells in the iris on day 5. In (d) and (e) arrows indicate the position of the pupillary margin and arrowheads indicate the ciliary body. In (e) there is a gap to the right where the tissue has been cut to flatten it. (f) Branching nerve and patches of stained cells in the choroid/sclera on day 6. (g) Single stained cells and patches of stained cells in the retina of the uninoculated eye on day 7.
incidence of virus isolation from the BS was similar to that for the TG1 and TG2 but with a delay in timing, first appearing on day 3 with a peak of 12 of 12 on day 5.

Silver staining
This showed bundles of fibres running from the back of the eye towards the ciliary body and branching into the iris and stroma (Fig. 2c).

DISCUSSION
Our observations on eye disease, isolation of virus from eye washings and presence of viral antigen in the corneal epithelium were similar to those previously reported after inoculation of the cornea or the snout with HSV (Tullo et al., 1983; Shimeld et al., 1985a, 1986).
After inoculation of the snout the sequence of isolation of virus from the TG, BS and the eye is further evidence that virus can reach the eye by zosteriform spread (Blyth et al., 1984; Shimeld et al., 1985a; C.M.P. Claoué, unpublished results). Indeed, section of the branches of the trigeminal nerve where they emerge from the infraorbital foramen considerably reduces the amount of virus reaching the eye after inoculation of the snout (Shimeld et al., 1987 and unpublished results). The similar incidence of virus in the TG1 and TG2 suggests that the inoculation site on the snout is innervated by both ophthalmic and maxillary branches of the trigeminal nerve. Thus virus may reach the eye via ophthalmic and indirectly via maxillary branches of the trigeminal nerve. As the SCG is frequently infected the sympathetic nerves provide a further route by which virus may reach the eye. Spread of virus to parts of the TG not directly supplying the inoculation site may occur via the BS (the ‘back door’ route; Tullo et al., 1982b). In the present study the sequence of isolation from the TG1 and TG2 together, BS and then TG3 provides further support for this idea.

The single fibres or bundles of fibres showing viral antigen in the iris, ciliary body and choroid/sclera after inoculation of the cornea or snout were similar to those demonstrated in uninfected mice by silver staining and in the rat iris by immunofluorescence with antibody to nerve growth factor (Finn et al., 1986). Therefore it seems most likely that the fibres stained by PAP were nerves. Since the eye has both a sensory and autonomic nerve supply and virus was often found in the SCG the fibres could have been of either type. The distribution and number of the main nerves suggests that they were the long ciliary nerves (Beatie & Stilwell, 1961).

It has been suggested that virus travelling from the inoculation site towards the central nervous system probably lacks an envelope and thereby is unable to leave the axon and infect adjoining Schwann cells (Lycke et al., 1984; Hill, 1987). In the present experiments virus was detected in the TG 1 to 2 days before antigen was seen in the nerves. The timing of the first appearance of stained nerves and the location of antigen (as if in Schwann cells) was similar after inoculation of the cornea or snout. Even at the earliest times of staining, antigen was often present along the whole length of the nerve. Therefore such staining after inoculation of either site most probably indicates virus travelling towards the eye after replication in neurons. It appears that the Schwann cells must become infected almost synchronously by virus emerging from axons at different points along the nerve. The spread from one Schwann cell to another that has been proposed for transport of virus along nerves (Johnson, 1964) would require much longer times than those observed before the whole length of the nerve contained antigen. Anderson & Field (1984) have also reported antigen in Schwann cells of the ciliary nerves after the intranasal inoculation of HSV in mice.

After inoculation of the cornea or the snout, staining of antigen was seen in patches of cells in the iris, ciliary body and choroid/sclera 1 to 3 days after antigen appeared in nerves suggesting that spread to these tissues also occurs via their nerve supply. Similar staining of cells in the iris and ciliary body has been seen in sections and flat mounts after inoculation of the snout with HSV (Claoué et al., 1987). Mydriasis was frequently associated with the presence of viral antigen in the iris and, as in the corneal epithelium (Shimeld et al., 1986), antigen appeared before clinical disease. The single iris with antigen 1 day after corneal inoculation was in an eye with a hyphaema probably resulting from perforation of the cornea at the time of scarification leading to direct infection of the iris.

In a few animals antigen was present in cells in the stroma 1 to 2 days after corneal inoculation probably arising from direct introduction of virus into the stroma during scarification. Staining was never seen in the stroma after inoculation of the snout or in the corneal nerves after inoculation at either site. Rather than lack of antigen this probably reflects limited ability of the PAP reagents to penetrate the stroma (the thickest tissue examined).

Previous studies (Whittum et al., 1984; Shimeld et al., 1985b) have shown that inoculation of one eye of the mouse with HSV may be followed by spread via the central nervous system to the other eye. Although not a major part of the present work, the PAP method allowed us to demonstrate viral antigen in the retina of the uninoculated eye 7 days after inoculation of the cornea. Thus the method can be applied to whole mounts of various parts of the eye, or indeed to any thin sheet of tissue, allowing rapid study of the distribution of antigen; a disadvantage is the difficulty in determining the depth of stained cells in the tissue.
In conclusion the PAP method has demonstrated the widespread nature of infection in the mouse eye following inoculation on the cornea or snout with HSV. It is also clear that the extent of such infection is dependent on spread of virus in ocular nerves. Direct evidence for such interpretation has not previously been available owing to the difficulty of demonstrating the presence of virus or its antigen in histological sections of such structures as ocular nerves. The whole mount technique applied to PAP staining may prove valuable in further studies on the pathogenesis of ocular infection with HSV or other agents.

We wish to thank Penny Stirling for photographic printing. This work was supported by the South Western Regional Health Authority, British National Committee for the Prevention of Blindness, Royal National Institute for the Blind, St. Dunstan’s and the Medical Research Council.

REFERENCES


(Received 16 June 1987)