Ocular lesions associated with dissemination of type 2 herpes simplex virus from skin infection in newborn rabbits

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The subcutaneous inoculation of the backs of New Zealand white rabbits 17 to 34 hr old with
10^5 50% tissue culture infection dose (TCID_{50}) of type 2 herpes simplex virus (HSV-2) induced cutaneous lesions within 24 hr, foci of disseminated infection in many organs (including the eye) on day 3 and thereafter, and the death of the animals on day 5 with infection of the central nervous system. Infectious HSV-2 could be isolated from the mononuclear cells and plasma of the peripheral blood, indicating the active role of both elements in the dissemination of the virus. Infectious HSV was also recovered from the corresponding sensory ganglia of the skin lesion (the cervicothoracic ganglia) as early as 2 days after the subcutaneous inoculation of the virus. About 40% of the animals developed ocular lesions consisting of retinal folds with or without degenerative changes. Iritis and choroiditis also developed in some eyes. Infectious HSV-2 could be isolated from 33% of the eyes on days 4 and 5. Thus the newborn rabbit may serve as a suitable experimental animal for the study of HSV-2-induced chorioretinitis in the human newborn. (INVEST OPHTALMOL VIS SCI 21:681-688, 1981.)

Key words: type 2 herpes simplex virus, skin infection, retinal lesions, disseminated infection, viremia, ganglion infection, newborn rabbits

Neonatal herpes simplex virus (HSV) infections have been recognized since Batignani’s description of herpetic keratitis in a newborn in 1934.1 For many subsequent years, HSV infection in the neonate was believed to eventuate characteristically in a fatal systemic disease. Recently, however, the development of advanced laboratory techniques has resulted in the recognition of a broader spectrum and more frequent occurrence of HSV infections in the neonate.

Ocular manifestations of neonatal HSV infection—conjunctivitis, keratitis, chorioretinitis, optic neuritis, cataracts, uveitis, and microphthalmia—occur in about 10% of neonates with disseminated infection and in 33% of those with localized infection.2 Type 2 HSV (HSV-2) is the major cause of HSV neonatal disease3 and the only agent found in herpetic chorioretinitis.4, 5 The dermatotropic nature of HSV-2 has been thought to be partly responsible for this unique association of HSV-2 with chorioretinitis,5 but the pathogenetic mechanisms by which the internal ocular structure becomes affected remain unclear.

Experimental animal models are invaluable tools for the study of pathogenetic
mechanisms of any disease, yet almost no attempts to construct animal models of neonatal herpetic chorioretinitis have been made in the past. Recently we have successfully produced retinochoroidal lesions in the newborn rabbit eye by using HSV-2 to infect the animal’s skin, which is major natural portal of entry for the virus in the newborn. In this paper we describe our model and the various lesions the virus produces in the newborn rabbit.

Materials and methods

Newborn rabbits. New Zealand white pregnant rabbits were acquired at midterm from a local commercial vendor. Each rabbit was placed in a separate cage with a breeding box. The newborn rabbits we used were between 17 and 34 hr old.

Virus. The Curtis strain of HSV-2 was grown in primary cultures of rabbit kidney cells maintained in a medium consisting of 95% Medium 199, 5% rabbit serum, and antibiotics. The final titer was $10^5$ 50% tissue culture infective dose (TCID$_{50}$) per milliliter.

Virus inoculation of the skin. With 26-gauge hypodermic needles, we made subcutaneous injections of 0.05 ml of virus suspension into each of two sites on the backs of the newborn rabbits, one on each side of the midline.

Collection of blood and tissue specimens for virus assay and histopathology. The rabbits were killed by intraperitoneal injection of sodium phenobarbital, and the visceral organs were exposed by an aseptic technique. For virus isolation attempts, 2 ml of cardiac blood was collected immediately from each rabbit in a syringe containing 10 U of heparin. Various organs (heart, lungs, thymus, liver, spleen, kidneys, adrenal glands, skin, brain, and eyes) were then removed aseptically, and a piece of each organ (except one eye and one adrenal gland of each rabbit) was fixed in Bouin’s fixative for histopathologic study. The remaining tissues, including the second adrenal gland and the second eye of each rabbit, were frozen at $-60^\circ$C until used for virus isolation attempts.

Virus inoculation from cardiac blood. As described previously, an equal volume of sterile physiological saline solution was added to the heparinized blood and separated the leukocytes in Ficoll-Hypaque gradient as described by Boyum.

The separated leukocytes were washed three times in 3 ml of phosphate-buffered saline (PBS) at pH 7.3. The washed leukocytes and plasma were then inoculated into two tubes of Vero cells. The tubes were incubated in a stationary position at 36°C, and the culture medium was changed completely 1 day later. We examined the cells for cytopathic effects (CPE) daily for 7 days. The virus isolated were identified as HSV by a neutralization test in which we used anti--HSV-2 immune serum.

Virus isolation from tissues. A 1 cc piece of each tissue was ground with a mortar and pestle, with 1 ml of crystalline alumina (90 mesh) added to facilitate the grinding. Tissue homogenate, prepared by adding 2 ml of a medium consisting of 95% Eagle’s minimum essential medium (MEM), 5% fetal calf serum, and antibiotics was centrifuged at 800 x g for 10 min at 40°C. The resultant supernatant fluid was considered to be a 1:10 dilution of the specimen, and further serial 10-fold dilutions were made with the medium. Vero cell tubes were then inoculated with 1 ml of each dilution, incubated in a stationary position at 36°C, examined for CPE daily for 7 days. The reciprocal of the highest dilution showing CPE was considered to be the infectivity titer of the tissue homogenate per milliliter.

Histopathologic studies. All the tissues fixed in Bouin’s solution were processed and embedded in paraffin. Five-micrometer sections, stained with hematoxylin and eosin (H&E), were then examined microscopically.

Measurement of skin lesions. The lesions produced by the subcutaneous injection of HSV were examined daily, and their diameters measured in millimeters. To calculate the average size of the lesions produced by HSV on a given day, we divided the sum of the diameters of all the lesions on all the rabbits on that day by the total number of skin sites injected.

Experimental results

Determination of optimal infective dose. To obtain the optimal infective dose of HSV-2 with which a high rate of ocular lesions, low mortality, and prolonged survival could be achieved, the following experiment was carried out.

Rabbits in each of the litters received a subcutaneous injection of either 1, 10, 10$^2$, 10$^3$, or 10$^4$ TCID$_{50}$ of HSV. Another rabbit in each litter served as a control and was inoculated in the same way with MEM, the diluent for the virus. The animals were examined...
daily for the development of skin lesions, systemic symptoms such as hindleg paralysis, and death. When death was imminent, the animals were killed and subjected to gross autopsy examination. Both eyes were also removed for histopathological examination.

All the animals inoculated with $10^4 \text{TCID}_{50}$ of the virus developed severe skin lesions by day 2 and died by day 4. With smaller inocula, the skin lesions developed more slowly, and the animals survived progressively longer; the fatality rate was 100% by postinoculation day 5 with $10^2 \text{TCID}_{50}$ and by day 7 with $10^3 \text{TCID}_{50}$. The fatality rate was still over 80% in the group receiving only $10 \text{TCID}_{50}$ and remained 10% in the group receiving as little as $1 \text{TCID}_{50}$. The incidence of retinal lesions was almost the same in the animals inoculated with $10^3$ or $10^4 \text{TCID}_{50}$ (35% or 33%, respectively) and was 25% in the ones inoculated with $10^2 \text{TCID}_{50}$. For this reason an inoculation dose of $10^3 \text{TCID}_{50}$ of HSV-2 was used throughout the experiments.

Dissemination of HSV from skin to visceral organs and eyes. Litters of newborn rabbits were inoculated subcutaneously with $10^5 \text{TCID}_{50}$ of HSV. On successive postinoculation days they were examined and killed. We collected specimens from various organs for histopathologic and virologic studies and removed blood and both cervicothoracic and trigeminal ganglia for virus isolation attempts.

Pathologic studies. All the animals showed skin lesions at the site of inoculation within 48 hr after the virus inoculation. The lesions were initially erythematous and indurated areas but were followed by typically grouped, herpetic vesicles (Fig. 1, a) that enlarged, coalesced, became crusted (Fig. 1, b), and were sometimes hemorrhagic prior to death. On postinoculation day 4, all the animals showed signs of systemic illness, including lethargy, irritability, restlessness, and hindleg paralysis; they had seizures and ate poorly, and all died by day 5.

Gross autopsy showed signs of disseminated viral infection. Livers and spleens were the sites of most of the lesions, which appeared as early as postinoculation day 3. The adrenal glands showed microscopic foci of
necrosis and hemorrhage in both cortex and medulla. Pneumonitis, myocarditis, and hemorrhagic nephritis also occurred in some animals. There were perivascular cuffing and encephalitis in the brain. Typical Cowdry type A intranuclear inclusions were found in the liver, spleen, the adrenal gland, and the skin.

In the eye, full-thickness retinal folds (Fig. 2a) with occasional focal necrosis (Fig. 2b) were observed in about 40% of the rabbits killed on postinoculation day 5. In only a few cases did we see iritis or choroidal involvement, and only a minimal inflammatory response was associated with either of these changes. The fact that none of the ocular changes was seen in normal animals, however, or in animals inoculated subcutaneously with
heat-inactivated HSV (56°C for 30 min) indicates the virus-specific nature of the lesions.

Virologic studies. Infectious HSV could be recovered only from the skin on postinoculation day 1; on day 2 it could be recovered from the skin in high titers ($10^3$ to $10^4$ TCID$_{50}$) and from the lungs in low titers (Fig. 3); and on or after day 3, it could be recovered in high titers from many organs. It could first be isolated from the eyes on day 4 and was present in one third of the eyes cultured on either day 4 or day 5. Titers of the virus in the eyes ranged from $10^2$ to $10^3$ TCID$_{50}$.

Isolation of HSV from blood. Infectious HSV could not be recovered from the blood before day 3 (Table I). It could be found in

Fig. 2b. Retinal fold with focal necrosis.
the blood of 38% of the rabbits on day 3, 44% on day 4, and as many as 78% on day 5.

On day 3 HSV could be recovered from the mononuclear fraction of the blood of two rabbits and from the mononuclear and plasma fractions of one rabbit. On days 4 and 5, however, it could be recovered more often from mononuclear cells and plasma. Overall, the virus was recovered from only mononuclear cells in five rabbits, only plasma in one rabbit, and both mononuclear cells and plasma in eight rabbits.

Isolation of HSV from sensory ganglia. Skin infection with HSV-2 was accompanied by corresponding sensory-ganglion infection during its early stages. As shown in Table II, infectious HSV could be isolated from the homogenate of the cervicothoracic ganglia of seven of eight rabbits as early as day 2 after the skin inoculation and from the same ganglia of almost all the rabbits thereafter (days 3 and 4). On all these days, the trigeminal ganglia were free of the infection.

Discussion

In this paper a new experimental animal model for the study of neonatal herpetic chorioretinitis has been described and characterized. The unique feature of the model is that the eye lesions are produced in the newborn rabbit by infecting the skin with HSV-2, a natural route of infection for HSV in newborn infants. In contrast to a previously reported animal model, in which a newborn rat was inoculated to produce eye lesions by an unnatural route (intracerebral), we could produce retinal lesions in 40% of our newborn rabbits by way of the skin.

In a study of human infants, about 30% with herpetic skin lesions developed chorioretinitis. As in these human infants, HSV-2 produced typical skin lesions in the newborn rabbits, multiplied at the skin sites, disseminated to various organs, including the eye, and produced chorioretinal eye lesions. The newborn rabbit model is thus quite suitable for the study of neonatal herpetic eye infection. Recently we have used it successfully to determine the protective effect of immunization and of antiviral agents on the neonatal herpetic eye infection.

In the newborn rabbit model, typical retinal lesions were observed only in the eyes of the animals with HSV-2 skin infection. No such retinal lesions were seen in the eyes of normal rabbits or of animals whose eyes and skin were inoculated with heat-inactivated HSV-2, which would seem to indicate that
Retinitis, HSV-2 skin infection

Table I. Isolation of HSV-2 from circulating mononuclear cells and plasma of newborn rabbits

<table>
<thead>
<tr>
<th>Postinfection day</th>
<th>No. of specimens</th>
<th>HSV-2 isolated from (no. of specimens)</th>
<th>Totals</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Mononuclear cells only</td>
<td>Plasma only</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
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<td>1</td>
<td>0</td>
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<tr>
<td>5</td>
<td>9</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Totals</td>
<td>5</td>
<td>1</td>
<td>8</td>
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</table>

the retinal lesions in the eyes of the HSV-infected newborn rabbits were virus-specific. No intranuclear inclusions typical of HSV could be found in these retinal lesions, however, although low titers of infectious HSV could be recovered from the whole eye. To define the exact relationship between the retinal lesions and the virus, therefore, further studies are clearly needed. Other workers have found HSV-like particles in the retina of an infant with bilateral herpetic chorioretinitis and in an experimental retinitis produced by HSV-2 in rats. Infectious HSV could be recovered from mononuclear cells of the peripheral blood and plasma of newborn rabbits as early as 3 days after the skin infection and was present in the eye 1 day later. It would seem therefore that in this model HSV reaches the eye from the skin lesions via the hematogenous route. In human infants, intranuclear inclusions were found in both vascular endothelial cells and the perivascular cuffing, and this also suggested hematogenous dissemination of the virus. It is not clear, however, which of the mononuclear cell subsets in the newborn rabbit are associated with HSV-2 or whether the virus multiplies in the mononuclear cells. In humans, HSV is known to multiply in both lymphocytes and monocytes.

Cervicothoracic ganglia of newborn rabbits were infected with HSV-2 as early as 2 days after their skins were inoculated. Hematogenous dissemination of the virus to these ganglia was unlikely because the trigeminal ganglia of the same animals were not affected. It would seem therefore that the virus reached the ganglia by a neuronal route. Field and Hill estimated that herpesvirus traveled toward the ganglia at about 2 mm/hr. If this figure were applied to our newborn rabbits, only 5 hr would be needed by the virus to reach the cervicothoracic ganglia, a distance of about 10 mm. It would thus be quite possible for the virus to infect these ganglia from a cutaneous site by a neuronal route.

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Table II. Isolation of HSV-2 from sensory ganglia of newborn rabbits

<table>
<thead>
<tr>
<th>Ganglia</th>
<th>Postinfection day</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Cervicothoracic</td>
<td>0/5</td>
</tr>
<tr>
<td>Trigeminal</td>
<td>0/5</td>
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</tbody>
</table>

Values are number of animals yielding HSV-2/number of animals tested.

REFERENCES