Endothelial lesions of rabbit cornea produced by herpes simplex virus

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Microscopic lesions of corneal endothelium produced by herpes simplex virus were studied in flat preparation of the endothelium following injection of the virus into the anterior chamber of the rabbit eye. The intraocular injection of the virus produced corneal opacity, characteristic endothelial lesions, and uveitis. These ocular changes were virus specific, and were accompanied by virus multiplication in the tissues. The virus was isolated readily from the aqueous humor of the infected eyes during the peak period of virus multiplication in the infected tissues. The endothelial lesions observed were: (1) derangement of endothelial cells, (2) discrete focal lesions (plaques), and (3) diffuse lesions. Eosinophilic intranuclear inclusions were seen with varying frequencies in the cells of both focal and diffuse lesions. Only the diffuse lesions appeared to be directly related to the production of corneal opacity.

Key words: herpes virus infections, corneal endothelium, corneal opacity, uveitis, intranuclear inclusion, herpes simplex virus, anterior chamber injections, virus isolation, viral inclusions, aqueous humor, iris, histopathology, rabbits.

Many animal viruses have been shown to produce corneal opacity when inoculated into the anterior chambers of rabbit eyes.1-4 The development of the corneal opacity induced by influenza A,5 Newcastle disease,5 vaccinia virus,6 or trachoma agent7 was shown to be related directly to the appearance in corneal endothelium of microscopic lesions characteristic for each microorganism.

Recently, the author has found that inoculation of herpes simplex virus (HSV) into the anterior chamber of the rabbit eye produced corneal opacity resulting from endothelial lesions of unique morphology. This report describes the characteristics of HSV-induced changes in the anterior segment of the rabbit eye with a particular emphasis on the endothelial lesions of the cornea.

Materials and methods

Rabbits. The experimental animals were young, healthy, male New Zealand white rabbits weighing from 4 to 5 pounds. Rabbits with "spontaneous" opaque cornea or other ocular abnormalities were not used.

Virus. The PH strain of HSV, in the form of a mouse brain emulsion, was subcultured in primary cultures of rabbit kidney cells. For this study, the supernatant fluid of the tissue culture medium from the tenth passage was used. It had...
a titer of $2.5 \times 10^6$ plaque forming units (PFU) per milliliter.

_Intraocular injection of virus._ Rabbits were anesthetized by an intravenous injection of sodium pentobarbital, and topical administration of Tetracaine HCl (Alcon Labs., Fort Worth, Texas) drops was also employed. A 26 gauge hypodermic needle attached to an empty 0.5 ml syringe was introduced through the conjunctiva and into the sclera 3 to 5 mm. behind the limbus. The needle was then directed obliquely through the sclera and into the angle of the anterior chamber; 0.2 ml. of aqueous humor was aspirated and the needle was withdrawn from the eye. With another syringe, a second insertion of a 26 gauge needle was made in the same manner, and 0.2 ml. of the desired concentration of virus was introduced slowly into the anterior chamber. Care was taken to prevent damage to the endothelium by the tip of the needle. By this technique, leakage of the inoculum was minimized after withdrawal of the needle from the anterior chamber.

_Collection of aqueous humor for virus titration._ Rabbits were killed by intravenous injection of sodium pentobarbital, and approximately 0.2 ml. of aqueous humor was aspirated after introducing a 23 gauge hypodermic needle into the anterior chamber through the central area of the cornea. The aqueous humor was then diluted to 1:10 in the growth medium for chick embryo cells described below, and stored at -75° C. until titration.

_Preparation of ocular tissue homogenate for virus titration._ The cornea with iris attached was excised aseptically from the eye and weighed. It was washed twice in 3 ml. of the growth medium, placed in 1 ml. of anti-HSV rabbit serum, and kept at room temperature for 30 minutes. Then the tissue was washed twice again in 3 ml. of the growth medium. It was minced to cubes of approximately 1 mm. size, and ground in a mortar with a pestle. One ml. of sterile crystalline albumin (90 mesh) was added to facilitate the grinding of the tissues. A 10 per cent tissue homogenate was made by adding growth medium in the amount 9 times of the wet weight of the cornea and iris. This was centrifuged at 4° C. for 10 minutes at 800 x g. The supernatant was collected and stored at -75° C. until virus titration.

_Titration of virus._ Primary cultures of chick embryo fibroblast cells were grown in disposable plastic Petri dishes, 100 mm. in diameter. The growth medium for the cells consisted of 10 per cent lactalbumin hydrolysate, 10 per cent heat-inactivated calf serum in Earle’s balanced salt solution, and 100 units of penicillin, 100 mg of streptomycin and 20 mg of Nystatin per milliliter. To titrate the virus, 10-fold dilutions were made of the specimen, and 0.2 ml. of each dilution was placed on each of duplicate plates of the cells. The plates were placed in a 5 per cent CO2 incubator at 36° C. for 2 1/2 hours; 6 ml of overlay medium was poured into each plate, and they were returned to the incubator after the medium had solidified. The overlay medium consisted of 0.5 per cent lactalbumin hydrolysate, 0.1 per cent yeast extract, 10 per cent heat-inactivated calf serum, and 0.6 per cent agarose (Mann Research Labs., N. Y.), in Earle’s balanced salt solution fortified with antibiotics.

Flat preparation of corneal endothelium. The entire sheet of endothelium, stained with silver nitrate, was separated from the cornea and flattened on a slide according to techniques described elsewhere. The dried sheet of endothelium was then stained with a 1:20 dilution of Harris’ hematoxylin for 2 hours. For staining the intranuclear inclusions of HSV the dried sheet of silver-stained endothelium was further fixed in Bouin’s fixative for 30 minutes and kept in 70 per cent ethanol overnight. Then it was stained with 1:20 dilution of Harris’ hematoxylin for 2 hours and with saturated aqueous eosin for 30 minutes.

Results

Production of corneal opacity by herpes simplex virus. Following the injection of $10^6$ PFU of the virus into the anterior chamber of the rabbit eye, corneal opacity became evident in 75 per cent of the eyes on day 2 and 95 per cent on day 6 (Figs. 1 and 2). The opacity was so intense that the pupillary outline could not be seen through the cornea. With $10^6$ PFU of the virus, only faint opacity was observed on day 2 or day 3 and intense opacity was seen on day 6 in 25 per cent of the eyes. No opacity was observed with $10^5$ PFU of the virus during the entire period of observation. In general, the opacity began to appear at the peripheral region of the cornea and spread toward the center. Neither heat-inactivated HSV nor uninfected culture medium of rabbit kidney cells produced the corneal opacity.

Microscopic lesions of corneal endothelium produced by HSV. This study was carried out in flat preparations of corneal endothelium stained with silver nitrate and counterstained with Harris’ hematoxylin. When normal corneal endothelium was stained in this manner, the cell boundaries were outlined with a de-
posit of brownish silver granules, and the nuclei were stained blue with hematoxylin (Fig. 3). The individual cells were uniform in both shape and size and were arranged in a regular mosaic pattern. The majority of the cells contained single oval or bean-shaped nuclei, and a few had two or three nuclei.

Fig. 1. Opaque cornea produced by herpes simplex virus.

The eyes inoculated with HSV showed the following characteristic microscopic lesions in the endothelium:

*Derangement of regular mosaic pattern of cell arrangement.* This was due to a moderate degree of irregularity in size and shape of the individual cells (Fig. 4). This change began to appear in the endothelium at the periphery of the cornea within 10 or 24 hours after inoculation of either $10^5$ or $10^4$ PFU of the virus respectively. With $10^2$ PFU, the change was seen at 72 hours or more after the inoculation (Table I).

*Discrete focal lesions (plaques).* These were small circular lesions consisting of cells of different sizes and shapes arranged in an irregular pattern (Figs. 5 and 6). They were seen in all corneas within 24 or 48 hours following injection of $10^6$ or $10^4$ PFU of the virus, respectively (Table I). Occasionally plaques could be seen in the deranged endothelium. The number of plaques per cornea was higher in the eyes inoculated with $10^6$ PFU of HSV than in
Fig. 3. Corneal endothelium of normal rabbit. (Silver and hematoxylin stain; x120.)

Fig. 4. Derangement of endothelium at the periphery of the cornea produced by herpes simplex virus. Many inflammatory cells are present. (Silver and hematoxylin stain; x120.)

those with $10^1$ PFU. However, there was no close correlation between the number of plaques produced and the number of infective virus particles inoculated. The plaques were more numerous at the periphery of the cornea than at the central area. Inoculum of $10^2$ PFU of the virus failed to produce plaques.

Diffuse lesions. These consisted of endothelial cells of markedly irregular size and shape affecting much larger areas of the endothelium than the discrete focal lesions (Fig. 7). They were seen only in corneas with distinct opacity (Table I).

In general, infiltration of inflammatory cells in the endothelium was more extensive around the peripheral regions of the cornea than the central area. The role of inflammatory cells in the pathogenesis of the lesions is not clear at present. Neither the lesions nor inflammatory cells were seen in eyes inoculated with heat-inacti-
Fig. 5. Discrete focal lesions (plaques) of corneal endothelium produced by herpes simplex virus. Derangement of endothelium is also present in left one-third of this picture. (Silver and hematoxylin stain; ×130.)

Fig. 6. A discrete focal lesion seen under higher magnification. Many eosinophilic intranuclear inclusions (arrows) are seen. (Silver, hematoxylin and eosin stain; ×300.)

Table I. Summary of corneal lesions produced by HSV

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<th>Inoculum (PFU)</th>
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*= Negative; ±, occasionally positive; +, almost always positive.

Table I. Summary of corneal lesions produced by HSV

Inoculum (PFU) & Corneal opacity & Deformation & Focal (plaque) & Diffuse  
$10^6$ & ± & + & + & +  
$10^4$ & ± & + & + & ±  
$10^2$ & ± & + & - & -  

*= Negative; ±, occasionally positive; +, almost always positive.

Demonstration of intranuclear inclusions in the endothelial lesions. In order to find whether the endothelial lesions were the site of virus multiplication, intranuclear inclusions were looked for in a flat preparation of silver-stained corneal endothelium counterstained with hematoxylin and eosin after fixation with Bouin's solution.
A majority of the cells in the plaques showed eosinophilic intranuclear inclusions, Cowdry type A, typical of HSV (Fig. 6). However, inclusions were seen less frequently in the cells of the diffuse lesions (Fig. 8).

*Other ocular lesions.* In addition to the corneal opacity and the endothelial lesions, HSV-infected eyes showed marked congestion and occasional hemorrhage at the limbus. The conjunctivitis was generally severe by 48 hours, and a moderate amount of purulent exudate was seen. The formalin-fixed section stained with hematoxylin and eosin revealed marked stromal edema with extensive inflammatory responses in the limbus, filtration angle, and iris. Occasionally, what appeared to be eosinophilic intranuclear inclusions were seen in the epithelial cells of the posterior surface of the iris. The lens appeared to be free of inflammation.
Fig. 9. Isolation of herpes simplex virus from ocular tissues and from aqueous humor of rabbit eyes following intracameral injection of varying amounts of the virus ($10^2$, $10^4$, and $10^6$ PFU). Each circle represents one eye.

**Virus isolation from ocular tissues and aqueous humor.** To correlate the progress of the ocular lesions with viral activity in the infected tissues, attempts were made to isolate and titrate the virus in both tissues (cornea, iris and portion of limbus), as well as in the aqueous humor at various times following injection of the virus. Groups of rabbits received intracameral injections of virus into both eyes with varying concentrations of HSV ($10^2$, $10^4$, or $10^6$ PFU). On days 1, 2, 3, 4 and 6, a group of three or more rabbits were killed, and both aqueous humor and ocular tissue homogenate were obtained for virus titration in the manner described in the section on methods. The results of this experiment are summarized in Fig. 9.

**Isolation of HSV from ocular tissues.** As little as $10^2$ PFU of the virus-initiated viral synthesis in ocular tissues when introduced into the anterior chamber, and peak titers of between $10^4$ and $10^6$ PFU were obtained on day 2. With the inoculum of $10^6$ PFU, the virus titer reached a peak on day 1. The virus was isolated from the tissues as late as 6 days after the infection in two out of eight eyes inoculated with...
10⁴ PFU of HSV, and in five out of six eyes inoculated with 10⁵ PFU. The isolates were confirmed to be HSV by neutralization tests with anti-HSV rabbit serum.

**Isolation of HSV from aqueous humor.** Following the injection of 10⁴ PFU of HSV into the anterior chamber of the rabbit eye, the titer of the virus in the aqueous humor dropped abruptly to 5 × 10² PFU at 10 hours, followed by an increase in titer at 18 hours. Peak titers of between 10⁴ and 10⁵ PFU were reached at 24 hours. The virus was isolated in rather high titers from all eyes up to 2 days after infection, from a half of the eyes on day 3 and 4, and from one of six eyes on day 6. When 10⁴ PFU of the virus were administered, positive isolation was obtained from the aqueous humor of seven of eight eyes on day 1, from five of eight eyes on day 2, and from none thereafter. From the eyes inoculated with 10² PFU of HSV, only one of six eyes showed virus on day 1 and day 3, three of six eyes on day 2 and none on day 4. The isolates were HSV as evidenced by positive neutralization tests with anti-HSV rabbit serum. In all cases, virus titers of the aqueous humor were substantially lower than those of the corresponding ocular tissues, ranging between 5 × 10⁻⁶ and 5 × 10⁻⁸.

**Discussion**

Various animal viruses and the agents of Chlamydia have been shown to be capable of producing corneal opacity when introduced into the anterior chambers of the eyes of laboratory animals. The development of the corneal opacity induced by the microorganisms was related directly to the appearance in corneal endothelium of microscopic lesions characteristic of each agent. In the present study, HSV inoculated into the anterior chamber of the rabbit eye also produced corneal opacity associated with characteristic endothelial lesions (derangement, plaques, and diffuse lesions) and uveitis. Among the endothelial lesions produced by HSV, only the diffuse lesions appeared to be directly related to the production of corneal opacity; the more extensive the diffuse lesions, the denser the corneal opacity. Both derangement and plaque formation in the endothelium, on the other hand, were seen in corneas without opacity. However, the derangement may well be an early stage of the diffuse lesions.

Maloney and Kaufman reported "endothelial loss" in rabbit eyes following injection of live HSV into the anterior chamber. Irvine and Kimura also described certain endothelial changes in experimental stromal herpes simplex keratitis in rabbits following the inoculation of RE strain of HSV on the scratched corneal surface. They noted changes ranging from apparent cell death to cell multiplication with mitosis, as well as multinucleated giant cells and focal proliferation of endothelial cells. However, they considered the lesions to be nonspecific signs of endothelial damage and regeneration since these lesions could be produced when either bovine serum albumin or sodium hydroxide was applied to the cornea. The lesions they described differ from those observed in the present study. This could be due to differences in the strain of virus used, the route of virus inoculation, or the staining technique for endothelium.

The present study showed that the cells in the plaques frequently contained eosinophilic intranuclear inclusions, while the cells in the diffuse lesions seldom contained inclusions. The results appear to suggest that the plaques represent virus infection of the cells whereas the diffuse lesions are mainly produced by nonviral mechanisms. Further studies are needed to confirm this possibility. Several other authors have also demonstrated intranuclear inclusions in occasional endothelial cells, and viral antigens in corneal endothelial cells have been demonstrated by the fluorescent antibody technique.

In previous experiments, it was shown that there were marked morphological differences among the microscopic endothelial lesions produced by Newcastle disease,
influenza A, vaccinia virus, or trachoma agent. The present study revealed that the morphology of the microscopic endothelial lesions produced by HSV, particularly of the plaques, was so characteristic that they could be differentiated from each of those produced by the other microorganisms mentioned above. The findings strengthen the previous view that the corneal endothelial lesions produced by various viruses may be morphologically unique and thus useful as a means of virus identification.

HSV apparently multiplied in various ocular tissues following its injection into the anterior chamber of the rabbit eye since (1) the virus could be isolated regularly from the tissues in higher titers than the titers of the original inocula, and (2) typical intranuclear inclusions of HSV were present in a number of ocular tissues. During the period of peak multiplication of HSV in the tissues, the virus was isolated in rather high titers from the aqueous humor of all eyes that had been inoculated with $10^6$ PFU of the virus, and from the majority of the eyes inoculated with either $10^6$ or $10^4$ PFU of the virus. Previous authors reported difficulty in detecting the virus in the aqueous humor after injection of PH strain of HSV into the anterior chamber of the rabbit eyes. Our constant success in isolation of the virus from the aqueous humor can be attributed to (1) the use of a more sensitive technique for virus isolation, and (2) the use of HSV which had been adapted to the rabbit tissues. Positive isolation of HSV from aqueous humor of rabbit eyes has been previously reported by others.

The experimental infection produced in this study may be a potentially useful experimental model for analyzing in vivo effects of various antiviral agents on HSV. Similar models have been proven to be ideal for studying in vivo effects of interferons on ocular lesions produced in rabbits by vaccinia, Newcastle disease, and herpes simplex viruses. Furthermore, HSV-induced endothelial lesions of the cornea provide an additional tool for the study of the pathogenesis of virus infections in the rabbit eye.

REFERENCES
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