Herpes simplex uveitis: An experimental study with the fluorescein-labeled antibody technique

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The direct fluorescent antibody technique of Coons was used to study Von Szily's classical experiment on herpetic uveitis in which inoculation of herpes virus into one eye of a rabbit resulted in the development of uveitis 10 to 14 days later in the opposite un inoculated eye. With the fluorescent antibody technique the virus could be demonstrated in the cells of the iris, ciliary body, corneal endothelium, and trabecular meshwork of the inoculated eye as long as 7 days after inoculation. From the fourth to the eighth day after inoculation the virus could be traced spreading along the posterior ciliary nerves of the inoculated eye toward the central nervous system. On the ninth to fifteenth day, fluorescent antibody staining demonstrated the presence of the virus proliferating in the retina but sparing the choroid of the un inoculated opposite eye. Careful sectioning of both optic nerves and the chiasm from the second through the nineteenth day after inoculation showed patchy viral involvement of the nerves and chiasm but failed to demonstrate a progressive spread of the virus infection by contiguous involvement of the glial elements in one nerve moving to the chiasm and up the opposite nerve to the retina. If the virus was inoculated into the vitreous cavity of the right eye, it could be found in the retina of that eye in 48 hours and followed as a progressive infection of the glial cells along the right optic nerve to the chiasm. Infection of the left retina, however, appeared at 9 days after inoculation and before passage of the virus up the left optic nerve could be demonstrated. The inability to demonstrate a continuous spread of the virus along the nerves to the opposite eye suggests an intermittent viremia as the probable route of spread to the opposite eye. Further studies are underway to demonstrate other routes by which the virus may spread in this experimental model.

In 1924, in an effort to produce experimental sympathetic ophthalmia in the rabbit, Von Szily inoculated herpes simplex virus into a dialysis cleft formed between the ciliary body and the sclera in one eye of rabbits. Although all of the animals developed uveitis in the inoculated eye, 15 per cent of the animals developed violent uveitis in the opposite un inoculated eye 10 to 14 days later. Gifford repeated Von Szily's experiments with similar results. Both Von Szily and Gifford suggested from histologic study of the inflammatory changes along the optic nerves and the chiasm in the animals that the virus reached the opposite un inoculated eye by direct transmigration via the optic nerves and chiasm. Goodpastur, in studying similar models, concluded that the herpes virus spread to the central nervous system by an intermittent viremia route.
system along the axis cylinders of the involved nerves, whereas Marinesco and Draganescu concluded from their studies that the virus spread along the "lymphatic channels" of the nerves.

Recent study of Von Szily's model by Kimura has demonstrated that if the PH strain of herpes simplex is inoculated into the anterior chamber in one eye of rabbits, severe uveitis develops in the opposite uninoculated eye 7 to 12 days later in 74 per cent of the animals. He also demonstrated that this was a herpetic uveitis by isolating the herpes virus from the optic nerve and retina of the inflamed uninoculated eye.

The purpose of the present investigation has been to study this experimental model with the direct fluorescent antibody technique of Coons to demonstrate the distribution of the herpes virus in the uninoculated eye when it develops uveitis, and to chart, if possible, the route by which the virus gains access to the uninoculated eye.

**Methods and materials**

**Virus.** PH strain ("O" strain in literature) of herpes simplex virus was used throughout this experiment. The present virus was the twenty-fifth passage in mouse brain (M25) and was stored as a 20 per cent suspension in skim milk at -60° C. The LD₅₀ in mice of PH M25 was 1 x 10⁻⁴⁵.

**Herpes simplex antibodies.** Specific antibodies were obtained from the following sources:

- **Rabbit immune sera.** Two to three kilogram pigmented rabbits were immunized by repeated weekly subcutaneous injections of the virus suspension for 20 weeks. The final immunizing dose was given by scratching the corneas with the virus suspension to boost the titer of neutralizing antibody. The immune sera titer ranged from 2.3 to 2.6 logs of protection against herpes simplex virus (PH M25) by the neutralization test performed in mice as described by Smadel. Individual serum was pooled before conjugation with fluorescein.

- **Human immune sera.** Convalescent sera from patients with herpes simplex keratitis were collected and pooled. This immune serum gave 2.5 logs of protection in the neutralization test.

- **Pooled human gamma globulin.** Poliomyelitis immune gamma globulin (lot No. G-4256) was supplied by the American National Red Cross. This sera gave 1.5 logs of protection.

**Conjugation of fluorescein and antibodies.** The globulin fractions of the rabbit and human immune sera were precipitated with half-saturated ammonium sulfate at 0° C. and cleared of ammonium sulfate by passage through a Sephadex column (G-25). Globulin fractions were conjugated with fluorescein isothiocyanate according to the method of Riggs. Conjugation was carried out at a ratio of 1 mg. of fluorescein to 20 mg. of protein. Unconjugated fluorescein was removed by passage down a Sephadex column (G-25). The fluorescein-labeled antibody was stored in 5 ml. aliquots at -20° C. until needed. Prior to use, each aliquot of conjugate was absorbed twice with various tissue powders (rabbit brain, whole mouse) to decrease nonspecific fluorescence.

**Inoculations and specimens.** Sixty-one rabbits (Black Dutch and New Zealand White) weighing 2 to 4 kilograms were inoculated with 0.03 ml. of a 20 per cent suspension of herpes simplex virus (PH M25) in the anterior chamber of the right eye. The animals were followed clinically and were killed 1 to 19 days after the inoculation. Immediately after death the eyes were removed along with tenon's capsule, the extraocular muscles, and the optic nerves back to and including the chiasm. In 15 animals the brain immediately adjacent to the chiasm was also studied. The removed tissues were embedded in gelatin, frozen immediately, and sectioned in a cryostat. Sections of the globes were made in the sagittal plane and frequently included the exit of the optic nerve. The optic nerves and retrobulbar tissues were usually sectioned at two to three different levels behind the globe. Sections of the chiasm and surrounding brain tissue constituted the most posterior sections examined in this study.

**Staining technique with the fluorescent antibody.** Specimens were covered with two to three drops of fluorescein-labeled antibody for staining, and incubated for 30 minutes at 37° C. in a moist chamber. They were then washed for 15 minutes in three changes of phosphate buffered saline, pH 7.2, and covered with a drop of glycerol and a cover slip. The preparations were examined with a Zeiss fluorescent microscope and photographs were taken on Super Anscochrome with a 35 mm. camera.

The specificity of the fluorescent antibody to demonstrate the presence of herpes simplex antigens was confirmed by (1) its failure to stain preparations not infected with herpes virus, (2) its ability to stain known infected preparations, and (3) the inhibition of staining by preincubation of known infected preparations with nonconjugated herpes immune globulin. Fluorescein-labeled globulins from animals in which we could demonstrate no neutralizing antibodies to the herpes virus also failed to stain known infected preparations.
There was no difference noted in the pattern of specific staining interpreted as herpes simplex antigens with either the human or the rabbit fluorescent antibody except that the rabbit antibody in some specimens produced a greater amount of diffuse nonspecific background staining.

Results

Confirmation of the herpetic uveitis developing in the uninoculated eye. Eleven out of 30 rabbits killed between the eighth and fifteenth days had clinical evidence of uveitis in the uninoculated (left) eye and showed specific fluorescent antibody localization of herpes simplex viral antigens in the retina of that eye (Fig. 1). The ganglion and bipolar cell layers appeared to be the first to show the presence of the virus, with the deeper layers of the retina becoming involved later (Fig. 2). At no time were viral antigens identified in the choroid of these eyes, although it was always greatly thickened and heavily infiltrated with inflammatory cells when uveitis was present. The thickened choroid is shown in Fig. 3. The herpes virus was identified in the ciliary body and iris in the uninoculated eyes in only two animals, one on the thirteenth day and the other on the seventeenth day after inoculation.

Fluorescent antibody localization of the herpes virus did not occur in the uninoculated eyes that did not develop clinical evidence of uveitis.

Studies to chart the spread of the herpes virus to the uninoculated eye. The study to elucidate the route by which the herpes virus injected into one eye reaches the other eye has so far been concerned with

Fig. 1. The number of uninoculated eyes (left) studied and the frequency of positive fluorescent antibody localization of herpes simplex virus in the retina and ciliary nerves of the eyes.

Fig. 2. Frozen section of the retina from an uninoculated left eye stained with herpes simplex fluorescent antibody. Note the specific staining of the nuclear inclusions as well as the diffuse staining in the ganglion and bipolar cell layers.
Fig. 3. Similar preparation to that used in Fig. 2 at a lower magnification. Note that the intense fluorescent antibody staining is confined to the retina. The choroid is heavily infiltrated with inflammatory cells.

Fig. 4. Chart of inoculated eyes (right) studied showing the incidence of herpes simplex virus localization in the iris and ciliary body with a specific fluorescent antibody.

Of the right eyes inoculated with the herpes virus, specific fluorescent antibody localization studies have shown the virus in the iris and ciliary body from the first through the seventh postinoculation day (Figs. 4 and 5). In several eyes the virus was even found proliferating in the endothelium of the cornea and the trabecular meshwork (Figs. 6 and 7).

The virus was found in the retina of the inoculated right eye less frequently up to the eighth day than thereafter. There was positive fluorescent antibody identification of the virus in the retina in 4 out of 24 inoculated eyes examined up to the eighth day after inoculation and in 9 out of 22 such eyes examined after the eighth day (Fig. 8). The data are analyzed in terms of before and after the eighth day because this was the first day on which virus appeared in the opposite uninoculated eye. Virus appearing in the retina of either eye on that and any subsequent day could have become infected by a route common to both eyes. The incidence of herpes virus infection of the retina of either eye after the eighth day, whether the anterior chamber was inoculated with virus or not, was quite similar. For example, of the inocu-
lated eyes studied from the eighth to the nineteenth day, herpes virus proliferating in the retina was found in 9 of 22 eyes studied, and in the uninoculated eyes it was found in 12 of 30 eyes (compare Figs. 1 and 8).

Examination of the ciliary nerves leaving the inoculated globes demonstrated a spread of the virus in a centripetal fashion to the brain through a contiguous infection of the neurilemmal cells of the nerves (Figs. 9 and 10). The virus infection could be followed down the ciliary nerves from the fourth through the eighth postinoculation day (Fig. 8). A similar contiguous spread of the virus in a centrifugal fashion from the brain to the opposite uninoculated eye was also searched for, but it could not be demonstrated in any of the 61 animals studied (Fig. 1).

Herpes virus was found in the optic nerves of both eyes sporadically from the
Fig. 7. Section of the anterior chamber angle from the same eye as in Fig. 6. The positive staining of the trabeculum and anterior iris showing endothelial cells with nuclear inclusions was interpreted as evidence of a trabeculitis rather than a mere phagocytosis of viral antigens.

Fig. 8. The inoculated eyes (right) studied showing the incidence of herpes virus localization in the retina and ciliary nerves.

third to the eighth postinoculation day and infection was noted more frequently on the inoculated side. After the eighth day the virus was found more consistently on both sides (10 out of 34 animals) (Figs. 11 to 13). A study of the optic nerve sections at various levels and in conjunction with study of their accompanying retinas did not indicate a progressive spread of the infection by contiguous involvement of cells from the anterior chamber of the inoculated eye to the retina and optic nerve of that eye and then to the chiasm and up the optic nerve to the opposite eye. For example, virus was found proliferating in the optic nerve of the inoculated eye without evidence that the retina of that eye had been involved. It was not clear in the animals why or how the infection had jumped the retina. Also, virus appeared in the retina of the uninoculated eye without evidence that the optic nerve of that eye had ever been involved.

That the virus can spread from an anterior chamber injection in one eye to the central nervous system rapidly without necessarily involving the intervening retina or glial cells of the optic nerve on the inoculated side is demonstrated by the fact that, in 7 animals studied from the second to the seventh day, there was positive fluorescent antibody localization of the virus in the meninges, connective
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Fig. 9. Posterior ciliary nerve adjacent to the right optic nerve sectioned and stained four days after inoculation of the virus into the anterior chamber of the right eye. Note the specific fluorescent antibody staining indicating the proliferation of herpes simplex virus in the neurilemmal cells of the ciliary nerve.

Fig. 10. Higher magnification of a preparation similar to that in Fig. 9. The intensely staining signet ring-shaped cell is an infected neurilemmal cell wrapped around an axon.

tissue, and brain tissue near the chiasm without involving intervening tissues.

The herpes virus is capable of spreading to the central nervous system by contiguous involvement of adjacent glial cells in the optic nerve as evidenced by a series of 8 animals studied in which the virus, when inoculated into the vitreous cavity of one eye, was noted to spread by contiguous infection of the glial elements of the optic nerve toward the chiasm. In 2 of the 8 animals the virus appeared in the retina.
Fig. 12. Section of the optic nerve infected with herpes virus and stained with specific fluorescent antibody. The virus is localized in the glial cells in one quadrant of the nerve.

Fig. 13. Higher magnification of the infected glial cells shows the brightly staining nuclear inclusions typical of this virus infection when stained with a specific fluorescent antibody.

of the uninoculated eye on the ninth day after inoculation but there was no evidence of viral involvement of the glial elements of the optic nerve on the uninoculated side.

Comments

This study with a specific herpes simplex fluorescent antibody confirms the classical work of Von Szily and demonstrates that the herpes simplex virus is the cause of the inflammatory reaction developing in the uninoculated eye of a rabbit when inoculated with herpes simplex virus in the fellow eye. The distribution of the proliferating virus in the secondarily involved eye indicates that it is principally a herpetic retinitis, as the viral antigens are located only in the retina, and the marked choroidal reaction is secondary to the massive retinal necrosis.

Since the specific staining did not take place in the pigmented tissues of the uninoculated eye, it was considered unlikely that sensitization to uveal pigment played an important role in this experimental model.

Why the uveitis in the uninoculated eye developed in only 37 per cent of the animals (11 out of 30) in this study between the eighth and the fifteenth day, as opposed to 74 per cent in Kimura's previous study, is not known. It is possible that the lower incidence here is related to the different virus pool used which came from a different mouse passage of the virus. Another difference may be in the susceptibility of the rabbits. In Kimura's previous study the animals were inoculated all at one time. In the present study it was necessary to inoculate the rabbits in small groups of 3 or 4 and to sacrifice individual animals at 2 day intervals because of the time required to section, stain, and study each specimen in detail. Unfortunately, this material cannot be stored, as the antigens deteriorate even in the frozen state.

In this particular model, it is difficult to draw any firm conclusions as to the routes by which the herpes simplex virus spreads to the central nervous system and to the
opposite uninoculated eye. It is evident that the virus can spread along the cranial nerves to the central nervous system by contiguous involvement of the neurilemmal cells as demonstrated in the ciliary nerves. This finding is in agreement with the recently published report of Johnson, who also used the fluorescent antibody techniques to demonstrate the centripetal spread of herpes virus to the central nervous system along the endoneural cells (neurilemmal cells and fibroblasts) following the subcutaneous injection of the virus in suckling mice.

Wildy, as quoted by Johnson, has also presented evidence for the centripetal spread of herpes virus along the nerves from the footpad in mice by titrating the virus at various levels along the nerve.

The present study demonstrates that it is possible for virus to spread from the anterior chamber of the eye to the retina of that eye and subsequently by contiguous infection of susceptible cells down the optic nerve to the chiasm. It also appears, however, that at the same time virus is being disseminated rapidly by other routes (along the ciliary nerves and probably via the bloodstream) to various parts of the central nervous system. By what route the virus reaches the opposite retina rather consistently in this model from the eighth to the fifteenth day is not yet clear. Herpes virus showed up in the retina of the uninoculated eye in 12 of our animals, 8 to 15 days after the anterior chamber injection in the opposite eye. Of the animals none demonstrated spread of the virus to the uninoculated eye by contiguous involvement of the optic nerves and chiasm, nor was there evidence that the virus reached the opposite eye via the ciliary nerves. It would appear that the virus reaches the uninoculated eye by some yet unelucidated route. It is highly probable that this route is by way of an intermittent viremia and that the retina of the uninoculated eye becomes susceptible 7 to 8 days after the initial injection. This postulate suggests some sensitization of the ocular tissue subsequent to the initial inoculation. The 7 to 8 day time delay is consistent with described immunologic models. A study of the various antibodies developing in this experimental model to the variety of antigens involved in the inoculated eye (virus, mouse brain, and rabbit eye tissue) may help clarify this point.

REFERENCES
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