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THE EFFECTS OF A NEUROPATHOGENIC CANINE HERPESVIRUS ON 
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DISSERTATION

Presented in Partial Fulfillment of the Requirements for 
the Degree Doctor of Philosophy in the Graduate 
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By


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The Ohio State University 
1969

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CHAPTER I

EFFECTS OF A PATHOGENIC CANINE HERPESVIRUS ON ESTABLISHED THYROID ADENOCARCINOMA CELL CULTURES

Introduction

In 1965 a canine herpesvirus (CHV) was isolated from neonatal and fetal puppies affected with a fatal septicemic disease (3,17,18,25) and from spontaneously degenerating primary canine kidney cell cultures (24). The virus has been identified as a herpesvirus (4,24,25) and its antigenic relationship to herpes simplex virus has been demonstrated (2). The pathogenicity of this virus has been substantiated in newborn puppies (3,27). Fatal disease indistinguishable from naturally occurring infection developed in neonatal puppies within 10 days after inoculation of CHV (3,27). Lesions were consistently found in organs and tissues of ectodermal and neuroectodermal origin (3,15,27). Encephalitis in CHV-infected puppies closely resembled encephalitides in man associated with herpes virus infections (15). The dog may, therefore, serve as a model for the study of pathogenetic aspects of herpes virus encephalitis.

Important information regarding CHV-neuroectodermal cell interactions may be obtained by in vitro studies of infected glial cell cultures and canine brain explants. It is essential, however, to first investigate the effects of CHV in a simpler cell culture system.
in order to establish a basis of reference since no detailed description exists in the literature of the sequential effects of CHV on cell cultures. The cytopathic effects of CHV have been described only briefly (4,5,19).

The objective of the present experiment was to investigate the sequential development of lesions produced by CHV in an established canine thyroid adenocarcinoma cell line by correlating data obtained by cytochemistry and immunofluorescence with findings of light and electron microscopy.

Materials and Methods

Virus.—The stock virus used in this study was canine herpesvirus strain F205 in its third passage in established cultures of canine thyroid adenocarcinoma cells. The TCID$_{50}$/ml was 1 X 10$^{6.12}$ as indicated by the cytopathic effect in established canine thyroid adenocarcinoma cells on the fifth day after inoculation. The source of the virus and some of its properties have been previously described (1,2,3,25).

Cells.—Canine thyroid adenocarcinoma cell cultures were prepared from a continuously transferred line by a method previously described (10). Cells were grown in 100ml prescription bottles and in Leighton tubes containing 20ml and 2ml of medium, respectively. The cell line was at the 366-370 transplant generations.

1Originally obtained from Dr. L. E. Carmichael, Veterinary Virus Research Institute, New York State Veterinary College, Cornell University, Ithaca, New York.
Inoculation.----When the monolayers were confluent the cultures were inoculated by removing the old medium, adding 2ml and 0.2ml of herpesvirus stock to 100ml prescription bottles and Leighton tubes, respectively, incubating 2 hours at 37°C, and adding 20ml of fresh medium to prescription bottles and 2ml of fresh medium to Leighton tubes.

Immunofluorescence.----Immunofluorescent studies were performed on cultures at different times after inoculation by the direct method. Fluorescein isothiocyanate labeled anti-CHV globulin was used\(^1,2\).

Cells were washed in phosphate buffered saline (pH 7.2), fixed in cold acetone, air-dried, flooded with conjugated serum, incubated at 37°C for 1 hour, rinsed in phosphate buffered saline (pH 7.2), mounted in buffered glycerin and examined with a microscope which was equipped with an ultraviolet light source\(^3\) and ultraviolet exciting filter\(^4\).

The following controls were used: (1) uninfected tissue culture cells, (2) infected tissue culture cells incubated with unlabeled anticanine herpesvirus globulin, and (3) infected tissue culture cells incubated with unlabeled normal canine globulin followed by incubation with

\(^1\)Originally obtained from Dr. L. E. Carmichael, Veterinary Virus Research Institute, New York State Veterinary College, Cornell University, Ithaca, New York.

\(^2\)Ammonium sulfate precipitated protein buffered with sodium carbonate (pH 9.0), conjugated with fluorescein isothiocyanate at the rate of 1 mg of dye to 60 mg protein, and passed through DEAE Sephadeex to remove unbound dye (Dr. L. E. Carmichael, personal communication).

\(^3\) BHO 200 W, Osram, Berlin, Germany.

\(^4\) UGI, E. Leitz, Incorporated, New York.
labeled anticanine herpesvirus globulin.

Acridine Orange Stain (AO).—The technique described by MAYOR was used (12). Best results were obtained when staining times with acridine orange were reduced to 7 or 8 minutes from the 10 minutes suggested by MAYOR.

Electron Microscopy.—Fixation of cells was accomplished in 100ml prescription bottles with buffered osmic acid. The fixative was replaced after 1 hour with 70% alcohol and the steps of dehydration and infiltration (propylene oxide, propylene oxide-maraglas, and maraglas in the refrigerator overnight) were carried out within the bottles. The cell sheet was scraped from the glass, centrifuged at 300g into a pellet, and embedded in maraglas. Sections were cut at 250 to 350Å, stained with uranyl acetate and lead hydroxide and examined with a Philips 200 electron microscope.

Collection of cells.—Coverslips were removed for staining at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 44, 46, 48, 60, and 72 hours after inoculation. Cell monolayers in prescription bottles were fixed at 12, 22, 24, 28, and 34 hours after inoculation for electron microscopy. May-Grunwald-Giemsa and hematoxylin and eosin stains were used in preparing cells for light microscopic evaluation.

Cell enumeration.—At least 500 cells per coverslip were counted and their lesions categorized from collections taken 1, 3, 5, 7, 10, 16, 20, 24, 48, and 72 hours after inoculation. Since the early cytopathic effects tended to occur in foci the following counting procedure was adopted. The '0' line of an eye-piece micrometer insert
was used to determine cells which were counted. Only cells in the path of the line were counted. Counting was begun at one edge of the coverslip. When the opposite edge of the coverslip was reached the slide was moved 1 cm and the count continued. This was repeated until at least 500 cells had been counted and the coverslip had been traversed at least 3 times. Counts were never discontinued until an edge was reached. During the counting procedure cells with lesions were counted and categorized according to their morphologic type.

Results

Various types of cellular lesions occurred at different intervals following inoculation. Foci of cells with a certain type of lesion predominated during particular periods of time. The time of first appearance was the criterion used to designate the lesion as an early, middle, or late manifestation of viral effect. The length of time a particular lesion predominated was used to determine the length of early, middle, and late phases. These procedures were adopted since the cytopathic effects were asynchronous and, except in the earliest phase, cells were found with lesions characteristic of any phase. Using these criteria, the effects of the virus on established thyroid adenocarcinoma cells were divided into three phases.

Early phase.——The earliest time at which lesions were regularly detected was 3 hours after inoculation. Before this time affected cells were found infrequently.

The first lesions were moderate enlargements of the nucleoli (1.5–2.0 times normal size). The swollen nucleoli became irregular in
outline and manifested separation of nucleolar contents (Fig. 1). Dense material representing the pars fibrosa of the nucleolus was clumped within the eosinophilic, homogenous areas or pars amorpha of the nucleolus. Accumulations of densely basophilic material, not associated with nucleoli, were randomly distributed throughout the nucleoplasm. The nucleolus in early stages appeared larger and more intensely red with AO than did nucleoli of non-affected cells. The majority of nucleoli from inoculated cultures fluoresced pale red or green-yellow. Both swollen nucleoli and nucleoli with irregular shapes were seen (Fig. 2). Not all of the nucleoli underwent these changes. In cells containing 3-5 nucleoli it was common to find 1 normal appearing nucleolus. In addition, nuclear chromatin clumped along the inner surface of the nuclear membrane. No antigen was demonstrated in these cells by immunofluorescence.

The early phase lasted approximately 3-4 hours and involved less than half the number of cells per coverslip, the number ranging from 35.1% to 40.8% in different cultures.

At 8 hours post-inoculation, cells were observed in which the number of nucleoli per cell was decreased. The remaining nucleoli were shrunken, hyperchromatic, and occasionally located next to the nuclear membrane. The majority of control cells and cells in cultures immediately following inoculation contained 3-5 nucleoli per cell whereas the majority of cells in cultures 8 or more hours after inoculation contained 1-3 nucleoli (Fig. 3). The number of such cells increased throughout the three phases. The remaining nucleoli were faintly orange-red or yellow-green and small when stained with AO.
Legends

Fig. 1. Thyroid adenocarcinoma cells 3 hours after inoculation. Several nucleoli (large arrows) are enlarged and irregular in shape as compared to normal nucleoli (small arrows). H&E, X 1250.

Fig. 2. Thyroid adenocarcinoma cells 3 hours after inoculation. Two cells in the center have swollen nucleoli (arrows) while the cell at the lower left contains shrunken and irregular nucleoli which fluoresced yellow-green instead of orange-red. Acridine orange, X 108.
Fig. 3. Canine herpesvirus cytopathic effect. The per cent of cells containing less than 3 nucleoli per cell increased throughout the experiment (72 hours) indicating a decrease in the total number of nucleoli. The curves were drawn by inspection.

Fig. 4. Mature viral particle (arrow) lies in close apposition to the cell membrane which has formed a pocket partially enclosing the virus. X 41,250.
Fig. 3

Canine Herpesvirus Cytopathic Effect

Fig. 4
Cells with these nucleolar changes were seen in the later part of the early phase and throughout the middle and late phases. In a few cells small, spherical, intensely red fluorescent particles of ribonucleic acid were observed near the nuclear membrane.

Ultrastructural evaluation of cells in the early phase of infection revealed viral particles in various phases of entry into the cells. Viral particles were partially enclosed by invaginations and outpouchings of the plasma membrane (Fig. 4). Complete viral particles were seen in membrane-bound vacuoles in the cytoplasm (Fig. 5). These vacuoles were located at various distances between the cellular and nuclear membranes. Viral particles not surrounded by a membrane were also observed in the cytoplasm.

The earliest consistent ultrastructural change associated with viral infection was in the nucleus. Normal appearing nucleoli contained accumulations of dense fibrillar material interspersed within a fine, granular matrix (Fig. 6). In contrast, portions of the nucleoli of affected cells segregated (Fig. 7). The dense, fibrillar material (pars fibrosa) was separated from the granular component (pars granulosa) in the nucleolus. The pars fibrosa subsequently separated into irregular dense clumps which were disseminated throughout the nucleus. The pars granulosa dissipated into the nucleoplasm. Not all nucleoli were affected. Chromatin was clumped along the inner nuclear membrane. These clumps were discrete, variable in density and size, and were demonstrated in all of the later phases of infection. Nuclear projections into the cytoplasm were prominent.
Fig. 5. Mature viral particle (arrow) enclosed by membranes (of a vesicle) in the cytoplasm. X 86,250.

Fig. 6. Normal nucleus demonstrating fine chromatin evenly distributed with 2 nucleoli present. Nucleoli contained dense fibrillar pars fibrosa (arrows) within finely granular pars granulosa. Nuclear membranes are distinct. X 17,700.
Middle Phase.—By 10 hours after inoculation, cells with a different type of lesion were noted. Small (approximately 1-3μ), discrete, eosinophilic, intranuclear inclusions were seen within the nucleus (Fig. 8). Following Bouin's fixation and hematoxylin and eosin staining, inclusions were primarily spherical, uniformly stained and separated from coarse chromatin by a clear halo containing a few chromatin granules. One or two larger masses of chromatin were often associated with the outer borders of the inclusion. The inclusions varied in numbers from 1 to 5 per cell and were scattered throughout the nucleus. The per cent of cells containing these bodies increased from 3% (10 hours) to 27% (34 hours) during the middle phase.

A second type of inclusion body, present in fewer cells, was also observed during the middle phase. A cell with these inclusions was characterized by the clumping of chromatin into masses around the inner nuclear membrane. The central nuclear area contained a few chromatin particles and large amorphous, finely granular, eosinophilic masses (Fig. 9). The eosinophilic inclusions appeared in two forms. One form occupied the entire central area of the nucleus and extended around the clumped peripheral chromatin. Chromatin granules were scattered throughout the inclusion. The second type occurred as a single aggregate of finely granular bodies separated from nuclear chromatin by a non-staining rim. All inclusions fluoresced a dark green with AO and were eosinophilic when re-stained with hematoxylin and eosin (Figs. 10 and 11). Both forms of inclusion were present in the middle phase. The second form was most common in the early stages while the first form of inclusion was more common in the later stages of the middle phase.
Fig. 7. Portion of the nucleus and cytoplasm of an infected cell. Nucleolar disruption characterized by fragmentation of the pars fibrosa (arrows) and dispersion of the pars granulosa into the nucleoplasm. Chromatin is accumulated in discrete clumps next to the nuclear membrane. Note nuclear projections and vesicles in the cytoplasm. A few viral particles (V) are present in the nucleus. X 12,500.

Fig. 8. Thyroid adenocarcinoma cells 10 hours after inoculation containing discrete intranuclear inclusions (arrows) separated from nuclear chromatin by a clear halo. H&E, X 1250.
Cells with this type of inclusion increased from 2% (16 hours) to 16% (34 hours) during the middle phase.

The last type of inclusion body observed during the middle phase was the Cowdry type A inclusion considered characteristic of cells infected with herpesviruses (13) (Fig. 12). These inclusions were oval to round, intensely eosinophilic and occupied the entire central nuclear area. They were separated from nuclear chromatin by a clear halo and fluoresced dark green with AO. Type A inclusions were present in 4% (16 hours) to 8% (24 hours) during the middle phase.

Antigen appeared throughout the middle phase in the form of large discrete clumps along the nuclear membrane and as more diffuse masses in the center of the nucleus (Fig. 13). The antigen along the inner nuclear membrane was clumped at irregular intervals and occasionally formed an even fluorescent rim accentuating the entire nuclear circumference. Areas of antigen within the central nuclear area were of similar size and conformation as the dark green fluorescent inclusions observed with AO and the eosinophilic bodies demonstrated with hemotoxylin and eosin.

Cells containing one or more of the three types of inclusions predominated until 34 hours. They composed 3% of the cells remaining on the coverslip at the beginning of the middle phase and increased to a maximum of 49% at the end of this phase.

During the middle phase of infection cells were characterized ultrastructurally by various stages of viral assembly. Viral particles appeared in groups in the vicinity of chromatin condensations and remnants of the pars fibrosa (Figs. 14 & 15). In these areas densely staining granules of viral precursor material were demonstrated and
Fig. 9. Thyroid adenocarcinoma cells 12 hours after inoculation demonstrating large, amorphous, eosinophilic inclusions (arrows). The nuclear chromatin is clumped against the nuclear membrane and dispersed throughout the inclusion. H&E, X 1250.

Fig. 10. Thyroid adenocarcinoma cell 12 hours after inoculation containing a dark green area of fluorescence at one pole (arrow). Degenerated nucleolus (Nu) was yellow green similar to the chromatin along the nuclear membrane, X 1325.
Fig. 11. Eosinophilic inclusion body in same cell as fig. 10 at one pole of the nucleus (arrow) represents the area of fluorescence observed after acridine orange staining. Bouin's fixation, H&E, X 1325.

Fig. 12. Thyroid adenocarcinoma cell 16 hours after inoculation demonstrating a Type A inclusion body. H&E, X 1250.
separated from the nucleoplasm by an irregular, less densely staining border. The viral particles were round or hexagonal and contained one of three types of cores (Fig. 15). The first type of core was electron-lucent giving the impression of a hollow capsid. Three or four dense formations symmetrically situated against the inner capsid wall composed the second type of core. The clear space between these formations delineated a cross- or star-shape. The last type of core consisted of a round dense nucleoid surrounded by a clear halo. The diameter of the viral particles was 65 to 85 μm. Dense nucleoid material was 30 to 40 μm in diameter. The capsomeric substructure of viral particles was discernable along the outer borders of capsids and formed a spoke-like pattern.

Envelopment of the capsid occurred within the nucleoplasm primarily in association with proliferating nuclear membranes (Fig. 15). The nuclear membranes extended into the nucleoplasm in addition to doubling and tripling in number (Fig. 15). Enveloped particles were demonstrated within the nucleus and vesicles in the cytoplasm but were not free in the cytoplasm. These enveloped particles measured 125 to 185 μm in diameter.

Thirty-four hours post-inoculation was considered to be the end of the middle phase of cytopathic effect. However, cells with lesions typical of the late phase had appeared during the mid-stage of the middle phase. There was a transition period during which lesions typical of both phases were present but cells with one or more of the three types of inclusions predominated. Typical herpesviral effect as seen by light
Fig. 13. Thyroid adenocarcinoma cell 16 hours after inoculation. Bright fluorescence in the center of the nucleus and along the nuclear membrane indicates CHV antigen. The majority of these cells were found during the middle and early late phases. X 1325.

Fig. 14. Affected thyroid adenocarcinoma cell demonstrating intense nuclear activity. The chromatin is clumped next to the nuclear membrane. An aggregate of granular viral precursor material (arrow) is visible near a fragment of pars fibrosa of the nucleolus. Note incomplete viral particles (V) and extracellular mature virus along cellular membrane. X 11,800.
microscopy of unstained cells (21) appeared during this transitional phase. A distinct cytopathic effect was first noted at 10 hours and small foci of rounded, highly refractile cells were observed at 16 hours.

Late Phase.—The cells predominating during this stage were shrunken and hyperchromatic. The nucleus was approximately one-half normal size, had a thickened nuclear membrane, and lacked well defined chromatin. Remaining nucleoli were shrunken, hyperchromatic, and situated near the nuclear membrane. The cytoplasm was shrunken and strongly eosinophilic. The nucleus fluoresced light green with AO and, in places, lacked a limiting membrane (Fig. 16). Chromatin was sparse and present in the form of small particles. The cytoplasm fluoresced a yellow-orange rather than red. Antigen-filled nuclei lacked definite borders and tended to blend with the cytoplasm. (Fig. 17). In addition, the cytoplasm contained particulate masses of antigen.

Cells with this type of lesion appeared to develop from the inclusion-containing cells which predominated during the middle phase. These cells were seen as early as 20 hours and their number increased from 15.% at 34 hours to 44.% at 72 hours.

Foci of shrunken, hyperchromatic cells were piled upon one another and retained little structural integrity. The number of cells present on the coverslip was markedly decreased due to detachment from the glass surface. Washing the coverslips dislodged many of the cells.

Electron microscopically, cellular degeneration and viral release characterized the late phase of infection. The nuclear membranes disintegrated and released viral particles and nucleoplasm into the
Fig. 15. Portion of nucleus demonstrating stages of viral assembly. Partially formed capsid is seen enclosing a less densely staining core (large arrow). Empty capsids and capsids containing various types of cores (small arrows) are next to an accumulation of viral precursor material. The outer envelope of one viral particle is being formed (double arrow). X 82,000.

Fig. 16. Degenerated TC cell 36 hours after inoculation. Notice partially disrupted nucleus and shrunken cytoplasm. X 1250.
Fig. 17. Degenerated cells 36 hours after inoculation. The entire nucleus had bright fluorescence which blended with fluorescent particles in the cytoplasm. X 1250.

Fig. 18. Portion of cytoplasm of degenerated cell demonstrating membrane-lined vesicles filled with mature viral particles. Smaller accumulations (arrow) of viral particles were closely enveloped by cytoplasmic membranes.
cytoplasm. Cytoplasmic vacuoles between the nuclear and plasma membranes contained numerous mature viral particles (Fig. 18). Dilated cisternae of ER contained 2 or 3 viral particles. Cytoplasmic organelles were severely degenerated. Mitochondria were swollen and disrupted. Numerous vacuoles and lipid inclusions were in the cytoplasm.

Discussion

The results of this study demonstrated that distinct sequential alterations occurred in cells infected with canine herpesvirus. The first alteration, nucleolar swelling with subsequent segregation and disappearance, was similar to lesions reported in cells infected with herpes simplex virus (6,11,13). JACOB (9) has described similar nucleolar changes in primary frog renal tumor cells which contained herpes-like particles. The apparent loss of nucleolar RNA as observed in this investigation with AO staining was analogous to the demonstration of nucleolar RNA decrease by tritiated uridine studies of herpes simplex-infected giant cells (8). Similar nucleolar changes have been demonstrated in tissue culture cells infected with other DNA and RNA viruses (7). These nucleolar lesions may be the result of partial suppression of cellular ribonucleoprotein production during the conversion of the cells' metabolic processes to the production of viral precursor products. The specificity of nucleolar lesions for viral infection, however, must be further substantiated and chemically defined since the lesion does not occur with all viruses (22) and similar nucleolar changes have been observed in association with
chemical injury (23).

The densely basophilic clumps in the nucleoplasm during and following nucleolar degeneration were similar in size and number to B bodies reported by LOVE and WILDY (11) in the nuclei of herpes simplex-infected HeLa cells. These authors stated that B bodies were dense particles (nucleolini) of nucleoli which had been extruded into the nucleoplasm during nucleolar disruption. B bodies reportedly consist of ribonucleoprotein as demonstrated with toluidine blue-molybdate staining. CARMICHAEL et al. (4) described similar, although larger, bodies in the nuclei of CHV inoculated primary cultures of dog kidney cells but did not describe the nucleoli of these cells. No spatial relationship between the basophilic clumps and the various types of eosinophilic inclusions of the nucleus was apparent.

The small, discrete, eosinophilic inclusions in the nucleus marking the beginning of the middle phase (10 hours) were similar to inclusions described in early phases of cellular infection with herpes simplex (13). The finding that this type of inclusion predominated during the middle phase and decreased as the numbers of the two other types increased may indicate that it was derived from the other forms by the coalescence of several particles. The small inclusions were not obvious with AO staining whereas the other two types were prominent. This suggests they are of a different chemical nature and can be demonstrated only by certain fixatives and stains.

The second type of inclusion which filled the nucleus was similar to that reported by SPERTZEL et al. (24) in CHV-infected dog kidney culture cells. Similar inclusions have been reported in herpes simplex
infected rabbit corneal cells grown in tissue culture (13). The less intense staining of the inclusion with AO suggests that the DNA is not as concentrated as that observed in Type A inclusions. At this stage centrally situated masses of antigen could be demonstrated in affected nuclei. It was not possible to definitely relate areas of viral antigen with the inclusions since different techniques and cells were involved. It should be noted, however, that the size and location of the fluorescent mass of antigen and the demonstration of viral particles in the central nucleoplasm indicate the possibility of the inclusions representing at least one stage of viral maturation.

The third type of eosinophilic inclusion was the type A inclusion often described as characteristic of herpesvirus infected cells. Its appearance as the last type of eosinophilic inclusion in association with degeneration of affected cells lead to the conclusion that it represented an end stage inclusion.

The number of inclusion bodies demonstrated in this study was greater than reported by others. DE RATULD and WERNER (18) did not produce inclusions in cultures of fetal canine lung fibroblasts inoculated with a CHV antigenically identical to CARMICHAEL's isolate although the virus multiplied in the cells. CARMICHAEL et al. (4) and CORNWELL et al. (5) reported only an occasional eosinophilic intra-unclear inclusion in dog kidney cell cultures. Possible explanations for these differences may be the susceptibility of the cells, virulence of the viral strain, and/or fixation and staining variations.

The process of viral maturation was similar to that reported for several other herpesviruses (16). There were various types of incom-
plete viral particles present in areas of viral synthesis. The "empty" capsid type of viral particle was encountered in the nucleus during the early phases of infection in areas rich in viral precursor material. Other capsids appeared to be enclosing the viral precursor as nucleoid material. It was not possible to attribute any functional significance to these coreless capsids. MICHAEL et al. (14) demonstrated similar coreless capsids of polyoma virus in a CsCl density band containing mouse cellular DNA and speculated that the coreless virions (pseudovirions) may transduce genetic markers of the host cell.

The final phase of infection was characterized by degenerated cells with disrupted nuclei and dense cytoplasm. The presence of vesicles containing viral particles in the degenerated cells indicated the completion of viral maturation. The mature virions were observed in the process of leaving affected cells.

Correlation of data obtained by cytochemistry and immunofluorescence with findings of light and electron microscopy made it possible to follow the replication cycle of CHV and its effects on an established cell line of natural host origin. By comparing these findings with those reported from previously investigated herpes virus-infected cell cultures it was concluded that CHV-replication and its sequential effects on cells were similar to results described in the literature for other herpes virus-cell interactions. Because of these similarities it is suggested that CHV-infected nervous tissues of natural host origin (in vivo and in vitro) may provide information which is applicable to encephalitides in man associated with herpes viruses. The results of this study also indicate that the nucleolus may be
important in the initial phase of viral replication. In order to establish the precise relationship of viral replication to nucleolar alterations further investigation is warranted.
Summary

The effects of a pathogenic canine herpesvirus were studied sequentially in established canine thyroid adenocarcinoma tissue culture cells. The lesions were defined as early, middle, and late manifestations of viral effects based on the time a lesion first appeared. The early phase (3 to 10 hours) consisted of nucleolar swelling followed by disruption. The nucleolar changes were characterized by loss of RNA as determined with acridine orange and segregation of the nucleolar components as demonstrated by electron microscopy. The middle phase (10 to 34 hours) was characterized by the appearance of three types of eosinophilic intranuclear inclusions. One type of inclusion appeared to transform progressively into another. The appearance of viral antigen in the nucleus as demonstrated by immunofluorescence, correlated with ultrastructural evidence of viral replication. The late phase (34 to 72 hours) was manifested by cellular degeneration and viral release into the cytoplasm.
REFERENCES


CHAPTER II

EFFECTS OF A PATHOGENIC CANINE HERPESVIRUS ON CANINE BRAIN CELL CULTURES
AND CEREBELLAR EXPLANTS

Introduction

Several members of the herpesvirus group have been associated with acute inflammatory and destructive diseases of the nervous system (1, 21). Herpesviruses have been demonstrated to be present and probably involved in both acute and latent or recurrent encephalitides in man (10, 13).

A recently isolated canine herpesvirus (CHV) (4) has been reported to produce acute encephalitis in the newborn puppy (24, 33) and has been associated with a latent disorder of the central nervous system in an adult dog (22). In addition CHV has been demonstrated to be antigenically related (2) to herpes simplex virus, an established agent of acute and latent encephalitis in man (10).

The lesions in the brains of infected puppies have been described (7, 24, 33) and the sequential effect of the virus on non-neural cell monolayers has been reported (16).

The response of the different cell types in nervous tissue to CHV and the method of replication of the virus in neuroectodermal cells have not been investigated. Unanswered questions are: 1) does the virus affect all cells of the nervous system?, 2) are affected cells attacked simultaneously or sequentially?, and 3) do neuroectodermal cells provide an adequate environment for viral replication?
To answer some of these questions canine cerebellar explants and brain cell cultures were employed in the present study. Objectives of this investigation were: 1) to investigate the sequential development of CHV cytopathic effects on canine cerebellar explants and attempt to determine the cell types affected and sequence of viral infection, 2) to study the interactions between CHV and astrocytes in cell cultures by correlating data obtained by cytochemistry and immunofluorescence with findings of light and electron microscopy, and 3) to compare the effects of the virus on cultured explants and cell cultures of nervous tissue with lesions reported in the brains of infected dogs.

The techniques for culturing fragments of the nervous system are well established (11, 12, 23, 32). They have made possible the study of significant structural and functional cellular interrelationships in vitro. The development of a technique for growing explants of canine cerebellar tissue on glass coverslips in Leighton tubes in our laboratory (30) has permitted explants to be grown in sufficient numbers for serial collections and observations. Events at the cellular level can be sequentially recorded and related to the cytopathic effect.

Materials and Methods

Cerebellar explants.—The tissue culture technique has been previously described (30). Briefly, cerebellar cortices of neonatal puppies were aseptically diced into small pieces, placed on glass coverslips in Leighton tubes, covered with medium, and incubated at 37°C.

Brain cell cultures.—The brain cell cultures used in these
experiments were prepared from the cerebral hemispheres of newborn puppies. The hemispheres were collected aseptically and washed three times in Hank's balanced salt solution (HBSS) containing 2mg per cent phenol indicator. Antibiotics were added to make a final concentration of 200 units of penicillin and 100μg per ml of streptomycin. The leptomeninges and choroid plexus were stripped from the brain, which was then minced with scissors to a gelatinous pulp.

The cells were dispersed by digestion in 4 to 5 volumes of 0.25 per cent trypsin in HBSS. This was done in an Erlenmeyer flask at approximately 22°C using a magnetic stirring rod. After 15 minutes the cell suspension was decanted through a layer of gauze into a beaker containing 2ml of bovine calf serum. The remaining fragments were again trypsinized for another 15 minutes and the process repeated until a total of 4 decants were collected in separate beakers. The first decant was discarded while the remaining 3 decants were centrifuged at 300g for 6 minutes and the supernatant discarded. The remaining pellet was resuspended in growth medium and centrifuged again at 300g for 6 minutes. After centrifuging, the supernatant was discarded and the sediment suspended in sufficient growth medium to give approximately 150,000 cells per ml. The cell suspension was distributed in aliquots of 0.2ml into sterile Leighton tubes containing 11 x 35mm glass coverslips and 20ml into 100ml prescription bottles. The containers were sealed with sterile white rubber stoppers and the cultures incubated at 37°C in the horizontal position.

The growth medium consisted of Hank's lactalbumin hydrolysate (HLH)
medium plus 8 to 10 per cent bovine serum, 0.5 per cent sodium carbonate, and 1.0 per cent antibiotics (200 units of penicillin and 100μg of streptomycin per ml). The HLH medium was composed of HBSS plus 500mg per cent lactalbumin hydrolysate and 2mg per cent phenol indicator. The medium was changed whenever the pH indicator began to indicate acidity.

**Virus.**—The stock virus used in this study was canine herpesvirus strain F205 in its third passage in established cultures of canine thyroid adenocarcinoma cells. The TCID\(_{50} / \text{ml} \) was \(1 \times 10^{6.12}\) as indicated by the cytopathic effect in established canine thyroid adenocarcinoma cells on the fifth day after inoculation. The source of the virus and some of its properties have been previously described (1, 2, 4, 28).

**Inoculation.**—Cerebellar explants were inoculated with virus after 7 or 14 days of growth by removing the old medium, adding 0.2ml of herpesvirus stock, incubating 2 hours at 37°C, and adding 0.3ml of fresh medium.

The brain cell cultures were inoculated when they became confluent by removing the old medium, adding 2ml of herpesvirus stock to 100ml prescription bottles and 0.2ml to Leighton tubes. After incubating for 2 hours at 37°C, 20ml of fresh medium was added to prescription bottles and 2ml to Leighton tubes.

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1 Originally obtained from L. E. CARMICHAEL, Veterinary Virus Research Institute, New York State Veterinary College, Cornell University, Ithaca, New York.
Acridine orange stain.—Fluorochrome acridine orange (AO) was employed for the demonstration of nucleic acids. Cultures were stained by the technique of MAYOR (18). Best results were obtained when staining times in AO were increased to 15 minutes from the 10 minutes suggested by MAYOR (18).

Immunofluorescence technique.—The direct method of immunofluorescence was employed to study brain cell cultures which were collected at various times post—inoculation. Fluorescein isothiocyanate labeled anti-CHV globulin was used (courtesy of L. E. CARMICHAEL). Cells were fixed in cold acetone and washed in phosphate buffered saline (pH 7.2), air-dried, flooded with conjugated globulin, incubated at 37°C for 1 hour, rinsed in phosphate buffered saline (pH 7.2), mounted in buffered glycerin and examined with a Leitz ultraviolet microscope equipped with an Osram HBO bulb and U51 and K510 filters. The following controls were used: (1) uninfected brain cell cultures (2) infected cell cultures incubated with unlabeled anticanine herpesvirus globulin (3) infected cell cultures incubated with unlabeled normal canine globulin followed by incubation with labeled anticanine herpesvirus globulin.

Electron microscopy.—The techniques of fixation, embedding, sectioning, and staining for electron microscopy have been described (16). All sections were stained with uranyl acetate and lead citrate

1Ammonium sulfate precipitated protein buffered with sodium carbonate (pH 9.0), conjugated with fluorescein isothiocyanate at the rate of 1mg of dye to 60mg protein, and passed through DEAE Sephadex to remove unbound dye (L. E. CARMICHAEL, personal communication).
and examined with a Philips 200 electron microscope.

Collection of cells for staining.—Explants were removed for staining at 3, 6, 8, 12, 16, 20, 24, 36, 48, and 72 hours after inoculation. Coverslips containing brain cell cultures were removed for staining at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 44, 46, 48, 60, and 72 hours after inoculation. Brain cell cultures in 100ml prescription bottles were fixed at 12, 22, 24, 28, and 34 hours after inoculation for electron microscopy. May-Grunwald-Giemsa (20), hematoxylin and eosin (5) and supravital methylene blue (30) stains were used in preparing cells for light microscopic evaluation. In addition, selected explants were impregnated according to Holmes' silver nitrate technique as modified by WOLF (32).

Cell enumeration.—The procedure used in the enumeration and categorization of cells has been previously described (16). At least 500 cells per coverslip were counted from collections taken 1, 3, 5, 7, 10, 16, 20, 24, 48, and 72 hours after inoculation.

Results

Canine cerebellar explants

Both explants infected on the seventh and fourteenth day of growth were investigated with the purpose of evaluating the various cell types which differed at different stages of development. Astrocytes were the predominant cell type in the outgrowth zone of both 7- and 14-day explants. Oligodendrocytes were numerous in 7-day explants but became scarce in 14-day explants. Well defined microglia
were not present in sufficient numbers to be evaluated. Various types of neurons were present in both 7- and 14-day explants, however, larger neurons (eg. Purkinje's cells and motor neurons) were more readily observed in the thinner 14-day explants.

Lesions were demonstrated first in astrocytes in the peripheral outgrowth surrounding the explant. Evidence of nucleolar degeneration was observed 6 hours after inoculation. Remnants of the dense portions of the nucleoli were dispersed throughout the nucleoplasm but the amorphous portions were not visible. RNA was decreased or absent in the nucleolus as demonstrated by AO staining. Altered nucleoli fluoresced yellow-green in contrast to the orange-red fluorescence of non-affected nucleoli.

Numerous eosinophilic intranuclear inclusion bodies were observed in astrocytes of explants 8 hours or more after infection (Fig. 19). The inclusions were either small, discrete aggregates irregularly distributed throughout the nucleus or larger, less defined, eosinophilic masses which completely filled the nucleus. Type A inclusions were also observed in the nuclei of affected astrocytes. By 12 hours the majority of the astrocytes in the more centrally located outgrowth contained inclusions.

The peripheral explant area consisted of foci of rounded, piled-up, degenerated cells at 12 hours. The number of foci increased progressively and at 24 hours the outgrowth no longer possessed the tapering, delicate border associated with non-infected cultures (Fig. 20). Instead, the cells were shrunken, hyperchromatic and clumped together (Fig. 21). Areas of the outgrowth were more reticulated in appearance
Fig. 19. Seven-day old explant culture of canine cerebellum (16 hours post-inoculation) illustrating inclusion bodies in astrocytes (large arrows) and an oligodendrocyte (small arrow) in the peripheral outgrowth. Hematoxylin and eosin stain; X 500.

Fig. 20. Uninoculated seven-day old explant culture of canine cerebellum. Dense area represents original explant while the lighter area is the outgrowth zone. Note delicate, lacy appearance of the border of the outgrowth. May-Grunwald-Giemsa stain; X 70.
at 24 hours when compared with control explants. This effect was due to closely packed protoplasmic astrocytes becoming fibrous in character. Processes were prominent and the perikarya were shrunken (Fig. 22). Gentle washing during staining often removed many of the cells comprising the outgrowth zone. At 36 hours, astrocytes with inclusion bodies were numerous within the original explant zone. These cells appeared the same as affected cells in the outgrowth.

The oligodendrocyte was the second cell type to show lesions (Fig. 19). Affected cells were observed in the outgrowth zone of explants 12 hours after infection and were demonstrated in all inoculated 7-day cultures after 12 hours. Oligodendrocytes had eosinophilic inclusions which filled swollen nuclei and often displaced the chromatin against the nuclear membranes. The intensity of eosinophilia in different areas of the inclusion varied with the central area being more eosinophilic than the periphery. Other oligodendrocytes contained no inclusions but were swollen and had clumped chromatin. Although oligodendrocytes were numerous in the cultures, few demonstrated inclusions. Even at their peak occurrence at 16 hours post-inoculation inclusions were present in only 6.2 per cent of the oligodendrocytes compared to 39.1 per cent of the astrocytes.

Large neurons in infected cultures were studied mainly with silver impregnated preparations. May-Grünwald-Giemsa and methylene blue supravital stains were used in interpreting the changes in granule cells. Fourteen-day explants permitted better observation of neurons in the dense central explant area than did 7-day explants.
Fig. 21. Seven-day old explant culture of canine cerebellum (24 hours post-inoculation) illustrating the effect of CHV. The border of the outgrowth consists of hyperchromatic, shrunken cells clumped together. May-Grunwald-Giemsa stain; X 70.

Fig. 22. Area of outgrowth from CHV-infected canine cerebellar explant containing astrocytes (arrows) with shrunken perikarya and processes creating a reticulated pattern. Hematoxylin and eosin; X 354.
Lesions involving the large neurons, Purkinje's cells and neurons of the cerebellar nuclei (Fig. 23), were first detected 36 hours after inoculation. At this time there was evidence of neuronal degeneration which was manifested in two ways. The first was an increased argyrophilia of the perikarya, shrinkage of dendrites, and swelling of the axon (Fig. 24). The second form of degeneration was marked swelling of the perikaryon and cell processes which was followed by granular dissolution (Fig. 25). Both the large neurons considered to originate from the cerebellar nuclei and Purkinje's cells manifested these forms of degeneration in 14-day explants. Inclusion bodies were not detected within nuclei of these two types of large neurons.

Granule and stellate cells underwent similar degeneration. There was shrinkage of the processes and perikaryon with eventual lysis. Inclusion bodies were not demonstrated with certainty. In hemotoxylin and eosin stained sections an occasional granule cell contained a central, lightly eosinophilic area suggestive of an inclusion body. The number of stellate and granule cells in infected cultures was noticeably less than in controls after 48 hours.

Brain Cell Cultures

Over 90 per cent of the cells in brain cell cultures were astrocytes. Other neuroectodermally derived cells were not present in significant numbers to be evaluated. Astrocytes of non-infected cultures were characterized by an oval basophilic nucleus which measured 15 by 8µ and usually contained 3-5 nucleoli (Fig. 26). The perikaryon was acidophilic and measured approximately 30 to 20µ. Processes extended
Fig. 23. Fourteen-day old canine cerebellar culture (uninoculated) showing a normal motor neuron. The numerous dendritic processes opposite the axon (A) are characteristic of neurons of the cerebellar nuclei. Holmes' silver impregnation; X 1250.

Fig. 24. Fourteen-day old canine cerebellar culture (36 hours after inoculation) showing a degenerated large neuron. Note increased argyrophilia in nucleus and shrinkage of dendritic processes (arrows). Holmes' silver impregnation; X 1430.
Fig. 25. Degenerated neuron from CHV-infected fourteen-day old canine cerebellar explant 48 hours after inoculation. Perikaryon and remaining processes are swollen and surrounded by satellite cells. Holmes' silver stain; X 1430.

Fig. 26. Uninoculated brain cell culture illustrating characteristic population of astrocytes. Cytoplasmic processes are distinct. Nuclei possess several nucleoli. One cell is in mitosis. Hematoxylin and eosin; X 600.
from all poles of the perikaryon to interdigitate with or course over other astrocytic processes.

Distinct lesions appeared in the astrocytes at different times post-inoculation. The time of appearance of a prominent cellular alteration was considered to represent the beginning of a phase of viral effect. The presence of a different type of lesion subsequently marked the beginning of a new phase although cells with lesions of the previous phase were still present. Three main phases of viral effect could be recognized and were designated as early, middle, and late. The length of time that a lesion predominated determined the length of the phase. These criteria were used since the cytopathic effect was asynchronous and cells with lesions characteristic of all phases were found at any time.

**Early phase.** ——Nucleoli were the first cellular structures to be affected. The number of nucleoli per cell decreased progressively (Fig. 27). After 1 hour post-inoculation cells with 3-5 nucleoli predominated whereas at 16 hours cells with less than 3 nucleoli were in the majority. The decrease in the number of nucleoli, although first demonstrated in the early hours after inoculation, was seen throughout the entire observation period (72 hours). In the later hours many cells did not possess any nucleoli.

Lesions within nucleoli were regularly detected 4 hours after inoculation of the cultures. Affected nucleoli enlarged (1.5 times normal size) and fluoresced yellow-green with AO in contrast to their red to orange-red appearance in non-affected cells (Fig. 28). Later
Fig. 27. Canine herpesvirus cytopathic effect. The per cent of cells containing less than 3 nucleoli increased throughout the experiment (72 hours) indicating a decrease in the total number of nucleoli. The curves were drawn by inspection.

Fig. 28. Astrocytes in brain cell culture 10 hours after infection stained with acridine orange. Normal nucleoli (small arrows) fluoresced orange-red and were round. Abnormal nucleoli (large arrows) fluoresced yellow-green and were irregular in shape. X 182.
PER CENT

80 - 60

CELLS WITH <3 NUCLEOLI

CELLS WITH 3-5 NUCLEOLI

CELLS WITH >5 NUCLEOLI

HOURS AFTER INOCULATION

CANINE HERPESVIRUS CYTOPATHIC EFFECT

Fig. 27
the shape of the nucleolus changed from a rounded structure with a sharply defined circumference to an irregular shape with hazy borders. The pars fibrosa and pars amorpha of the nucleus were separated. The pars fibrosa fragmented into densely staining basophilic bodies which were irregularly distributed throughout the nucleoplasm. The pars amorpha appeared to undergo dissolution into the surrounding nucleoplasm. Occasionally a nucleolus remained partially intact but became shrunken, hyperchromatic, and lacked normal detail and staining characteristics. Such nucleoli were generally located in apposition to the nuclear membrane.

Lesions characteristic of the early phase involved from 10.3 per cent to 12 per cent of the cells and predominated until the sixteenth hour after inoculation.

Another cellular change during the early phase (as early as 6 hours after inoculation) was a clumping of chromatin throughout the nucleoplasm and along the nuclear membrane. The chromatin aggregates fluoresced a bright green with AO in contrast to the less intensely fluorescent dark green nucleoplasm.

The nucleolar changes were also a striking feature of the early phase on electron microscopic examination. The even granular pars amorpha interspersed with dense segments of the pars fibrosa observed in normal cells (Fig. 29) was altered in infected cells. The alteration consisted of a separation of the pars fibrosa from the pars amorpha (Fig. 30) with subsequent fragmentation of the pars fibrosa and dispersion of the pars amorpha into the nucleoplasm. In addition chromatin was clumped along the nuclear membrane. Viral particles in various
Fig. 29. Nucleus of an astrocyte in uninoculated cell culture illustrating a normal nucleolus (Nu). Finely granular pars amorpha is interspersed with segments of pars fibrosa (arrows). Chromatin is evenly distributed. X 18,000.

Fig. 30. Nucleus and cytoplasm of an astrocyte in the early phase of infection. Viral particles (V) are enclosed in a vacuole in the cytoplasm. Nucleus contains clumped chromatin and a segregated nucleolus. The pars fibrosa of the nucleolus is fragmented (small arrow) and separated from the pars amorpha (large arrow). X 23,100.
stages of entry and enclosed within membrane-lined vacuoles in the cytoplasm were demonstrated in affected cells (Fig. 30).

Middle phase.----At 16 hours after inoculation cells with eosinophilic inclusion bodies predominated. The types of inclusions were placed in one of three groups based on their relative size and staining characteristics. The first group consisted of inclusions which were small (2-5μ), discrete, moderately eosinophilic, and partially separated from nuclear chromatin (Fig. 31). These were the most common type of inclusion until the forty-eighth hour. At their peak occurrence (20 hours) 18.5 per cent of the cells contained inclusions and 78.8 per cent of the inclusions were this type.

Inclusions characteristic of the second type were larger (sometimes filling the nucleus), less discrete, lightly eosinophilic, and interspersed within the nuclear chromatin (Fig. 31). They occurred either singly or in small groups. These inclusions were first observed at 10 hours and the number of cells containing them increased throughout the middle phase and into the late phase. In the early part of the late phase 40 per cent of the cells contained inclusions. Over 50 per cent were of the second type.

The third type of inclusion was the type A form. The inclusion was densely eosinophilic and filled the major part of the nucleus. It was separated from the peripherally located chromatin by a clear halo (Fig. 32). The type A inclusion was the least common form. It was observed as early as 7 hours and was present throughout the middle and late phases.
Fig. 31. Intranuclear inclusions in CHV infected astrocytes 16 hours after inoculation. Note discrete (small arrows) and large, amorphous types (large arrows) of inclusions. Hematoxylin and eosin; X 1250.

Fig. 32. Type A intranuclear inclusion body in CHV-affected astrocyte 20 hours after inoculation. Chromatin is condensed next to the nuclear membrane. Hematoxylin and eosin; X 1250.
The various types of inclusions were also demonstrated with AO. They were dark green, finely granular, and, with the exception of the diffuse type of inclusion, separated from the nuclear chromatin by a non-fluorescent rim.

The presence of viral antigen was demonstrated in the cells by immunofluorescence during the later hours of the early phase and throughout the middle phase. Two general patterns of distribution were noted. The first consisted of particulate accumulations of viral antigen in the cytoplasm and around the nuclear membrane (Fig. 33). The second distribution pattern was particulate masses around the nuclear membrane and within the nucleoplasm only (Fig. 34). Both forms were noted in approximately equal numbers of cells. Antigen-containing cells occurred in several foci irregularly distributed on the coverslip.

Lesions characteristic of the middle phase predominated until 48 hours after inoculation. Cells with lesions typical of the late phase appeared during the later part of the middle phase, representing a transition period during which both middle and late type lesions were present. Typical herpesviral cytopathic effects appeared during this transition period and consisted of small foci of rounded, piled-up cells. The cytopathic effect was evident between 12 and 16 hours after inoculation and was marked at 24 hours.

Intranuclear stages of viral maturation were demonstrated in cells of the middle phase by electron microscopy (Fig. 35). Viral formation occurred in areas of the nucleoplasm which contained dense,
Fig. 33. Astrocyte from infected monolayer culture (12 hours after inoculation) illustrating specific fluorescence in nuclear and cytoplasmic areas following exposure to labeled anti-CHV globulin. The viral antigen which appeared green occurs as particulate matter accumulated in clumps along nuclear membrane and in cytoplasm. Immunofluorescent method; X 1700.

Fig. 34. Specific, particulate fluorescence in the nucleus of an astrocyte from CHV-infected culture (16 hours after inoculation). Viral antigen appeared as green fluorescent particles within the nucleus. Immunofluorescent method; X 1825.
Fig. 35. Portion of nucleus of infected astrocyte. Various forms of capsids (arrows) are seen in an area of dense, granular, viral precursor material. Note clumped chromatin, membrane proliferation, and nucleolar remnant (Nu). X 45,000.

Fig. 36. Infected astrocyte illustrating nucleus with clumped chromatin, membrane proliferation, and disruption of nuclear membrane. Crystal-like arrangements of viral particles (arrows) are seen in cytoplasm with dispersed nucleoplasm. Numerous vacuoles are present in the cytoplasm. Several mitochondria are swollen and disrupted (M). Lipid bodies are prominent (L). X 18,000.
granular aggregates of precursor material. This material often appeared to be partially enclosed by developing viral capsids. The capsids were round or hexagonal. Their capsomeric substructure was evident around their circumference as a spoke-like pattern. Three types of capsids were observed. The most common type was a capsid enclosing three or four dense bodies arranged around the inner capsid wall. The central area was clear and star- or cross-shaped. A second type of capsid contained a round central mass separated from the capsid wall by a clear halo. Capsids lacking an electron-dense core represented the third form.

Membrane proliferation was prominent in areas of viral maturation and appeared to be continuous with the nuclear membrane. These membranes contributed an outer envelope to the capsids. Membrane enclosed capsids measured 130 to 180μ in diameter while capsids without an outer envelope measured 65 to 80μ in diameter.

**Late Phase.**—The lesions predominating in this phase consisted of hyperchromatic and shrunken cells. The nucleus was shrunken and lacked chromatin and its membrane was thickened and irregular. Distinct nucleoli were not discernable. The cytoplasm was densely eosinophilic with a purplish zone near the nucleus. Cytoplasmic processes were few in number and cells were distinctly separated from one another. The cells could be detached easily from the glass coverslip by washing. Many of the cells (60 per cent) became detached from the coverslip by washing at 72 hours after inoculation. Acridine orange staining demonstrated that nuclear DNA had extruded into the cytoplasm and that
the nuclear membrane was incomplete. Antigen was observed in the nucleus, perinuclear region, and cytoplasm of shrunken cell processes.

Electron microscopic evaluation revealed disruption of nuclear membranes, chromatin clumping and dispersion into the cytoplasm, and the presence of numerous viral particles (Fig. 36). Crystal-like arrangements of viral particles were observed in degenerated nuclei and in perinuclear regions containing extruded nucleoplasm. The cytoplasm showed signs of degeneration and contained numerous vesicles of various sizes. In addition, mitochondria were swollen and their membranes disrupted. Lipid bodies appeared in increased amounts. Viral particles enclosed in vesicles and free in the cytoplasm were noted between the nuclear and plasma membranes. Virus was also seen in the cytoplasm of shrunken cell processes.

Discussion

Two factors had to be considered when the viral effects in brain explants and cell cultures were compared with lesions in the brain of dogs infected with CHV. First, the relative quantity and distribution of the cell population in the explants and cell cultures was different from brain tissues. Second, the virus had access to all cellular components in the in vitro systems. This exposure is modified by the blood-brain barrier in the living animal. The brain explant culture represents practically all the elements of brain tissue with the exclusion of a vascular supply. Despite differences in the tissue systems there were several similarities in the lesions produced by CHV.
Lesions in vitro began as small foci which enlarged and eventually coalesced. PERCY et al. (24) and WRIGHT and CORNWELL (33) reported that lesions in the brains of young puppies also occurred as discrete foci. The pronounced involvement of glial cells observed in this study was compatible with astrocytic degeneration reported by PERCY et al. (24) and inclusion bodies within glial cells described by WRIGHT and CORNWELL (33). The occurrence of neuronal degeneration observed in vitro has been described in vivo in the brains of naturally and experimentally infected puppies. PERCY et al. (24) reported degeneration of neurons manifested by pyknosis of Purkinje's cells and fragmentation of many nuclei of the inner granular layer of the cerebellum. DERATULD and WERNER (7) reported pyknosis and neurolysis of Purkinje's cells and CORNWELL and WRIGHT (33) described areas of necrosis involving mainly the Purkinje cells and granule cells of the cerebellum.

The pathogenesis of canine herpes encephalitis is unknown. The mechanism of dissemination of the virus within nervous tissue and the cell types sequentially involved in vivo has not been reported. Our in vitro studies of CHV infected explants and cell cultures of nervous tissue demonstrated that glial cells were predominately affected and that viral maturation occurred within at least one type of glial cell. The presence of virus in processes of astrocytes from cell cultures as demonstrated by electron microscopy and immunofluorescence suggested one possible mechanism of spread of the virus along astrocytic processes. The spread of herpes simplex from cell to cell has been described. STOKER (27) reported that herpes simplex virus was transferred from
one cell to another without entering the extracellular space.

The role of neurons is less clear. The degeneration of these cells as reported in the present in vitro study and by others in in vivo studies (7, 24, 33) may be secondary to widespread infection of glial cells by CHV. The loss of glial cells would seriously impair normal neuronal functions. However, primary infection of neurons can not be overlooked. The lack of inclusion bodies alone is an insufficient criterion for the absence of direct infection by a virus (26). It may indicate, however, that neurons are not a preferential site of viral replication. It is not essential for a virus to replicate within a cell to inflict permanent injury to cellular organelles. Viral induced inhibition of cellular metabolism or toxic effects of viral coat proteins may be adequate noxae for cell damage.

The marked effect of CHV on glial cells in brain explant cultures was studied in greater detail in brain cell cultures. Cell cultures prepared from the cortices of newborn puppies consisted of over 90 percent astrocytes, the cell type predominately affected by CHV in explants. The earliest lesions in astrocytes (6 hours post-inoculation) were confined to the nuclei. Chromatin clumped and the partes amorpha and fibrosa of the nucleoli segregated. Nucleolar alterations were prominent throughout the experiment (72 hours). The precise relationship of these nucleolar changes to viral infection has yet to be established. Similar nucleolar alterations were observed in CHV-infected thyroid adenocarcinoma cells (16) and in HeLa cells infected with herpes simplex virus (17). JACOB (15) has described nucleolar changes in primary frog
renal tumor cells which contained herpes-like viral particles. Although other DNA and RNA viruses are associated with altered nucleoli (6), not all viruses initiate these changes (26). In addition, disruption of nucleoli has been observed in cells subjected to chemical injury (27).

The most characteristic effect of CHV on glial cells was the development of intranuclear inclusion bodies. These inclusions were considered as a definite indication of CHV-infection. Acridine orange staining demonstrated DNA in inclusions which fluoresced differently from cellular DNA. The possibility that DNA in inclusions represented viral DNA was supported by the presence of viral antigen and viral particles in various stages of maturation in the majority of nuclei during the peak time of inclusion body formation. Therefore, it was concluded that inclusion bodies were a manifestation of viral replication. The developmental and morphological characteristics of intranuclear inclusion bodies in glial cells were identical to those in CHV-infected thyroid adenocarcinoma cells (16) and to those in herpes simplex-infected non-neural cell cultures (19).

The viral replication cycle as determined by the methods used in this study corresponded in morphologic characteristics and developmental sequence to that reported for the same virus in thyroid adenocarcinoma cells (16) and for other herpes viruses in non-neural cell cultures (25). The presence of crystal-like formations of viral particles in cultured astrocytes indicates high concentrations of virus and suggests that the metabolic environment of neuroglial cells is particularly suitable for CHV reproduction. The effect of the virus
upon astrocytes was, however, detrimental to cell survival and resulted in cell death within 72 hours after infection.

Canine neuroglial cells in tissue culture represented excellent host cells for the study of CHV-cell relationships. They were also the preferential site of viral replication in brain explant cultures where the chance of exposure of all cell types was nearly equal. The cell cultures provided a unique opportunity for the continuous observation of pathologic processes after exposure to the virus. The sequential progression of infection in explant cultures and the selective appearance of inclusions within neuroglial cells could be followed and valuable information applicable to the pathogenesis of herpes virus encephalitis was obtained.

**Summary**

The effects of *Herpes canis*, an encephalitogenic agent of the canine species, on canine cerebellar explants and brain cell cultures were investigated. A progression of the infection from cell to cell in brain explant cultures could be followed resulting in degeneration and widespread cell death within 72 hours after inoculation. Astrocytes were recognized as the preferential cells for viral replication. Nerve cells were irreversibly damaged but manifested no definite inclusion bodies indicative of viral replication.

The sequential effects of *Herpes canis* on a neuroectodermal cell type (astrocytes) were studied in brain cell cultures by correlating the results of acridine orange cytochemistry and immunofluorescence
with the findings of light and electron microscopy. The progression of lesions within astrocytes was divided into three phases. The early phase was characterized by viral entry and nucleolar segregation consisting of separation of the pars amorpha from the pars fibrosa which subsequently fragmented. Three forms of inclusion bodies appeared during the middle phase and viral antigen and viral particles in various stages of maturation were demonstrated within the nuclei of affected cells. The late phase consisted of cell degeneration and viral release.

The two in vitro systems (brain explants and cell cultures) offered an excellent opportunity for detailed and repeated observations of the interactions of a neuropathogenic herpes virus with the various neuroectodermal cellular elements obtained from the natural host. Many of the findings appear to be directly applicable to pathogenetic principles of canine herpes virus encephalitis.
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