Canine herpes-induced retinal dysplasia and associated ocular anomalies

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Thirty-eight newborn Beagle puppies from eight litters of a specific pathogen-free colony maintained in isolation were inoculated with canine herpesvirus. Pups were killed between one and 30 days after inoculation. Histopathologic studies were carried out on the eyes and other tissues in conjunction with fluorescent antibody and viral isolation studies. Evidence of ocular inflammation manifested by panuveitis with the presence of intranuclear inclusion bodies was usually seen by the fourth day after infection. Eyes with severe inflammation showed peripheral anterior synechiae, cataract, and keratitis. The presence of the virus was confirmed by viral isolation from ocular tissues and fluorescent antibody studies. Developmental anomalies included retinal dysplasia with fold and tube formation of the neural retina, retardation of retinal maturation, and areas of necrosis and reorganization were seen. The retinal pigment epithelium showed initially patchy depigmentation and vacuolization and, subsequently, folding hypertrophy and duplication as well as areas of widespread atrophy and patchy loss. In some animals ectopic retina was found within cystic spaces of the optic nerve. These experiments confirm the ability of canine herpes infection in neonatal pups to produce severe ocular inflammation with subsequent retinal dysplasia and associated ocular anomalies.

Retinal dysplasia, or maldevelopment of the retina, is a relatively common histopathologic finding in congenitally blind eyes enucleated in childhood. It may occur as a sporadic finding without evidence of systemic anomalies, or may be associated with certain chromosomal abnormalities and hereditary diseases. Additional known causes in animals are certain viral diseases, including blue tongue virus, bovine diarrhea-mucosal disease, adenovirus, and lymphocytic choriomeningitis virus.

The true incidence of congenital malformations occurring as a result of prenatal or neonatal viral infection in humans or other species is unknown. It seems likely that they are a significant factor, since the number of human pregnancies complicated by systemic maternal viral infections exclusive of the common cold has been estimated to be 5 per cent. Herpesvirus is recognized as an ubiquitous agent, and has been shown both clinically and experimentally to be capable of crossing the placenta.
Table I. Occurrence of ocular lesions in pups infected with canine herpes virus histopathologic lesions

<table>
<thead>
<tr>
<th>Days after infection</th>
<th>No. of animals</th>
<th>Keratitis</th>
<th>Cataract</th>
<th>Iritis cyclitis</th>
<th>Choroiditis</th>
<th>Optic neuritis</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-4</td>
<td>8</td>
<td>1/8</td>
<td>—</td>
<td>3/8</td>
<td>2/8</td>
<td>3/8</td>
</tr>
<tr>
<td>5-7</td>
<td>19</td>
<td>6/19</td>
<td>3/19</td>
<td>14/19</td>
<td>14/19</td>
<td>19/19</td>
</tr>
<tr>
<td>8-11</td>
<td>5</td>
<td>—</td>
<td>—</td>
<td>3/5</td>
<td>3/5</td>
<td>3/5</td>
</tr>
<tr>
<td>16-30</td>
<td>6</td>
<td>—</td>
<td>1/5</td>
<td>2/6</td>
<td>2/6</td>
<td>2/6</td>
</tr>
</tbody>
</table>

Table II. Distribution of lesions within the neural retina and the retinal pigment epithelium in pups infected with canine herpes virus

<table>
<thead>
<tr>
<th>Days after infection</th>
<th>No. of animals</th>
<th>Neural retina</th>
<th>Retinal pigment epithelium (RPE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-4</td>
<td>8</td>
<td>Embryonal configuration, early differentiation, disorganization of cellular architecture in external retinal layer (ENL) in 2/8 animals.</td>
<td>Patchy depigmentation, vacuolization, inflammation, necrosis, infolding, and migration</td>
</tr>
<tr>
<td>5-7</td>
<td>19</td>
<td>Retinal cleavage up to equator. Occasional animal with embryonal retina. Inflammation, necrosis, disorganization and formation of spherical cellular clusters in the ENL. Partial and full-thickness folds and tubes.</td>
<td>Patchy depigmentation, vacuolization</td>
</tr>
<tr>
<td>8-11</td>
<td>5</td>
<td>Cleavage to ora, disorganized ENL, with the appearance of cellular clusters with central lumen. Full and partial thickness folds, fused folds, and tubes.</td>
<td>Atrophy, proliferation, intraretinal migration</td>
</tr>
<tr>
<td>16-30</td>
<td>6</td>
<td>Retina mature, atrophy of all retinal layers, 2 and 3 nuclear layers, folds and tubes, one layer rosettes in external nuclear layer</td>
<td></td>
</tr>
</tbody>
</table>

In recent years, considerable data has been compiled regarding herpesvirus-induced congenital abnormalities.14-21, 23

The present study was undertaken in order to determine the ocular changes occurring in the course of canine herpetic infection developing in neonatal pups and in particular to investigate the effect of this virus on the developing retina.

Materials and methods

Thirty-eight newborn, Beagle pups, from eight litters of a specific pathogen-free colony maintained in isolation were used. The neonatal pups were inoculated with an isolate of canine herpesvirus (CHV), strain F-205.24 The dosage of virus was between 10^3 and 10^4 median tissue culture infective doses (TCID₅₀) per pup. Twenty-six pups were inoculated via the oral-nasal route and 12 were inoculated intraperitoneally. Pups were infected between one and four days after birth and were euthanized by intracardiac administration of pentobarbital at intervals from the first through the thirtieth days after inoculation (Table I). Uninoculated animals free of infection were killed at similar intervals and served as controls. Both globes from each pup were enucleated immediately after death for histologic examination. Necropsies were performed on each animal, including gross and histopathologic examination of visceral organs and brain.

Fluorescent antibody examination (direct technique) and viral isolation studies were routinely performed on brain (CNS), liver, spleen, and kidney as previously described.23, 24 For immunofluorescence studies, hyperimmune antiserum against the CHV was prepared in specific pathogen-free Beagle dogs by repeated intravenous inoculations with partially purified virus. When a satisfactory antibody response had occurred, dogs were anesthetized and exsanguinated. Serum was harvested from the clotted blood and a crude immunoglobulin fraction prepared by ammonium sulfate fractionation. A conjugate was prepared
Fig. 1. Severe mononuclear infiltration is seen within the iris five days post inoculation with canine herpesvirus (CHV). Hematoxylin and eosin (H & E), ×200.

with fluorescein isothiocyanate (Baltimore Biological Laboratories, Cockeysville, Md.) according to standard methods, and the crude immunofluorescent reagent then passed through a column (2.5 x 10 cm, per 4 ml. of conjugate) of DEAE cellulose equilibrated with 0.1 M Tris buffer, pH 8.5. The pale yellow fraction eluted in the void volume with this buffer was stored at −20°C in 3 ml aliquots and used as the staining reagent. Tests for specificity of the conjugate were initially done on frozen sections of kidney tissue from a puppy that had died following experimental CHV infection. Controls consisted of normal dog kidney tissue sections, sections of liver from a dog infected with canine adenovirus-type 1, and spleen sections from a distemper-infected dog. These tests revealed brilliant specific staining of the CHV-infected tissue, but virtually no fluorescence in the control preparations. Included in each series of frozen eye sections from CHV-infected dogs were sections prepared from normal eyes (negative controls) and sections of renal tissue from a CHV-infected dog (positive control). At least five serial sections from each globe were examined. Photomicrographs were taken automatically on color film (Anscochrome 500), using a Zeiss Photomicroscope with a fluorescence attachment. Approximately twofold enlargements of the color slides were made into black and white reproductions.

Globes for routine histology were fixed in 10 per cent phosphate-buffered formalin. Twenty-five paraffin sections, six microns in thickness, were cut on a microtome and stained with Harris' hematoxylin and eosin and periodic-Acid Schiff (PAS). Eyes were removed from selected animals at 2, 7, and 10 days after inoculation for immunofluorescent and viral isolation studies. In these specimens the eye was divided immediately after removal into two peripheral calottes and a papillomoptic nerve segment. One of the calottes was cut into small pieces and inoculated onto monolayers of primary dog kidney cell cultures. Inoculated cells were examined daily for histologic evidence of cytopathic effect (CPE). Frozen sections of the eye were cut from the remaining calotte and papillomoptic nerve segment and stained by the direct fluorescent antibody technique (FA).

Results

Clinical and histopathologic findings. All of the infected animals were asymptomatic until three to five days after inoculation. Clinical signs observed included nasal discharge, abdominal tenderness, and dyspnea, and have been previously described. The histopathologic findings and the results of fluorescent antibody and viral isolation studies on tissues other than the eye have been previously described.
Histopathologic evidence of ocular inflammation. Ocular inflammation is usually not apparent until the fourth day after infection, in contrast to the systemic involvement which is histologically evident on the second day after inoculation. By the fifth day after inoculation, all the animals show histologic evidence of ocular inflammation. A predominantly mononuclear infiltration with a few polymorphonuclear leukocytes is seen in the uveal tract and optic nerve of all the infected animals. The typical picture is that of a panuveitis of approximately uniform severity throughout the iris, ciliary body, and choroid (Fig. 1).

Eyes having severe iritis show peripheral anterior synechiae, cataract, and keratitis (Fig. 2). Intranuclear inclusion bodies are often seen (Fig. 3). Following the tenth day, the severity of the inflammatory changes decreased significantly. Minimal chronic inflammatory cell infiltration usually involving the choroid and optic nerve is detected as late as the twenty-ninth day after infection (Table 1).

Ocular inflammation, when present, is usually bilateral. In only three animals is the involvement unilateral and in each of these cases the involved eye shows minimal signs of inflammation. No correlation is apparent between the severity of involvement and the route of inoculation (i.e., intraperitoneal or oral-nasal).

Virology and fluorescent microscopy correlation. At two and seven days after infection, many foci positive to FA staining are seen in the iris, ciliary body, and retina (Figs. 4, a, b, and c). Viral CPE was seen in dog kidney monolayers inoculated with these ocular tissues with up to $10^{-2}$ dilution. In ocular tissue removed from animals 10 days after infection only a few fluorescent foci in the iris, choroid, and retina are seen. At this time viral CPE could only be detected up to the $10^{-3.5}$ dilution.

Developmental Abnormalities. Optic Nerve. In two animals killed 10 days after infection, islands of retinal tissues are seen within cystic spaces in the intraocular portion of the optic nerve. This ectopic retina is composed of two embryonal layers arranged around a central lumen (Fig. 5). Neural retina. The changes were noted in the retina after infection are (1) retardation of maturation; (2) folds and tube formation; and (3) intraretinal cellular changes which consist of chronic non-granulomatous inflammation, necrosis, and reorganization.

The neural retina of the control and experimental animals is incompletely developed at birth and shows an embryonal configuration. Gradual differentiation of the retina proceeds in the control animals and by eight days of age maturation of the retina has advanced to the area of the ora serrata. In most control animals the retina is fully differentiated by 10 or 11 days after birth. In contrast, experimental animals at ages 10, 11, 29, and 30 days show only partial differentiation of the retina. No apparent clear cut relationship between the degree of ocular inflammation and retardation in retinal development is apparent.

In two animals, 7 and 10 days of age, the retina is undifferentiated with a few folds noted, but no dysplastic retinal rosettes are present.
Areas of retinoschisis internal to the nuclear layers of the retina are seen posterior to the equator in these eyes (Fig. 5).

The earliest histopathologic changes in the neural retina are those seen in two animals on the third and fourth day after infection, and consist of minimal disorganization and loss of cellular orientation in the external retinal layers. Following the fifth day after inoculation all the animals show diffuse necrosis and disorganization of the neural retina with infiltration mainly of the outer retinal layers by lymphocytes and plasma cells. In addition, intense round cell infiltration in the choroid and a subretinal exudate containing chronic inflammatory cells is seen in these eyes.

Two major histopathologic patterns of retinal changes occur simultaneously in the infected eyes: (1) folds and (2) inflammation, necrosis, and disorganization and reorganization. The formation of full-thickness retinal folds usually occurs with marked subretinal exudate. When the retina retains the embryonal configuration, these folds are accordingly composed of the retinal neuroblasts (Fig. 6). In eyes examined following the tenth day after infection, many areas can be found in the retinas of the animals where the base of the folds has fused to form tubes. When the retinal development progresses to a mature configuration of internal and external nuclear layers and ganglion cell layer, the full-thickness folds are composed of the three nuclear layers. The partial thickness folds containing two nuclear layers, the external and internal nuclear layers, are also seen. These folds and tubes are distributed approximately equally in the anterior and posterior portions of the retina.

The second major pattern which can be discerned, occurring simultaneously with the folds, consists of intraretinal infiltration by plasma cells and lymphocytes, necrosis, disorganization and reorganization. In the eyes of the animals killed earliest after infection—5 to 8 days—the predominant change in the retina is widespread inflammation, necrosis, and cellular disorganization. These changes are most severe in the external retinal layers. Focal areas of intraretinal necrosis are positive on FA staining (Fig. 4, A). On the sixth to eighth days...
Fig. 4. A, Local fluorescence in the outer retinal layers seven days after infection (fluorescent microscope, x200). B, Fluorescence in the RPE seven days after infection (fluorescent microscope, x400). C, Fluorescence in the iris seven days after infection (fluorescent microscope, x200).
Fig. 5. Cystic spaces (C) and rosette-like structures composed of neuroblasts (R) within the cystic nerve head. Note areas of retinoschisis (RS) and the embryonal configuration of the retina in an eleven-day-old pup, seven days after inoculation. H & E, x50.

Fig. 6. Two nuclear layers around a central lumen forming a tube (T) and around an open invagination with fold (F) formation eleven days after infection. H & E, x200.
Fig. 7. Intraretinal rosette-like structures composed of clusters of cells arranged around a central lumen limited by a structure resembling the external limiting membrane (arrows) and processes within the lumen. Fourteen days after infection. H & E, ×400.

Fig. 8. Atrophic retina with rosette-like structures 30 days after infection. Note pigmented cell (arrow) within one of the rosettes. H & E, ×200.

after infection, several sections revealed the presence of small spheroid clusters of retinal cells in the external retinal layers. Eosinophilic material resembling rod outer segments is noted within the central lumen of these rosette-like structures. No evidence of an external limiting membrane can be identified by light microscopy at this stage.

Following the eleventh day, the histologic findings remain similar with approximately the same degree of disorganization, necrosis and inflammation, and clustering of photoreceptor-like cells as described above. The retina, after 11 days, is increasingly atrophic with fewer of neural cells observed in all layers throughout its circumference. Intraretinal clusters of rosette-like structures continue to be seen. These are composed of cells arranged around a central lumen. At this stage, a structure resembling the external limiting membrane in addition to outer segment-like elements is projecting into the lumen (Figs. 7 and 8). The structures are most commonly seen at, or anterior to, the equator.

Retinal pigment epithelium. The earliest change in the retinal pigment epithelium (RPE) is noted in eyes removed on the fifth day after inoculation, and consists of patchy depigmentation and vacuolization. These areas show fluorescence with FA staining (Fig. 4). In eyes with severe intraocular inflammation, necrosis of the RPE is evident (Fig. 9). These necrotic foci occur in areas of chorioretinal inflamma-
Fig. 9. Destruction of the retinal pigment epithelium (RPE) by inflammatory cells. Seven days after infection. H & E, x100.

Discussion

In humans, most neonatal systemic herpetic infections are stated to be acquired through the birth canal. Ocular changes described in these patients include acute and sometimes necrotizing keratitis, cataract formation, uveitis, retinitis, chorioretinal scarring, optic neuritis, and optic atrophy. Infants born with signs of established herpes infection rarely survive and are presumed to have had transplacental transmission of the virus. The abnormalities in the latter group vary according to the state of development at the time of infection. Microcephaly, porencephaly, hydrancephaly, periventricular calcification, microphthalmia in association with cataract, and retrolental masses are described. In experimental prenatal herpes infection, microcephaly, optic, and auditory vesicle anomalies were noted. In addition, in a preliminary study, foci of retinal dysplasia were described in pups infected with canine herpesvirus.

Acute viral infections may interfere with the development of organs in various ways. Malformation has been presumed to occur as a result of chromosomal damage of the germinal cells. The inflammatory cell in-
filtration and ensuing necrosis which are associated with viral infections cause disturbances in cellular migration and subsequent differentiation of tissues resulting in altered embryogenesis. Regressive changes occurring in organs after the inflammation has cleared have been cited as one mode of pathogenesis for resultant agenesis. Examples of developmental ocular abnormalities which may have a pathogenesis bearing some relation to that occurring with viral infections have been described in the dog following irradiation and in relation to autosomal recessive inheritance.

The retina in the beagle pup, in contrast to most of the other ocular tissues, is immature at birth. In the present study, panophthalmitis was found at the acute stage of the infection. The ocular structures that were relatively mature at the time of infection, including cornea, uveal tract, and optic nerve, showed scarring and atrophy. The lenses showed cataractous change. In the retina and in certain instances in the optic nerve, altered development occurred. The retinal changes appeared to be the result of: (1) the formation of retinal folds as a result of inflammation and subretinal exudation, with subsequent gliosis, fusion to form tube-like structures and (2) interruption of normal cellular migration and orientation with abortive attempts of primitive retinoblasts to orient themselves and differentiate into photoreceptor cells in rosette-like clusters. The intraretinal rosettes observed appeared to result from aberrant attempts at repair in areas that were undergoing active differentiation at the time of the insult. This type of change has been described not only after viral infections, but following irradiation of the immature retina. We interpret these changes to indicate that once a critical amount of necrosis occurs within the developing retina, regardless of specific insult producing it, orderly differentiation does not continue.

Other virus-induced systemic abnormalities, in addition to retinal dysplasia, have been described following infection in the immature animals. The most common site appears to be the central nervous system. Modified blue tongue virus will give rise to porencephaly and hydrancephaly in sheep. Lymphocytic choriomeningitis was reported to cause cerebellar hypoplasia. Bovine viral diarrhea-mucosal disease virus causes similar changes in cattle, while cerebellar dysplasia due to canine herpesvirus has been previously described.

The presence of a healthy RPE has been cited as an essential prerequisite for normal development of the neural retina. It has been suggested that detachment of the neural retina from the pigment epithelium or damage to the RPE results in the loss of the "organizing effect" of these cells with consequent abnormal retinal development. In the present experiments we observed depigmentation and vacuolization followed variously by necrosis, atrophy, and proliferation of the RPE during the course of the infection. In areas where three layer tubes or folds of neural retina developed, the RPE was sometimes found to be redundant in a similar manner. In other instances, it retained a normal configuration. No relationship could be found between the degree of development of the retinal cells in the folds and tubes and the appearance of the underlying RPE.

During the healing stages occurring 16 to 30 days after infection, intraretinal, pigment-containing cells apparently derived from the RPE were seen. Not infrequently these cells were located in the center of a one-layer rosette. It seems possible that these cells may play a role in induction of abortive photoreceptor differentiation and rosette formation, yet numerous areas were found by serial sections to be lacking an "organizer" cell. In these instances such a cell may have been present at an earlier time and disappeared, or the rosettes may form in their absence.

REFERENCES
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