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A STUDY OF MYELINATING CANINE CEREBELLAR TISSUE
CULTURE DURING NORMAL DEVELOPMENT AND
FOLLOWING EXPOSURE TO CANINE
DISTEMPER VIRUS

DISSERTATION
Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
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*******
The Ohio State University
1966

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CHAPTER I

GENERAL CULTURAL CHARACTERISTICS OF CANINE CEREBELLAR EXPLANTS

Introduction

The cultivation of nervous tissue in vitro dates back to the notable investigations of Harrison who reported the growth of neurons and their processes in explants from tadpoles cultivated in frog lymph (17, 18). Numerous subsequent reports concerning the neuron and its growth characteristics in culture have been made (4, 10, 14, 16, 19, 22, 24, 25, 26, 27, 28, 29, 30, 35, 36, 37, 39, 44, 45, 47, 50, 53, 56, 57, 62, 63, 66). During the past 15 years an added emphasis has been placed upon the study of the previously neglected neuroglia in tissue culture (2, 4, 19, 20, 22, 25, 26, 27, 28, 40, 43, 44, 46, 48, 49, 50, 54, 55, 56, 57, 59, 65, 67). Following the discovery by Peterson (51) that myelin may develop in vitro, considerable attention has also been given to the cultivation of myelinated axons from both peripheral and central nervous tissue (1, 6, 7, 8, 9, 21, 44, 52, 53, 66).

The objectives of this investigation were to simplify the existing methods for culturing nervous tissue explants which would also permit the development of myelin and to characterize the cellular components during culture growth by means of different optical methods and various staining or impregnating procedures.
In order to subsequently study the effects of the virus associated with demyelinating canine distemper encephalitis (61), cultures were prepared from canine cerebellum.

Materials and Methods

Canine cerebellar cortex was employed as the tissue source. The dogs ranged in age from three days to two weeks. Each dog was anesthetized with ether and the brain aseptically removed and placed in warm (37 C) sterile Gey's balanced salt solution (BSS) which contained 200 units of penicillin and 100 mg of streptomycin per ml. The intact brain was rinsed with three changes of Gey's BSS without antibiotic and the cerebellum and brain stem were removed to a sterile petri dish where the cerebellum was dissected free. The meninges were carefully removed and the cerebellum was cut transversely into three sections, each approximately 3 mm in thickness. The medullary white matter was dissected from each section, leaving three strands of cortex with cross-sectional dimensions of 3 by 3 mm. These segments of cortex were cut into explants in the depressed portion of a Maximow slide which had been rinsed in Gey's BSS. Explants averaged 1.5 x 1.5 x 1 mm in size. The explants were placed in a stoppered bottle with complete media and stored at 37 C until ready for explantation.

The coverslips (Gold Seal, #2, 11 x 35 mm), employed for receiving the explants, and the culture tubes (Leighton) were
specially treated prior to the time of explantation. The method was as follows:

1. Ether-Alcohol Rinse—Ether (one part) and absolute ethyl alcohol (three parts). Rinse two times.
2. Detergent Wash—(Haemosol\(^1\)). Wash for 30 to 60 minutes. Allow coverslips to soak and brush-clean Leighton tubes.
4. Acid Rinse—Rinse in 0.01 N HCl.
5. Water Rinse—Rinse three times in distilled water.

All glassware including coverslips was sterilized by dry heat (400 F) for two hours. Gey's BSS was added to the coverslips before explantation. The coverslips were stored at 4 C for 12 to 24 hours and then warmed to 37 C prior to use.

Two explants were placed directly on each coverslip, which was gently pushed into the flat part of the tube containing 0.4 ml of complete medium. Each tube was rotated carefully to ensure contact of the explant with the medium and then placed in a stationary rack at 37 C. The complete medium used was that reported by Bornstein (6) and consisted of balanced salt solution (Gey's was substituted for Simms'), 35 per cent; fetal bovine serum, 40 per cent; bovine serum ultrafiltrate, 25 per cent; and glucose, 600 mg per cent. The pH of the final medium ranged from 7.2 to 7.4. The medium was changed twice a week by adding 0.3 ml to each tube after the complete removal of all old medium.

\(^1\)Meinecke Company, Baltimore, Maryland.
Following each change the tubes were rotated to establish contact of the explant with the medium.

It should be emphasized that no adhesive material, such as rat-tail collagen or chicken plasma clot, was used to produce adherence of the explants to the coverslip. This was done to improve the results with various stains that were employed during the study of canine distemper virus in explant tissue culture (61).

Cultures were examined daily with bright light and phase microscopy. With bright light microscopy the cultures were routinely studied in the Leighton tube. For more detailed studies, examinations were also made in Maximow coverslip assemblies. For examination with phase microscopy a Leitz Ortholux microscope was used with a variable phase condenser and positive, normal contrast phase objectives. Time-lapse cinematographic studies were also performed on selected cultures. For this purpose the cultures were assembled in a Rose culture chamber and photographed on a Sage ² cinematographic stage equipped with a 16 mm Bolex camera (Model No. H16) and a Leitz Ortholux microscope. A filming speed of eight frames per minute was used.

Tinctorial procedures employed for examination of glia included May-Grünwald-Giemsa (41) and Gallego's stains (38), and the silver impregnation described by Costero (12, 13). For demonstration of neurons and their processes the methylene blue supravital stain (4, 14, 56) and Holme's silver nitrate impregnation, as modified by Wolf (66), were used.

Results

Evaluation of the culture method

Explants adhered well and began to flatten out on the coverslip with beginning growth after 12 to 24 hours. Three-tenths ml of medium were adequate for all medium changes subsequent to the 0.4 ml used at the time of explantation.

The most critical phase of the culture growth was during the first two to three days when the explant was developing its initial outgrowth. This stage of growth was directly related to the degree with which the explant and the medium were in contact. Because of the small amount of medium employed for the initial explantation (0.4 ml) and the relatively high elevation of the explant above the coverslip, explants would frequently become dehydrated and subsequently necrotic if not properly exposed to the medium. To avoid this, each culture tube was rotated twice a day for three days following explantation.

Optimum culture growth was obtained when brains from puppies three to seven days of age were used. Cultures prepared from older nervous tissue (over two weeks) grew slower and were less adaptable to in vitro conditions.

Culture characteristics

Neuroglia.—The initial cellular outgrowth from the explant consisted of astrocytes, oligodendroglia, and mesenchymal cells. Large astrocytic processes, which extended out onto the glass coverslip, were accompanied by oligodendroglia. In focal areas around the explant, sheets of fusiform, mesenchymal-type cells
containing a granular cytoplasm also contributed to the beginning outgrowth. Isolated cells of this type could, in addition, be seen among the astrocytic processes. The early outgrowth produced a tapered zone which was densely cellular and became less accentuated as the explant flattened out.

The outgrowth, which extended peripherally from the tapered zone, was quite cellular and was often one cell layer thick. The individual cell types were not always possible to distinguish. Polygonal cells identified as astrocytes predominated but a large number of oligodendroglia and some mesenchymal cells were also present. Many astrocytic fibers and a lesser number of neuronal processes from the explant extended between these cells. At this stage of growth small sheets of mesenchymal cells often occupied the most peripheral part of the outgrowth, sometimes almost surrounding it. These cells were fusiform in shape. Their nuclei measured 21 by 10 microns and the dimensions of the perikarya were 42 by 14 microns.

With advancing outgrowth the densely cellular area adjacent to the tapered zone expanded peripherally with the cells developing more pronounced processes. The majority of the cells had a lacy appearance and resembled protoplasmic astrocytes. Within the outer part of this zone, areas sometimes developed that were less heavily populated and consisted of a loose network of well-differentiated astrocytes (Fig. 1, 2). This type of cell growth was most common in young cultures and in some explants it extended completely around the explant. Astrocytic and neuronal
Fig. 1. Seven-day-old explant culture of canine cerebellum illustrating the pattern of cellular outgrowth (J1335-5). May-Grünwald-Giemsa stain; x 32. The distribution of outgrowth from the explant (A) includes the tapered zone (B), the densely cellular zone which is only a few cell layers thick (C), the zone containing a loose network of cells (D) and a peripheral zone consisting of large fusiform, mesenchymal cells (E).

Fig. 2. Twelve-day-old explant culture of canine cerebellum showing the degree of astrocytic argyrophilia (J1917-38). Costero's silver impregnation; x 315. Well-differentiated fibrous astrocytes, which are moderately argyrophilic, form a loose network of processes in the outgrowth. The small intensely argyrophilic cells are predominantly granule cells.
processes originating within the explant also passed through this zone to the periphery. Mesenchymal cells often were present adjacent to this area in the most peripheral part of the outgrowth (Fig. 1). The majority of the astrocytes in the loosely constructed area closely resembled the fibrous type and they could be well demonstrated with the May-Grünwald-Giemsa and Gallego's stains. With the former method the nucleus was moderately eosinophilic and oval in shape, with dimensions of 15 by 8 microns. Usually two, and frequently more, nucleoli were present. The perikarya stained a light blue and measured 24 by 11 microns. Dense processes extended from all poles of the perikaryon to form the complete network typical of this zone. Astroglial processes could be particularly well demonstrated with Gallego's stain. Very fine filaments, frequently overlooked by other methods, were usually revealed by this technique. Astrocytes were also moderately argyrophilic following impregnation with either of the silver methods employed (Fig. 2). With increasing age the densely cellular area (mainly protoplasmic astrocytes) adjacent to the tapered zone usually replaced the less dense areas of growth. This eventually resulted in a uniform cell sheet that surrounded the explant.

The peripherally located sheets of fusiform mesenchymal cells were present until approximately the third to fourth week of culture growth when they were usually replaced by large, flat polygonal cells having few processes. In some cases, however, a mesenchymal collar of cells remained indefinitely. A gradual
transition of the more centrally located astrocytes with typical morphologic features into the large polygonal cells could be detected, indicating that the latter were probably astrocytic in type.

With increasing age a dense zone of radiating astrocytic fibers surrounded the explant and tended to separate it from the cellular part of the outgrowth.

Oligodendroglia accompanied the astrocytes in the initial outgrowth surrounding the explant (Fig. 3, 4). They increased in number during the first few days of growth and then began to disappear from the outgrowth after one to two weeks. Oligodendroglia could be easily distinguished from astroglia in stained preparations and in living cultures with phase microscopy. Morphologically, they had a spherical to oval cell body, almost all of which was nucleus. With phase microscopy the cell body was golden brown in color and was surrounded by a light halo. There was a small accumulation of cytoplasm that usually did not completely surround the nucleus. Very small delicate processes emanated from the perikaryon without a hillock formation. The processes appeared stretched and frequently were attached to astroglial fibers (Fig. 4). There was a tendency for oligodendroglia to be more concentrated in some areas of the outgrowth than in others.

Following fixation and staining with the May-Grünwald-Giemsa method, the perikarya of oligodendroglia measured 8 by 6 microns. The cytoplasm stained lightly basophilic and the nucleus moderately to darkly eosinophilic (Fig. 3, 4). The nucleus was
Fig. 3. Five-day-old explant culture of canine cerebellum demonstrating the cells present in the early outgrowth (K357-5). May-Grünwald-Giemsa stain; $x$ 125. The small, darkly stained cells with fine processes situated between the astrocytic fibers or lying parallel with them are predominantly oligodendroglia. A few granule cells are also present, but are difficult to distinguish at this magnification. Macrophages with a foamy-appearing cytoplasm and eccentric nuclei are distributed throughout the growth (arrows).

Fig. 4. A higher magnification ($x$ 500) of the central part of Figure 3 illustrating several oligodendroglia (O). The fine, delicate processes of these cells are mostly attached to the astrocytic fibers. Astrocytes (small arrows) and macrophages (large arrows) can also be detected.
usually homogeneous, but nucleoli could occasionally be detected. The processes were lightly eosinophilic. Oligodendroglial perikarya were also moderately argyrophilic, particularly with Costero's method. The intensity of the argyrophilia was greater than with the cell bodies of astrocytes.

With time-lapse studies oligodendroglia exhibited the typical tug-of-war stretching of their processes that has been described by others (2, 40). Pulsation of the perikaryon, as reported (40, 48, 50, 54, 56), was not detected, but the number of cells examined for this activity was limited. In comparison, astroglia had a high degree of membrane activity.

Macrophages were first detected in the central and marginal parts of the explant after three to five days of growth. These cells rapidly increased in number during the following week (Fig. 3). They first exhibited a round shape and contained numerous cytoplasmic granules that gave them a dark brown color with bright light and a bright glowing effect with phase microscopy. The nucleus was rarely visible because of the granular cytoplasm.

With the May-Gr"unwald-Giemsa stain the nucleus was moderately to darkly eosinophilic. It was invariably eccentric in position and was usually oval or crescent-shaped conforming to the contour of the cell membrane (Fig. 3, 4, 5). Occasionally, folded or double nuclei occurred. One to two nucleoli were usually present. The cytoplasm varied in appearance. Many of the cells were large (30 x 26 microns) and contained a markedly foamy
Fig. 5. Five-day-old explant culture of canine cerebellum showing neuronal and glial components of the outgrowth (J1223-13). May-Grünwald-Giemsa stain; x 500. A large, flat astrocyte with few processes (large arrow) and more differentiated astrocytes (small arrows) form a framework which contains macrophages (M) and granule cells (G). The macrophages have a basophilic granular cytoplasm which in one cell is radially distributed as fine filaments. The two granule cells have a basophilic nucleus, a randomly coursing process extending from the cytoplasmic pole, and a fine straight process originating from the nuclear end of the cell.

Fig. 6. Nineteen-day-old explant culture of canine cerebellum demonstrating two cells with the morphologic characteristics of microglia (J1223-47). Gallego's stain; x 800. These cells were located on the glass beyond the peripheral outgrowth zone. Their rod-shape is similar to that demonstrated with microglia. With phase microscopy these cells have properties (cytoplasmic granularity) similar to well-defined macrophages (Fig. 3, 4, 5).
cytoplasm (Fig. 3, 4). The remaining macrophages had a basophilic cytoplasm which ranged from pale to very dark (Fig. 5). Cytoplasmic granulation was variable in these cells. Macrophages were, in general, moderately argyrophilic with Costero's method.

During the second week of culture many of the macrophages extended spinous processes from their cell body for varying lengths which altered their spherical shape to some extent (Fig. 5). Also, macrophages which had migrated to the periphery of the outgrowth zone, or beyond and onto the glass, occasionally continued to change in shape. Some had an elongated form which resembled microglia as demonstrated in sections of central nervous tissue (Fig. 6). Cells of this type were variable in size. Some had perikarya and processes comparable to astrocytes, but the majority were smaller. One differentiating feature of these cells, as seen with phase microscopy, was their intense glowing effect due to the marked cytoplasmic granularity.

Ependymal cells were occasionally detected in cultures and were easily recognized by their rapidly beating cilia. These cells could be identified in cultures even after several weeks. Several patterns of cell arrangement were identified. Most commonly the cells were arranged in sheets or bands, resembling strips of ependymal epithelium. Occasionally, ependymal cells formed a vesicular structure with centrally oriented cilia. These formations sometimes contained free cells that were in a constant tumbling movement because of the ciliary activity.
Neurons.—Four principal morphologic types of neurons were distinguished in explant cultures of canine cerebellum.

During the first week of growth two kinds of neurons could be consistently demonstrated with the methylene blue supravital method. One type was characteristic of the granule cell as reported by others in tissue culture (48, 56) (Fig. 7, 8). The outer embryonic granular cell layer of the cerebellum has been considered to represent its place of origin (48). These cells were routinely demonstrated at the margin of the explant after a few days of culture growth. They were also detected in the middle and outer parts of the outgrowth as the culture became older. With age these cells decreased in number and after four to eight weeks were usually absent. Following fixation and staining the cells usually had a pear-shaped perikaryon that measured 13 x 6 microns. The nucleus, which measured 8 x 6 microns, frequently occupied a tapered end of the cell body and a small amount of cytoplasm formed the remainder of the perikaryon (Fig. 5). With the May-Grüwald-Giemsa method the nucleus stained darkly eosinophilic to basophilic and was usually dense and homogeneous. One to two faintly stained nucleoli could occasionally be detected. The cytoplasm was less basophilic and compared in color to that of astrocytes (Fig. 5). The nucleus of granule cells was also markedly argyrophilic, whereas the cytoplasm impregnated lightly and was comparable to other cells in the outgrowth (Fig. 2). The cellular characteristics were best appreciated with methylene blue supravital preparations in which the cell,
Fig. 7. Twenty-three-day-old explant culture of canine cerebellum illustrating the typical staining characteristics of granule cells with methylene blue (J1223-51). Methylene blue supravital stain; x 315. The granule cells stain very intensely with this method. The sperm-like shape is typical of this cell type.

Fig. 8. Twenty-three-day-old explant culture of canine cerebellum depicting two large stellate cells and several granule cells stained with methylene blue (J1223-51). Methylene blue supravital stain; x 315. The stellate cells, which are similar to Golgi type I neurons, have an affinity for the stain equal to that of granule cells. Note here and in Figure 7 how the densely stained chromatin retracts from the nuclear membrane leaving a light circular zone.
particularly the nucleus, stained dark blue (Fig. 7, 8). With this stain the nuclear chromatin contracted from the nuclear membrane leaving a light unstained halo. Many cells had a sperm-like appearance due to a prominent process originating from the cytoplasmic pole of the perikaryon (Fig. 7). This process sometimes bifurcated a short distance from the cell body in the form of a "Y" or "T" (Fig. 5). Granule cells frequently had, in addition, a delicate process which originated without a hillock from the nuclear pole of the cell body (Fig. 5). Cells with two or more processes also occurred, but the perikaryal size remained relatively constant. With positive phase microscopy the granule cell generally appeared gray-black.

The second type of neuron actually represented a group of cells that were concluded to probably consist of both small stellate cells, from either the inner or outer layer of the cerebellar cortex, and large stellate cells of the granular layer (grosse Sternzellen or Golgi cells). These cells were usually larger than the granule cell and frequently had large prominent processes (Fig. 8). During early culture growth they were located in the peripheral part of the explant and in the dense glial outgrowth surrounding the explant. After three to four weeks these cells were also occasionally present in the mid-outgrowth. The perikaryon and processes of these cells stained intensely with the methylene blue stain (Fig. 8). They could not, however, be adequately demonstrated with the May-Grünwald-Giemsa stain or by
silver impregnation. In most cultures the population of this cell was one-fourth to one-third that of the smaller granule cells.

After the second to third week of culture growth, when the explant had thinned out, two other neuronal types could be detected. One was seen less frequently and morphologically resembled the histological descriptions of large motor neurons of the cerebellar medullary nuclei (Fig. 9). This cell type was usually randomly distributed throughout the explant. They stained uniformly and moderately with methylene blue. May-Grünwald-Giemsa staining was usually unsuccessful because the density of the explant was too great. The cellular characteristics could be best demonstrated with silver impregnation due to the marked argyrophilia of this cell type. These neurons were large, multipolar and had perikarya that measured 27 x 31 microns. Well-defined neurofibrils could often be demonstrated in the cell body and dendrites. The axons were generally long and as thick as any in the culture. The attachment of the axon to the perikaryon, or less frequently as an extension of a large process, could also often be demonstrated.

The remaining neurons were identified as Purkinje cells. The perikaryon was invariably located within the explant and measured 27 x 14 microns. In the earlier stages of growth (one to two weeks) the cell body could only occasionally be identified by methylene blue staining due to lack of explant penetration. However, later (after two to three weeks) the cells often stained well. The perikaryon stained with moderate intensity, much less than either
Fig. 9. Thirty-one-day-old explant culture of canine cerebellum illustrating a large neuron with features different from the Purkinje cell (J1223-69). Wolf's modification of Holme's silver impregnation; x 1250. This cell was identified as probably representing a large neuron from a cerebellar medullary nucleus. The many dendrites radiating from the surface of the perikaryon and prominent neurofibrils in both the cell body and dendrites were typical features of this cell type. Compare with Figure 10. The axon is out of focus in this picture.

Fig. 10. Twenty-four-day-old explant culture of canine cerebellum showing the typical morphologic features of the Purkinje cell in culture (J1223-52). Wolf's modification of Holme's silver impregnation; x 1250. Two typically oval-shaped Purkinje cells are seen with their dendrites located at the pole of the cell opposite to that of the axonal attachment (arrows). The dendrites of the upper cell are slightly out of focus.
the stellate or granule cells. The axonal hillock, however, occasionally stained a deep blue with this method. In contrast, the attachment of the axon with the hillock was often very faint and difficult to visualize with silver impregnation. After two weeks in vitro the Purkinje cells stained weakly with the May-Grünwald-Giemsa stain. Many cells, however, had a slight peripheral concentration of Nissl substance that stained deeply basophilic. After four weeks in culture a definite, discrete Nissl substance was distributed evenly throughout the cytoplasm of many Purkinje cells. By this time the perikarya also had increased in size. With both methylene blue and silver impregnation long axons were demonstrated. They took a random path through the explant but frequently doubled back on themselves in a serpentine manner. They also sent off branches which occasionally could be seen to extend toward the cell body and even make contact with it or with the proximal axon. Other processes grew into the outgrowth. The dendritic growth was similar to that routinely demonstrated in tissue sections with the Golgi method (Fig. 10). The only difference was that in tissue culture the length and extent of the arborization was reduced. The morphological identification of the Purkinje cell at this stage of growth, however, was unquestionable. The Purkinje cells varied in number but often 20 to 30 could be counted in a single explant. They also were quite frequently arranged in a row reminiscent of the distribution in tissue sections of the cerebellum (Fig. 11).
Fig. 11. Twenty-three-day-old explant culture of canine cerebellum demonstrating the frequent arrangement of Purkinje cells in culture (J1223-50). Wolf's modification of Holme's silver impregnation; x 125. The general outline of a cerebellar folium with its typically arranged Purkinje cells (dark oblong bodies) can be detected in this culture.

Fig. 12. Twenty-three-day-old explant culture of canine cerebellum illustrating an axonal enlargement (bulb) of a large neuron (J1223-50). Wolf's modification of Holme's silver impregnation; x 500. The axon of a large multipolar cell (probably from a cerebellar medullary nucleus) has developed an axonal enlargement (large arrow) a short distance from the cell body. Note the reduction in diameter and argyrophilia of the axon as it approaches the perikaryon. A separate neuronal process (small arrow) passes over the cell where it goes out of focus in this picture.
The degree of Purkinje cell argyrophilia varied with culture age. Young cells were weakly argyrophilic and the cytoplasm had a reddish color. The large round nuclei of these cells were readily detected, however. Older Purkinje cells had increased argyrophilia. The neurofibrils were fine and not prominent in the perikaryon but were frequently visible in the dendrites. Also, large, spherical nuclei containing a single nucleolus were usually visible in these cells (Fig. 10).

Another feature, which was consistently detected in most silver impregnated cultures, was the occurrence of bulbous enlargements or thickenings along many of the neuronal processes (Fig. 12, 13, 14, 15). The majority of the processes were considered to be axons and their collaterals. The enlargements were darkly impregnated, often oval in shape, and were detected most frequently in the dense part of the explant. With good light penetration, the internal structure was seen to consist of a fibrillar material. The continuity of the neurofibrils from each side of the enlargement into the adjacent axonal segment was often detected (Fig. 13). A connection of axons, which had enlargements, with perikarya of Purkinje cells or other large neurons could be demonstrated with methylene blue stain and following silver impregnation (Fig. 12). The former method usually gave the most conclusive results since the area of the axonal hillock generally impregnated weakly with silver, particularly in the case of Purkinje cells. The cell body of involved neurons occasionally had evidence of degeneration, which included swollen or pyknotic
Fig. 13. Thirty-one-day-old explant culture of canine cerebellum showing two enlargements along one neuronal process (J1223-69). Wolf's modification of Holme's silver impregnation; x 1250. Notice the distinct neurofibrils at the junction of the axon with the bulbs (arrows) and the variation of the axonal diameter on either side of the right enlargement.

Fig. 14. Twenty-three-day-old explant culture of canine cerebellum illustrating several small processes originating from an enlargement occurring at the junction of two larger processes (arrows) (J1223-50). Wolf's modification of Holme's silver impregnation; x 1250. The faintly visible nucleus in the upper right of the bulb is that of an underlying glial cell.
Fig. 15. Thirty-one-day-old explant culture of canine cerebellum demonstrating a neuronal process with an unusual form of enlargement (J1223-50). Wolf's modification of Holme's silver impregnation; x 1250. In addition to the distinct bulb there is thickening of the adjacent part of the neuronal process (arrows).

Fig. 16. Twenty-four-day-old explant culture of canine cerebellum showing a neuronal process containing small segments that failed to impregnate with silver (J1223-52). Wolf's modification of Holme's silver impregnation; x 1250. These segments (arrows) often are granular and appear slightly distended.
nuclei that were eccentrically located. Also, the cytoplasm had an increased argyrophilia with accentuation of the neurofibrils. The axonal segment distal to the enlargement was usually thicker than the portion immediately proximal to it (Fig. 13). In addition, the proximal axon often continuously decreased in diameter from the bulb to the perikaryon (Fig. 12). No evidence of nuclei or other structures was ever detected with light microscopy to indicate that these structures might be cells. Furthermore, electron microscopic studies conclusively demonstrated that they were axonal in nature (34). Some enlargements also had a myelin sheath that was continuous with the adjacent axon. This was demonstrated with bright light examination of myelin stained preparations (60) and following electron microscopic examination (34).

The enlargements varied somewhat in their morphologic features and distribution. Occasionally, two and even three occurred along one process (Fig. 13). Most were oval to fusiform in shape. One or more branches of small diameter sometimes extended laterally from the bulb (Fig. 14). Other atypical formations also occurred (Fig. 15).

Axons and collaterals of Purkinje cells and neurons of cerebellar nuclei impregnated strongly with silver. The axonal diameter appeared to increase gradually with the age of the culture. There was, however, still considerable variation (0.2 to 2.0 microns) in cultures with mature-appearing neurons. The average axonal thickness in older cultures was about 0.9 microns.
The neuronal processes of both neurons were invariably long and branched frequently. In addition to being present in the explant, axons and collaterals also extended into the outgrowth, often to the extreme periphery.

Well-impregnated neurites of all diameters exhibited a periodic loss of density along their length and occasionally at places of branching. These areas often had a granular, and sometimes swollen, appearance. Some were completely devoid of silver (Fig. 16). The latter were detected more frequently with processes of small diameter. In addition, the larger processes (1 to 2 microns) occasionally had a longitudinal separation of the neurofibrils for a short distance which caused the affected portion to have a slightly increased diameter. These areas were distinctly different, however, from the above described focal alterations in impregnation.

Neuronal processes could also be detected with the methylene blue stain in the explant and the outgrowth. The peripheral growth of these processes often extended beyond the middle of the outgrowth and frequently exceeded that detected following silver impregnation. This finding indicated that neurons other than Purkinje cells or neurons of medullary nuclei contributed to the source of this growth. The cells of origin for these peripheral neurites were identified as stellate neurons in some instances and Purkinje cells in others, but in the majority of cases no related cell body could be identified.
Discussion

The method employed for culturing explants of canine cerebellum was quite reliable and consistently allowed myelination of axons (60). This technique had several advantages over other methods that have been reported for growing explants of nervous tissue in vitro. First, time was saved by eliminating the need for any of the adhesive materials. Second, a large number of cultures could be prepared in a short period and they could be stored in a minimum of space. Third, cultures could be easily examined microscopically in the Leighton tubes. Fourth, medium change was rapidly accomplished and, finally, the use of various stains was facilitated by eliminating collagen which stained and obscured cellular detail, particularly the early outgrowth surrounding young explants. Collagen, as employed by others (5, 15), was not used in order to facilitate staining and evaluation of preparations relating to the investigation of canine distemper virus in tissue culture (61). A minor problem associated with this method was the occurrence of some explant contraction in aged cultures. This resulted in a zone of radiating astrocytic fibers around the explant.

The morphologic characteristics and behavior of neuroglial growth in canine cerebellar cultures were, in general, similar to those reported for other species or types of central nervous tissue (2, 4, 9, 19, 22, 44, 50, 53, 56). There were some differences, however, regarding the times reported for beginning outgrowth. Berg and Källen (corpus callosum) (2) and Borghese
(hypothalamic nuclei) (4) did not detect the first cellular migration until four days or so after explantation. The time was shorter in other reports (9, 19, 22, 40, 50, 53, 56). Other described variations existed in cultures of different central nervous tissues regarding the migratory patterns of neuroglia and the morphologic characteristics of oligodendroglia. Hild (19, 22), Okamoto (50), and Borghese (4) reported that fusiform mesenchymal cells were the first to migrate from the explant. Others (9, 12, 26, 40, 48, 53) reported neuroglial elements were the earliest cells to extend as outgrowth. Murray (44) indicated that neuroglia (mainly oligodendroglia) followed microglia as the initial out-wandering cell. In the present study beginning growth began after 12 to 24 hours when oligodendroglia accompanied a predominantly astrocytic outgrowth. Mesenchymal cells, existing in small sheets or as individual cells within the neuroglial growth, were also detected. The oligodendroglia had perikarya that were spherical or oval in shape and were generally characterized by a limited amount of cytoplasm and fine delicate processes. These results agreed with some reports (4, 43, 50, 56), but were somewhat dissimilar to others (2, 19, 40, 48, 67) in which oligodendroglia had more abundant cytoplasm and prominent cell processes that even branched extensively in some cases (48, 67). One proposed explanation for this variation (43, 59, 67) was that the cellular characteristics might change under the influence of different culture conditions. Also, the cells may continuously change shape in culture (2). The former proposal could help
explain the differences found in the present study in which the commonly used collagen or plasma clot were not employed. This same principle might also clarify the differences reported for the migratory behavior of glial cells in culture.

The pulsatile activity, reported as a feature of the living oligodendroglial cell, was not detected in this investigation. Since only limited cinematographic studies of this cell were conducted, a definite interpretation could not be made regarding this activity. Oligodendroglial pulsation is apparently not a necessary criterion for identification since this feature is not consistently present in all cells (2, 4, 48, 50). The finding that oligodendroglia began to decrease in number, and eventually disappeared from the outgrowth with increasing culture age, was in general agreement with other studies (2, 44, 48).

Morphologically, oligodendroglia were similar to granule cells. This sometimes presented a problem in interpretation. Comparable findings have also been reported by other investigators (56). Several differentiating criteria were used in this study to enable proper identification of these two cells. Oligodendroglia occurred in highest numbers during the first week of culture growth; were golden-brown with a peripheral halo (phase microscopy); had a spherical to oval cell body containing a small accumulation of cytoplasm and had delicate, straight processes. Granule cells were also numerous after about one week but, in contrast to oligodendroglia, they persisted for four to eight weeks in culture. This neuron generally had a dark gray to black color
with phase microscopy and had no halo. Morphologically, the processes of granule cells were larger, longer and often had a meandering course; the cytoplasm was also more abundant than in oligodendroglia. In addition, the typical sperm-shape was a feature seen only with the granule cell. The May-Grünwald-Giemsa and methylene blue supravital stains provided additionally useful criteria for differentiation. The former method stained granule cell nuclei darkly eosinophilic to basophilic which enabled easy identification. Oligodendroglial nuclei stained moderately to darkly eosinophilic. The different amounts of cytoplasm in the two cells provided an additional means of identification. Oligodendroglia did not stain with methylene blue, whereas the granule cell stained intensively.

The major part of the outgrowth consisted of cells that were variable in morphology, but which mainly had characteristics of astroglia. The ones most dissimilar were the large fusiform cells usually located in the periphery of the outgrowth. These cells had no definite processes and frequently were joined together in sheets. They have generally been interpreted as mesenchymal cells originating from pial and/or vascular elements within the explant (4, 14, 22, 50, 56, 57).

During the third to fourth week of culture the mesenchymal cells were usually replaced by large, flat polygonal cells which were considered to be mostly astrocytes. The outgrowth at this time closely resembled that described by Hild in cultures of mesencephalic nuclei in which few mesenchymal cells were present
In the present study cellular interpretation was based upon several principles. First, the general tinctorial properties of the large polygonal cells were comparable to the typical astrocytes present in the outgrowth. Second, when pure cultures of meninges were compared with this cell type there was a distinct difference. Meningeal cells had an appearance and growth pattern very similar to that of the mesenchymal cells described previously. Finally, a transition of the large polygonal cells into well-differentiated astrocytes could be detected with stained preparations in both the periphery of the outgrowth and at its junction with the inner loosely populated zone present in young cultures. Other investigators have observed a similar transformation with time-lapse cinematography (46, 48).

Cells with features similar to microglia, which have been demonstrated in tissue culture following silver impregnation (12, 13, 64), were only occasionally detected. They usually occurred on the glass peripheral to the outgrowth and corresponded to Hortega's pseudopodic transitional form. Other round cells that accompanied the microglia were suggestive of the round-form stage of microglial development in vivo (58). It was concluded that the different forms detected merely represented morphological variations of the macrophage as has been described previously (12, 13, 42, 64). The reason for the low number of microglia in the present study may have been largely a result of the culture method employed. Other investigators, who have successfully demonstrated well-differentiated microglia in tissue culture following silver
impregnation, have used some adhesive material, usually plasma clot (12, 13, 64). Wells and Carmichael (64) found that these cells were better differentiated when cultures were prepared in a plasma clot as opposed to growing them on glass. Jacoby (31) stated that when macrophages migrated through a plasma clot they were usually elongated in shape. He also noted, however, that all forms did occur in cell monolayers on glass.

The various arrangements of ependymal cells in canine tissue culture corresponded with those previously described (20, 48). There was, however, no noted association between the presence of ependyma and the degree of myelination as concluded by Wolf (66).

Neurons with characteristics similar to those in this study have been reported in cerebellar tissue cultures from various species (9, 21, 24, 56, 66). Different neuronal types have been reported but their identification is often difficult to establish. There was one neuron, the Purkinje cell, upon whose characteristics several workers agreed (23, 24, 56, 66). There has been, however, some reported variation regarding the morphology and location of this cell in culture. In most studies, including the present one, these cells always existed within the explant, or at its edge, and remained relatively well differentiated during the period in vitro (9, 21, 24, 56, 66). In the present investigation the cellular arrangement (in rows) of Purkinje cells was also quite definite. A similar characteristic was recently reported by Hild (24). This feature facilitated identification, and emphasized the degree to which the architecture of the cerebellar cortex was
maintained in vitro. In other instances large neurons from the cerebellum (Purkinje cells) (14, 26, 27, 28) or from other areas of the central nervous system (14, 16, 27, 28) were reported to be located in the outgrowth.

A second large nerve cell was identified as probably originating from the medullary gray matter of the cerebellum. The opportunity for this cell type to be incorporated in the explants used at the time of culture preparation appeared to be very good since many of these medullary neurons were quite diffusely arranged in tissue sections and were frequently situated close to the cortex. In culture, these neurons were generally larger than Purkinje cells and had multiple dendritic processes originating from the surface of the perikaryon. They were also slightly more argyrophilic than the Purkinje cells. Both cell types stained moderately with methylene blue. Neurons with similar features in tissue culture have been described as large motor neurons of the cerebellar nuclei (24, 66) and as Purkinje cells (56).

The characteristics of the granule cell in canine tissue culture were compatible with the findings of several investigators (48, 56). This cell stained quite intensely with methylene blue; was markedly argyrophilic; had a typical appearance with phase microscopy; and had various shapes, one of which was a very characteristic sperm-like form. In contrast to the present results, and other reports (48, 56), Hild (24) concluded that granule cells were rare in cultures of neonatal kitten and rat cerebellum. He also did not consistently detect cells of the stellate or Golgi
types. Hild's investigations were based on the use of phase microscopy and silver impregnation; he did not mention using methylene blue. A separate view regarding the morphologic characteristics and location of the granule cell in culture was held by Wolf (66). His description of the granule cell following silver impregnation closely resembled the focal enlargements of neuronal processes which were detected in the present investigation. There was a marked similarity between the two descriptions with regard to the shape, degree of impregnation, number and location.

It has been proposed that cells having the features described in this study for granule cells originate from the outer embryonic granular cell layer of the cerebellum (48). Cells from this layer in newborn puppies are larger and have greater mitotic activity than those from the inner granular cell layer. This would tend to support the proposal advanced above. Cells of the inner granular cell layer have also been described in tissue culture of cerebellum and their morphologic characteristics resembled those present in stained brain preparations (48). Cells with similar appearance and behavior were occasionally detected in both the explant and outgrowth during the present investigation. They had no processes and usually underwent degeneration after a few days of culture growth. These cells were, however, carefully considered in the identification of oligodendroglia which had some similar features.
The last neuronal type varied in its morphologic features and included both the small and large stellate cells. They characteristically stained intensely with methylene blue and apparently impregnated weakly with silver since none were recognized with this method. The majority of these cells had a relatively large perikaryon and prominent processes. No definite axons were detected. These features were similar to the Golgi I neuron of the cerebellar cortex (32, 33). Other cells in this group had characteristics identical to those described by Pomerat and Costero (36) which they identified as stellate cells. These had fine parallel processes that extended in opposite directions from a relatively small, angular perikaryon. Cells demonstrated by these authors as Golgi II neurons were also occasionally found.

One basic principle regarding the interpretation of neurons in culture deserves special emphasis. The cortex of the neonatal canine cerebellum used for tissue culture purposes in this investigation was still in the process of development. The Purkinje cells, the Golgi cells, and the inner granular cell layer had not fully matured at this time. Likewise, the embryonic granular cell layer was prominent and contained a relatively high mitotic index. During the maturation process following birth, when this cell layer is gradually lost, it participates in the development of neurons of the molecular layer (stellate cells) and in the formation of the inner granular cell layer (32, 33). The ventricular matrix apparently is, however, the main source of cells of this latter layer (33). Considering these factors and the fact that cells
cultivated in vitro generally tend to be less differentiated than their counterparts in vivo, it was not surprising that neurons were sometimes difficult to identify. Neuronal differentiation in tissue culture was attained to the highest degree by the Purkinje cells and the large medullary neurons which corresponded to the situation in the cerebellum at the time of explantation. Granule and stellate cells tended to be less well differentiated in culture which also undoubtedly reflected their stage of development at the time of culture preparation.

No definite conclusion could be reached regarding the nature of the local areas of weak impregnation on neuronal processes.

Enlargements of neuronal processes occurred in the majority of the cultures containing Purkinje cells and other large neurons. Most of these enlargements were round to oval in shape but various other formations were also detected. Similar appearing structures have been reported as axonal swellings in tissue culture of the cerebellum of kittens and newborn rats (24) and as granule cells in cerebellar explants of neonatal mice (66). Hild (24) described these structures as axonal swellings, or varicosities, but gave no explanation for their existence. He recognized their fibrillar appearance and found them most frequently located along the Purkinje cell axon a short distance from the cell body. Bielschowsky (3) reported enlargements of Purkinje cell axons in association with a number of diseases in man which included multiple sclerosis, juvenile paralysis and amaurotic idiocy. A glial
obstruction to normal axonal growth was proposed as the cause of
the abnormality. Lateral branching of processes from the enlarge­
ments also were reported and the author considered the whole
phenomenon to be a regenerative function on the part of the axon.

Cajal (11) also described this manifestation in a thorough
discussion of degenerative phenomena related to cerebellar
traumatisms. Following the production of experimental lesions,
which severed axons of Purkinje cells in young dogs and cats, he
described necrotic, degenerative and metamorphic changes of the
proximal segment. Following necrosis at the point of trauma,
axons became degenerated up to the level of collateral branching,
often to the most proximal one. In the absence of collaterals,
the entire axon could become involved with the eventual existence
of an isolated cell body. During degeneration, varicosities oc­
curred along the length and at the end of the affected axonal
segment.

After degeneration and disappearance of the extracollateral
axon, Cajal described a hypertrophy, or increase in diameter, of
the remaining collaterals and the axonal segment proximal to them
(metamorphic change). This thickening involved the axon up to a
point of constriction, which usually occurred adjacent to a node
of the myelin sheath near the cell body, and extended distally
along the collaterals to the place of secondary branching. An
affected collateral often coursed back toward the perikaryon which
gave the entire structure a U-shaped appearance.
True regenerative phenomena were never detected by Cajal in the metamorphic part of the axon and collaterals of the Purkinje cell or in other cerebellar neurons. A regenerative process was considered to occur but was concluded to involve only the internal environment of axons and their ramifications. Cajal did rarely detect indications of new branches, which were rudimentary in character and eminated from thickened processes, but he remained uncertain whether these were true growths or pre-existing collaterals.

The various manifestations of process involvement described by Bielschowsky (3) and particularly by Cajal (11) in cerebellar sections were compatible with the findings in cerebellar cultures during the present investigation. In the majority of preparations the changes greatly resembled the stage of metamorphosis, or compensation, as defined by Cajal. If this were the case then the majority of the thickened neuronal processes in vitro represented a reconstructed axon; one composed of the original proximal segment but with a new distal portion (previous collateral). It would also be necessary to interpret the growth of neuronal processes as an extension of preexisting collaterals and not a regeneration "de novo" on the part of the originally traumatized axon. The significance of the above change in tissue culture upon axonal behavior, and even myelin formation, could not be determined but it was obvious that it should be considered in interpreting in vitro results of neuronal development and activity.
Summary

Explants of canine cerebellum were successfully grown directly on glass coverslips without the use of any adhesive material such as collagen or plasma clot. The outgrowth surrounding the explant consisted predominantly of astrocytes along with smaller numbers of oligodendroglia, macrophages, fusiform mesenchymal cells and neurons. Oligodendroglia had small perikarya and fine, delicate processes that were often attached to astroglial fibers. This cell began to disappear from the outgrowth after one to two weeks. Macrophages, which were first detected after three to five days, were large, round and often had a vacuolated cytoplasm. Transformation into rod-shaped microglia was also detected, usually peripheral to the outgrowth on the glass. Fusiform, mesenchymal cells most frequently occupied a position peripheral to the astroglial outgrowth and occasionally completely surrounded it. These cells were replaced by large polygonal astrocytes after three to four weeks.

Four general types of neurons were identified. These included granule cells (originating from the outer embryonic granular layer); small and large stellate cells, which included Golgi cells; large neurons of the cerebellar medullary nuclei and Purkinje cells. Granule cells and stellate cells were found in the explant and outgrowth and stained intensely with methylene blue. The former cell type also had a strong affinity for silver impregnation. Neurons identified as originating from cerebellar nuclei were larger than Purkinje cells, had a greater argyrophilia
and possessed multipolar dendrites. Both cell types were detected only within the explant. Purkinje cells remained well differentiated throughout growth in vitro.

The axonal and collateral processes of Purkinje cells, and the other large neurons, frequently were thickened or had focal bulbar enlargements. This change was concluded to represent a compensatory process resulting from the axonal trauma encountered when the tissue was prepared for explantation.
References


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CHAPTER II

DEVELOPMENT AND CHARACTERIZATION OF MYELIN IN TISSUE CULTURE OF CANINE CEREBELLUM

Introduction

The development of myelin in vitro was first observed in tissue cultures of peripheral nervous tissue by Peterson (43) who detected myelinated axons in explanted dorsal root ganglia from the chick embryo. Hild (20) first described in vitro myelination of central nervous tissue in cultures of kitten cerebellum. Since these investigations many other reports concerning the growth and maintenance of myelin in tissue cultures of both the central and peripheral nervous system of various animal species have appeared in the literature (3, 6, 7, 8, 21, 22, 23, 32, 33, 34, 35, 38, 39, 44, 45, 47, 53, 54).

The formation of myelinated axons in cultures of canine cerebellum has been reported by Hild (21) and Perier (38). They both described the formation of the myelin sheath as being continuous without segmentation. A similar observation was made by Bornstein and Murray in cultures of rat and cat cerebellum (8). Hild considered the axon to be instrumental in the formation of the myelin sheath, whereas Perier implied that glial cells also participated. During the present study, when myelinating explant cultures of canine cerebellum were grown in order to provide an
in vitro model for future investigations relating to the study of
demyelinating canine distemper encephalitis, segmentation of the
myelin sheath with the formation of nodes was consistently detected.
Since this finding has not been previously reported in myelinated
cultures of the dog cerebellum and has received only limited
attention in cultures of central nervous tissue of other species
(34, 39, 44), a detailed account of this development in tissue
culture was considered warranted.

A modification of previous methods for growing myelinating
tissue cultures was employed during the present investigation.
This was done in order to satisfactorily study stained preparations
infected with the canine distemper virus (50).

The objectives of this study were to evaluate a modified
method for growing myelinating tissue cultures of canine cere­
bellum, to compare the manner of myelin development with earlier
reported descriptions and to further characterize the morphological
features of the myelin sheath, especially the characteristics of
segmentation, in tissue culture.

Materials and Methods

The culture method used in this study has been reported
(49). It consisted basically of growing explants of canine cere­
bellum on glass coverslips in Leighton tubes. Culture examinations
were made with bright and polarizing light microscopy in Leighton
tubes and in Maximow coverslip assemblies as previously described
(49). Time-lapse cinematographic recordings were also made of
selected myelinated cultures.
The stains employed for the visualization of myelinated axons included the methylene blue supravital stain (49) and Baker's acid hematein phospholipid method (14). Wolf's modification of the Holme's silver nitrate technic (53) was used to selectively impregnate neuronal processes.

Results

Myelinated axons were regularly detected in 60 to 75 per cent of prepared cultures after approximately 17 to 28 days of growth. Isolated instances of myelination were detected, however, as early as seven days of culture growth. Occasionally a group of cultures prepared from a common tissue source had a lower percentage of myelination (20 to 30 per cent). The reason for this variation was undetermined.

Thin segments of myelin, which varied in length, appeared abruptly during the second to third week in the culture. These sheaths were often difficult to detect, even at 430 times magnification. After about three to four weeks of growth, myelinated axons had increased in size and could usually be detected by examining the explants in Leighton tubes with 100 times magnification. They were most easily demonstrated with bright light microscopy and appeared as two relatively straight parallel lines which sometimes were several hundred microns long (Fig. 17). Interruptions in the sheath could also be detected in living cultures but they were most easily demonstrated in preparations stained for myelin. Myelin could, in addition, be identified with
Fig. 17. Twenty-eight-day-old explant culture of canine cerebellum demonstrating several living myelinated axons (J885-33). Bright light microscopy; x 430. Note the random path that the axons take in culture and the two dense, parallel lines that characteristically outline the limits of the myelin sheath. Segmentation of the myelin sheath cannot be detected in this photograph.

Fig. 18. Seventeen-day-old explant culture of canine cerebellum showing axonal enlargements (bulbs) which are covered with a myelin sheath (J1223-46). Baker's acid hematein method; x 1250. The myelin sheath over the enlargements is continuous with the adjoining narrow axonal segments. The myelin sheath sometimes terminates at the proximal end of the bulbs (arrows) when they are located near the perikaryon.
polarized light and the degree of birefringence increased with the thickness of the sheath.

The myelin sheaths appeared in the best state of development in cultures that contained minimal necrosis and which had a dense, uniform, glial, tapered zone immediately surrounding the explant (49). The myelinated axons were most frequently detected in this glial mat but also occurred within the explant itself. They were usually oriented parallel with the edge of the explant but occasionally were seen to run perpendicularly to the border of the glial mat and then abruptly turn back. Axons possessing a myelin sheath were never seen to extend beyond the tapered zone.

With advanced age, or in cultures containing an excess of necrotic tissue, the myelin sheaths frequently became irregular and often had out-pouchings or distensions of various size. This morphologic feature was similar to reports made by others (8, 20, 21). Also, large focal distensions of otherwise normal-appearing myelin sheaths occurred (Fig. 18) which resembled the bulbous enlargements described previously with silver impregnation (49).

The manner in which the myelin sheath developed with respect to position along the length of the axon was primarily investigated with methylene blue-stained preparations. This method had the advantage of staining both the axon and myelin sheath. In most instances the myelinated portion of the axon was considerably thicker than the unmyelinated part. This enabled the extent of the sheath to be accurately determined. In cases when the myelin covering was thin and difficult to define after staining,
the supravital process was followed microscopically by exposing the culture to methylene blue in a Maximow slide assembly. The stained, myelinated strands were not consistent in their position along the axon. Some occupied the central part of the axon while others were located closer to the cell body. The sheaths occurred as segments that were separated by unmyelinated areas of varying lengths. In no instance did the perikaryon and myelin sheath make contact. Also, the terminal part of the axon always remained unmyelinated.

Myelination was limited to axons of the Purkinje cell in all cases in which the associated perikaryon could be identified. The Purkinje cell was the most frequently occurring large neuron in canine cerebellar explants but other large neurons, which were considered to have originated from the cerebellar medullary nuclei, also occurred (49). The axons of these latter cells are capable of myelination in vivo (24, 25) and the possibility of their participation in the myelinating process in vitro could not be excluded.

A feature that was detected consistently in all myelinated cultures was the occurrence of unmyelinated areas which interrupted the continuity of myelin sheaths. Many of these areas greatly resembled the nodes of Ranvier of the peripheral nervous system (27, 32, 33, 34, 45) while others were much longer. They were first observed in living explants with bright light microscopy and later clearly demonstrated in cultures stained for myelin (Fig. 19, 20, 21). The unmyelinated areas of the sheath differed considerably in length and ranged from 0.5 to 70 microns. The mean length
Fig. 19. Seventeen-day-old explant culture of canine cerebellum depicting a myelin sheath with two nodes and a short internodal segment (J1223-46). Baker's acid hematein method; x 1250. A faint line between the left node (arrow) illustrates the gradual reduction of the myelin sheath.

Fig. 20. Seventeen-day-old explant culture of canine cerebellum illustrating an increase in phospholipid at the prenodal ends of the myelin sheath (arrows) (J1223-46). Baker's acid hematein method; x 1250. Observe the gradual thinning of the myelin sheath as it extends into the node from the right side.
Fig. 21. Seventeen-day-old explant culture of canine cerebellum demonstrating a branching of a myelinated axon (J1223-46). Baker's acid hematein method; x 1250. A node is present on the main axon and at the point of branching. A gradual reduction in density of the myelin sheath can be seen at both nodal areas (arrows).
of these areas along different axons, however, varied between 2 and 6 microns. Many axons had more than one such area (Fig. 19). Seven were counted along one myelinated sheath. There was a general tendency for axons larger than 175 to 200 microns to have two or more interruptions of the myelin sheath.

The myelinated internodal segments had no particular pattern regarding length. Segments as small as 4 microns and as large as 270 microns were detected. Also, internodal differences of 20 to 75 microns along any one particular axon were common. There appeared to be no relationship between internodal length or number of nodes and the diameter or length of the myelinated axon. However, there tended to be a negative correlation between axonal diameter and the length of unmyelinated intervals.

At typical nodal formations, myelin sheaths usually became somewhat narrower in diameter than in the middle of the internodal segment (Fig. 19, 21). Also, at this junction there sometimes was a focal increase in the amount of phospholipid (Baker's acid hematein method) (Fig. 20). The myelin sheath did not always stop abruptly at the node. There often was a gradual thinning of the sheath into the nodal area (Fig. 19, 20, 21). This was seen with bright light microscopy as well as in stained preparations. Occasional branching of myelinated axons was detected (Fig. 21). The sheath of the main process was interrupted by a node at the point of branching.

Time-lapse cinematographic recordings showed myelinated axons to generally be less active than other cell processes in the
culture. There were two instances, however, when increased axonal movement was detected. One involved a peristaltic-like action or a longitudinal movement of the axoplasm of myelinated axons containing swellings or varicosities. A second type of movement was detected on only one occasion as a rhythmic axonal pulsation. This occurred in two different myelinated axons which were situated side by side in a 28-day-old culture. The photographic recording was made over a period of 2 hours and 27 minutes at 37 C. Short lengths of the axon participated in the activity. The pulsations were regular and, after being speeded-up by film projection, consisted of a rapid reduction in the axonal diameter which frequently brought the two lines representing the myelin sheath together. The actual time of pulsation was about four minutes. Subsequent time-lapse cinematographic recordings of cultures of comparable age failed to reveal a similar activity.

Discussion

Myelination of axons was successfully accomplished by the method employed for growing canine cerebellar explants in vitro (49). In most cases 60 to 75 per cent of the cultures myelinated but periodically a much lower percentage of myelination occurred. The reason for this variation was not determined.

The time required for development of myelinated axons in tissue culture of canine cerebellum ranged from 17 to 28 days. This was similar to the findings of other investigators who cultured cat, rat and dog cerebellum (8, 20, 21) and rat spinal
cord (44). In comparison, the mouse cerebellum appeared to require less time to myelinate in vitro (53).

A general impression was obtained concerning the manner of myelin formation in vitro by examining living cultures and using the methylene blue supravital method. During early myelination thin segmented sheaths appeared suddenly in culture and often occupied the central or proximal part of the axon. With advancing age more axons became myelinated and the thickness of myelin sheaths increased. Whether the myelin sheath also extended longitudinally along the axon with age could not be definitely determined but there was a suggestion of this occurring. The observation, with myelin stained preparations, that long intervals often separated myelinated segments in thin (young) axons tended to support this idea.

Neuroglial activity could not be evaluated during the myelinating process because of the extreme density of the explant. It is generally accepted, however, that the myelin sheath develops in vivo and in vitro from a mutual participation between neuroglia and the nerve process (31, 40, 41, 42, 47). The participating glial cell is widely considered to be the oligodendrocyte (9, 15, 26, 30, 48). Some, however, have suggested a similar myelin-forming activity for the astrocyte (2) or for an atypical glial cell associated with remyelinating axons following demyelination (10, 13). Hild (20, 21) interpreted the myelin sheath in tissue culture as a product of the axon itself without any oligodendro-glial participation. This conclusion was based upon the finding
that sheaths were sometimes seen in areas of the culture where no contiguous oligodendroglia could be recognized. Astrocytes, adjacent to the sheath were detected, however, but these cells were not considered to be engaged in the process of myelination. Hild (22) later concluded that a certain minimum concentration of various glial elements around an axon appeared to be a necessary condition for the development of myelin in vitro. He also felt that the axon itself participated in the process. Yonezawa and co-workers (54) implied that oligodendroglia were responsible for the myelination of rat cerebellum in vitro since their biological activity coincided with the development of myelin sheaths. Other investigations of tissue culture of the central nervous system have indicated a relationship between glial cells and the myelin sheath (3, 7, 8, 39, 47).

Schwann cells have conclusively been demonstrated to participate in the formation of myelin in cultures of peripheral nervous tissue (spinal ganglia). Murray (32, 33, 34) reported that the spiral myelin sheath was formed following a rotation of the Schwann cell about the axon. Also, the different internodal segments along nerve processes appeared to develop myelin independently of each other.

The existence of nodes along myelinated axons of the central nervous system is well established. These structures were first described by Tourneux and Le Goff in 1875 (51) but have been associated with the name Ranvier more frequently than with the original discoverers. Moreover, Ranvier actually disputed the
presence of such nodes in the central nervous system (4). Several other early investigators, including Cajal (4, 11), later confirmed the findings of Tourneux and Le Goff. For a review of the literature to 1928 see Bielschowsky (4). More recently, light and electronmicroscopic studies of nodes in the central nervous system have been reported (1, 5, 10, 12, 16, 17, 18, 19, 28, 30, 31, 36, 37, 40, 41, 48, 52).

There was an early lack of agreement concerning the formation of myelin in tissue culture of the central nervous system. Hild (20), who first described myelin development in tissue cultures from the central nervous system (cat cerebellum), reported that myelin first occurred along the axon in small segments which later developed into a continuous uninterrupted sheath without the formation of nodes. This same observation (lack of segmentation) was later made by Hild (21) and Perier (38) in cultures of canine cerebellum. In contrast to Hild, Bornstein and Murray (8) indicated that myelinated axons in cultures of the rat and cat cerebellum abruptly appeared without first forming small segments. The myelin sheaths were described as being continuous and uninterrupted. These results did not agree with the findings of the present study in which segmentation was detected. More recently reports have been made of nodes occurring in tissue cultures of cat (39) and rat (34, 39) cerebellum and rat and mouse (34, 44) spinal cord. In none of these tissue culture studies, however, has a detailed descriptive and comparative account of these structures, and the intervening internodal segments, been given.
In the present investigation unmyelinated nodal areas were detected in all myelinated cultures examined. The nodes were comparable to those illustrated in tissue culture and sections of central nervous tissue. In addition unmyelinated segments were observed that exceeded the length of those described in the above reports. The measurements cited by these investigators using brain sections generally varied from 1.5 to 7 microns. In canine cerebellar tissue cultures the mean length of unmyelinated intervals in different axons ranged between 2 to 6 microns, but extremes of 0.5 to 70 microns were also detected. It was difficult to establish what length of interruption constituted a node. There were no important morphological differences, except size, between long and short unmyelinated intervals.

No definite conclusion could be reached concerning the prenodal increase in phospholipid as detected in tissue culture. The separation of the myelin lamellae and the formation of terminal loops containing cytoplasm at this part of the sheath, however, may in some way be related to this finding. Also, the gradual fading of the myelin sheath at the node, as detected in both living and stained tissue culture preparations, corresponded well to the ultrastructural illustrations of this area (10, 31, 37, 39, 40, 52).

The characteristics of the internodal segments did not completely agree with the studies of others using sectioned material of the central nervous system. For example, there was no correlation between the internodal length and diameter of the axon
as was reported for mammals by Bodian (5) and Hess and Young (17). This relationship is known to be characteristic for the peripheral nervous system (27). The finding by Hess and Young (19) of internodal lengths as low as 200 microns in new-born rabbits were comparable, however, and might be more applicable to the present study since young dogs were used for culture. Hess (16) concluded that a correlation between internodal length and fiber diameter should not necessarily be expected in young animals since internodal segments increased considerably in length with age. This was especially true for nerve processes that attained a large diameter at maturity. Internodal lengths, similar to those in the present study, were also reported in sectioned material by Cajal (4) in man (150 to 240 microns), by Nakai (36) in young kittens and adult rats (200 microns) and by Maturana (31) in the optic nerve of adult Anurans (60 to 150 microns). In tissue cultures of rat spinal cord, continuous myelin strands of 10 microns to over one millimeter were found (44). Also, as in the present study, nodes occurred at very irregular intervals.

The recorded axonal activity that showed peristaltic-like action or longitudinal movement of various varicosities was comparable to the observation made by Hild (20, 21). He detected similar configurations in both living and stained preparations and concluded that they represented axonal expansions. With time-lapse cinematographic studies the swellings or herniations underwent constant changes with respect to their shape and location. Perier (38) illustrated structures with similar characteristics.
Bornstein and Murray (8) stated that slight irregularities or expansions of myelinated axons occurred, shifted in position along the axon and even sometimes disappeared. They also considered that large distortions of the myelin sheath were indicative of degeneration. In the present investigation the development of herniations or swellings of the myelinated axon frequently occurred when the culture was subjected to noxious influences (explant necrosis, intense prolonged light) or often after advanced culture age. Large focal enlargements also were occasionally detected along otherwise normal myelinated axons. These findings indicated that the irregular formations of neuronal processes might represent a compensatory response to injury encountered at the time of explant preparation (49) or later during culture growth.

The significance of the rhythmic axonal pulsation, which has not previously been reported by others, could not be evaluated. The length of the pulsations approximated those reported for oligodendroglia (29) but whether or not any relationship existed is speculation. It has been postulated that the axon of the peripheral nervous system (in vivo) may constrict at points along its length during the process of myelination in order to facilitate the spiraling of the developing myelin sheath (46). A related process might also be expected to occur in vitro. It must be emphasized, however, that the great majority of the myelinated axons photographed during this study had very low activity.
Summary

A modified method, in which explants were grown directly on glass coverslips in Leighton tubes, was employed for growing myelinated axons in tissue cultures of canine cerebellum. Myelination occurred in 60 to 75 per cent of the prepared cultures.

Myelin suddenly appeared after 17 to 28 days in the form of thin segments which were variable in length. The myelin sheaths developed along the central or proximal part of the axon and became thicker with age. No conclusions could be reached regarding the participation of neuroglia in the process of myelination since the culture density was too great.

Nodes similar to nodes of Ranvier of the peripheral nervous system were detected in all myelinated cultures. They varied considerably in length with the mean ranging from 2 to 6 microns. The internodal length was variable and independent of the axonal diameter or length.

The movement of myelinated axons, as observed by time-lapse cinematography, was low but varicosities, which frequently occurred when cultures were subjected to noxious influences or after advanced culture age, produced a peristaltic-like or longitudinal movement of the axoplasm. A second, less common type of axonal activity was characterized by a rhythmic segmental pulsation of the axon.
References


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CHAPTER III

THE EFFECTS OF CANINE DISTEMPER VIRUS ON EXPLANT TISSUE CULTURES OF CANINE CEREBELLUM

Introduction

Explant tissue cultures of the central and peripheral nervous system have been employed in the study of several disease processes. Most of this research has centered around the investigation of immunologic or toxic factors associated with demyelination (1, 5, 6, 7, 8, 9, 46, 51, 52, 65). In contrast, relatively little attention has been given to the effects that infectious agents have on nervous tissue cultured in vitro. Fernandes and Pomerat (18) described a neuronotropic effect of fixed rabies virus on explant tissue cultures of canine and feline cerebellum. Hogue (28) also reported a specific affinity of poliomyelitis virus for neurons of cultured human central nervous tissue. Other investigations involving cultures of nervous tissue include studies of scrapie (19, 27), hog cholera (25, 26), PPLO (53), and Hemophilus pertussis (17).

The cultural characteristics of the canine distemper virus have been studied in several tissues grown in vitro (3, 12, 13, 14, 21, 22, 24, 29, 36, 50, 58, 59, 61). Investigation of this virus in cultures of the central nervous system, however, has been limited. Klapötke (37) employed explants of embryonic mouse brain
to maintain the distemper virus serially through 26 passages over a seven-month period. He gave no description of a cytopathic effect (CPE) in the cultured cells. Gibson (21) studied the effects of the Lederle and Snyder Hill strains of canine distemper on trypsinized cultures of whole canine brain. He found viral effects 30 days following inoculation with both strains of the virus. Cytopathic changes, which consisted of inclusion body formation, plaques of cell degeneration and syncytium formation, were similar to those reported in cultures of extraneural tissues (3, 12, 13, 14, 58, 59, 61).

In order to have a convenient and controllable in vitro model for the study of the virus associated with demyelinating canine distemper encephalitis, explant tissue cultures of canine cerebellum were used in the present investigation. The objectives of this study were to determine the value of this tissue culture method as an in vitro model for the investigation of canine distemper, and to compare the effects of the distemper virus on cultured cerebellar explants with brain lesions of the disease in the dog.

Materials and Methods

The tissue culture method used has been previously described (60). It consisted basically of growing explants of canine cerebellum on glass coverslips in Leighton tubes.

The Lederle strain of canine distemper virus was used and was originally obtained from the spleen of an infected disease-free dog (21). The virus was subsequently passaged two times in
trypsinized, monolayer cultures of whole dog brain after which it caused CPE 15 to 20 days post-inoculation. During the later phases of the study the virus was passaged for a third time in order to obtain a more rapid CPE. The time required for CPE was reduced to eight days.

The culture procedure used for serial passage consisted of inoculating monolayer cultures prepared from whole brains of dogs under one week of age. The brain was minced and trypsinized (0.25 per cent in Hank's balanced salt solution—BSS) for 30 to 45 minutes in a magnetic mixer at room temperature. The cell suspension was passed through sterile gauze, centrifuged at 90 g for six minutes and resuspended in Hank's BSS. After a second centrifugation the cells were suspended in complete medium and quantitated (200,000 per ml). The complete medium consisted of a 0.5 per cent solution of lactalbumin enzymatic hydrolysate in Hank's BSS, 89 per cent; calf serum, 10 per cent; NaHCO₃, 1 per cent from an 8.8 per cent stock solution; penicillin, 200 units per ml; and streptomycin, 100 mg per ml. Twenty ml of cell suspension were added to each 100 ml prescription bottle used for preparation of harvest material and 2 ml to Leighton tubes containing coverslips, which were employed for staining and evaluating culture results. Cultures were inoculated when a monolayer formed after four to five days. The viral inoculum consisted of a 1:1 ratio of canine distemper virus (10 per cent splenic suspension or tissue culture harvest) in Hank's BSS. Uninfected tissue culture harvest was diluted in the same manner for inoculating control
monolayers. After an incubation of one and one-half hours at 37 C, maintenance medium was added (Lactalbumin hydrolysate in Hank's BSS, 94 per cent; calf serum, 5 per cent; NaHCO₃, 1 per cent from an 8.8 per cent stock solution; penicillin, 200 units per ml; and streptomycin, 100 mg per ml). Cultures were transferred (using 0.25 per cent trypsin) each week until the establishment of CPE. At this time the cells were suspended in their media by scraping them off the glass. The suspensions were stored as a harvest pool at -70 C until ready for inoculation of explant cultures of canine cerebellum. Companion harvests of uninoculated cultures were also stored in the same manner for use as control material.

Cerebellar explants were inoculated with virus after four to seven days of growth. The inoculums (control and infected) were prepared by suspending the stored tissue culture harvest in Gey's balanced salt solution (BSS) at a ratio of 1:1. After removal of all medium from each Leighton culture tube and the addition of 0.3 ml of the inoculum, the cultures were placed at 37 C for one and one-half hours. The inoculum was then removed and replaced by 0.3 ml of complete medium which consisted of Gey's BSS, 35 per cent; fetal bovine serum, 40 per cent; bovine serum ultrafiltrate, 25 per cent; and glucose, 600 mg per cent. The cultures were examined daily with bright light microscopy and periodically with phase microscopy as previously reported (60). Cultures, selected at various times following inoculation, were also stained by the May-Grünwald-Giemsa method (43) and impregnated
according to the Holme's silver nitrate technic as modified by Wolf (66).

Immunofluorescent studies were made on cultures at different times post-inoculation by using the direct method. The globulin used for conjugation was precipitated from canine serum with one-half saturated \((\text{NH}_4)_2\text{SO}_4\) and then resuspended in distilled water. The serum originated from dogs that were hyperimmunized with distemper virus from the same pool initially used for culture inoculation (splenic suspension). The protein content of the precipitate was determined by the biuret method after which it was conjugated with fluorescein isothiocyanate at the rate of 0.03 mg of conjugate per mg of protein. Unconjugated fluorescein isothiocyanate was removed by passage through a Sephadex column.\(^1\)

Control and inoculated coverslip cultures of canine cerebellar explants were washed in phosphate buffered saline (pH 7.2), air-dried and fixed in acetone at -70°C for 15 minutes. The cultures were subsequently air-dried, flooded with conjugated serum and incubated at 38°C for 30 minutes. The coverslips were mounted in glycerol adjusted with phosphate buffered saline (pH 7.2) and examined with a Leitz ortholux microscope which was equipped with an ultraviolet light source (Osram HB 200) and a UG1 filter.

Results

The first cells to become affected following virus inoculation were the large polygonal astroglia and fusiform mesenchymal

\(^1\)Pharmacia, Uppsala, Sweden.
cells in the peripheral outgrowth surrounding the explant. The initial sign of infection was the occurrence of small, granular intracytoplasmic inclusion bodies. The inclusion body formation occurred eight days post-inoculation when the inoculum passaged three times was used. Since the sequence of events that occurred in infected cultures was the same regardless of the passage used for inoculation, all time intervals mentioned will represent those following the use of this third passage. By the 10th to 12th day the intracytoplasmic inclusions had become larger and were variable in shape. Some were oval while others were slender and irregular. Also, at this time syncytial formations (giant cells) were first detected in focal areas at the periphery of the outgrowth (Fig. 22, 23). Both astroglia and mesenchymal cells participated in the giant cell development.

Changes in the more centrally located outgrowth had also occurred by the 12th to 14th day in infected cultures. The cells in the outgrowth, which normally appeared as a sheet of closely packed protoplasmic astrocytes, became more fibrous in character. The perikarya were reduced in size and the processes were prominent. This gave the area a reticulated appearance (Fig. 24). There was also a piling-up of cells, especially in the periphery of the outgrowth, which was contributed to largely by the formation of syncytia. Inclusion body formation was still predominantly confined to cells in the peripheral outgrowth.

Fifteen days post-inoculation, syncytial giant cells occurred in most all areas of the peripheral outgrowth. These
Fig. 22. Twenty-eight-day-old explant culture of canine cerebellum (23 days post-inoculation) illustrating the formation of syncytia of astrocytes (arrows) in the peripheral outgrowth (J2315-26). May Grünwald-Giemsa stain; x 32.

Fig. 23. A higher magnification of Figure 22 (upper right) showing two affected astrocytic cells; x 315. The syncytial giant cell on the left contains seven nuclei with varying stages of inclusion body development. Also, observe the variation in size and shape of intracytoplasmic inclusions in both cells (arrows).
Fig. 24. Forty-three-day-old explant culture of canine cerebellum (36 days post-inoculation) demonstrating the reticulated pattern detected in infected cultures (K201-70). Wolf's modification of Holme's silver impregnation; x 50. Several syncytial formations can be seen in the outgrowth (arrows) with a contracted, degenerated one also present (large arrow). The fibrous astrocytes throughout the outer three-fourths of the outgrowth have contracted perikarya and prominent processes.

Fig. 25. Twenty-eight-day-old explant culture of canine cerebellum (23 days post-inoculation) illustrating the early stages of intranuclear inclusion body formation (black arrows) and one large cytoplasmic inclusion filling most of the cell (white arrow) (J2315-26). Living culture, phase microscopy; x 640. The fine granular material of early inclusion body development is arranged in a definite pattern within the nucleus. The spherical, dark nucleoli are separated from the inclusion material and are often pushed peripherally with large inclusion formation (see Fig. 26).
formations were mainly composed of astrocytes since most of the mesenchymal cells were by this time replaced by astrocytic growth. The early stages of intranuclear inclusion body formation were also detected in these cells and in isolated astrocytes located in this area (Fig. 25).

The development of inclusion bodies in the cytoplasm, and subsequently in the nuclei of astrocytes in the inner outgrowth extending to the explant, followed the above changes. Syncytial giant cells were also present. The reticulated nature of this zone, as well as the piling-up of cells, increased slightly with time following inoculation. Throughout the outgrowth, individual or groups of astrocytes had signs of degeneration (Fig. 24). These included condensation and/or obliteration of the processes and perikarya, and nuclear pyknosis. Many of these cells contained either intracytoplasmic or intranuclear inclusions, or both. One characteristic feature of infected cultures was, however, that the majority of the cells did not rapidly degenerate and die but appeared capable of co-existing with the virus for an indefinite period of time. This was even true for cells that had both intracytoplasmic and intranuclear inclusion bodies.

By the 28th post-inoculation day practically all of the glial cells in the outgrowth and explant had inclusions. Many typical astrocytes in the outgrowth had inclusions only in the nucleus (Fig. 26). Intranuclear inclusions of glial cells were especially well demonstrated in the dense part of the explant with silver impregnation since the inclusion material did not impregnate
Fig. 26. Fifty-seven-day-old explant culture of canine cerebellum (52 days post-inoculation) showing the marked intranuclear inclusion body formation in astroglia located in the outgrowth (J1007-160). May-Grünwald-Giemsa stain; x 315.

Fig. 27. Forty-eight-day-old explant culture of canine cerebellum (36 days post-inoculation) demonstrating the high percentage of glial nuclei within the explant that contain inclusion bodies following virus inoculation (J1007-14). Wolf's modification of Holme's silver impregnation; x 500. The glial nuclei appear black with the centrally located inclusions being unimpregnated and light in color.
and appeared light in color (Fig. 27). Giant cells also occurred at this time in the explant.

Degeneration and necrosis of glial cells in both the explant and outgrowth continued to increase with the duration of infectivity but many cultures were maintained for as long as 52 days following inoculation without extensive cell destruction.

The manner in which inclusion bodies developed in the nucleus and cytoplasm differed. Cytoplasmic inclusions first occurred as small individual aggregates that were homogeneous and dense with stained preparations. They rapidly became larger; some almost completely occupied the cytoplasmic portion of the cell (Fig. 23, 25). Whether this was the result of a fusion of the smaller particles could not be determined. The inclusions stained basophilic to eosinophilic with the May-Grünwald-Giemsa stain.

In contrast, intranuclear inclusions first appeared as an accumulation of very fine particles (Fig. 25). These particles became larger and more prominent with age and eventually appeared to fuse into one dense mass. The fine structure of this development could regularly be detected with phase microscopy but only occasionally with stained preparations. With May-Grünwald-Giemsa preparations the early stages of development usually appeared as faint basophilic bodies which later became more intensely blue (Fig. 23, 26).

The effect of the distemper virus on oligodendroglia and macrophages (or microglia) could not be properly evaluated. Oligodendroglia had largely disappeared from the culture by the
second week, coinciding with the occurrence of CPE. This cellular behavior has been described in tissue cultures of the central nervous system in other reports (2, 46, 47).

Macrophages were still present at the time of the CPE but their numbers had decreased. The presence of macrophages in normal and infected cultures was largely dependent upon the amount of open space that was present in the cell sheet surrounding the explant. Since the majority of cultures developed a densely cellular outgrowth of astroglia, macrophages gradually decreased in number and were located mainly on the glass at the periphery. For this reason this cell type could not be adequately studied in the majority of infected cultures.

The study of large neurons in infected cultures was mainly done with silver impregnated preparations. The May-Grünwald-Giemsa and methylene blue stains were also used in interpreting the changes in granule cells. Differences between control and infected cultures were first detected in the large neurons (Purkinje cells and neurons of the medullary nuclei) during the third to fourth week following inoculation. At this time infected cultures had fewer axons and perikarya. They also had more evidence of neuronal degeneration. Degenerative changes in neurons were manifested in two ways. One was an increase in the argyrophilia of the perikarya and dendrites with an accentuation of the neurofibrils. The nucleus was usually eccentric and sometimes greatly distended by vacuolation. This type of degeneration was most frequently associated with the large neurons considered to originate from the
cerebellar nuclei and in Purkinje cells of older cultures. A second type of degeneration predominantly involved the Purkinje cell before distinct neurofibrils were detectable. This consisted of a shrinking or, in some cases, a swelling of the perikaryon which was followed by granular dissolution. Since these cells had less prominently impregnated neurofibrils in comparison to the medullary neurons, the degenerated forms had a red to red-black appearance with the Holme's method (Fig. 28). Inclusion bodies were not detected within perikarya of these two types of neurons. Neuronal processes were still detected in cultures up to 25 days post-inoculation when few perikarya were demonstrable. These processes eventually underwent dissolution by the 28th day, however. Uninoculated control cultures of the same age had a high percentage of normal-appearing Purkinje cells and processes.

Granule and stellate cells had signs of degeneration which were best detected in the outgrowth. This involved a dissolution of the processes and swelling of the perikaryon with eventual pyknosis and lysis. This change was especially well demonstrated with the methylene blue supravital method (Fig. 29, 30) and less so with silver impregnation. These cells stained very pale with the May-Grünwald-Giemsa stain in older cultures. The number of granule cells in infected cultures was comparable to controls up to the third week post-inoculation. In fact, the population in infected cultures sometimes appeared to exceed that in controls with some preparations. Inclusion bodies were detected most frequently in the nucleus of granule and stellate cells. They were
Fig. 28. Twenty-four-day-old explant culture of canine cerebellum (19 days post-inoculation) illustrating a degenerated Purkinje cell (J2315-20). Wolf's modification of Holme's silver impregnation; x 1250. The perikaryon is pyknotic and the nucleus is eccentric. A clump of irregularly arranged neurofibrils can be seen in one area of the cytoplasm (large arrow). The two dendritic processes to the left and the axon (A) are thin and frayed in appearance. The axon terminated in the lower right of the picture (small arrow).

Fig. 29. Forty-two-day-old explant culture of canine cerebellum (35 days post-inoculation) demonstrating degeneration and intranuclear inclusion formation in granule cells in the outgrowth (K201-83). Methylene blue supravital stain; x 315. Five degenerated cells can be seen, three of which contain lightly stained intranuclear inclusions (arrows) in the dark nuclei. Degeneration of processes can be especially well seen in one cell (upper right). Other cells have mainly lost their processes. Compare with Figure 30.
Fig. 30. Forty-two-day-old explant culture of canine cerebellum (uninoculated control) showing three normal granule cells (K201-84). Methylene blue supravital stain; x 315. The granule cells (arrows) contain well-formed processes and an evenly stained dark nucleus.

Fig. 31. Thirty-nine-day-old explant culture of canine cerebellum (30 days post-inoculation) illustrating the specific fluorescence of two intranuclear inclusions following treatment with labeled anti-distemper globulin (K953-15). Immunofluorescent method; x 1872. The viral antigen, which stained green, is evenly distributed throughout the inclusion. The nucleoli (arrows) stained a lighter green and are separate from the inclusion material.
readily demonstrated with silver impregnation or the methylene blue stain (Fig. 29).

Cultures that were inoculated with canine distemper virus either failed to myelinate or demyelinated shortly after the development of myelin sheaths. This change was dependent, to some extent, on the passage of virus used as the inoculum since each varied in the time required to produce a CPE. The demyelinating effect on myelinated cultures preceded detectable neuronal changes by approximately one week. In infected cultures the myelinated axons became irregular and contained many varicosities or distensions along their length. This was followed by a disruption and dissolution of the sheath.

The immunofluorescent studies of cerebellar explants infected with distemper virus revealed that both intranuclear and intracytoplasmic inclusions contained viral antigen. The first evidence of viral antigen occurred as small, green intracytoplasmic inclusions that were also detectable with the May-Grunwald-Giemsa stain. Intracytoplasmic inclusions had a uniform distribution of antigen since the complete inclusion body fluoresced evenly regardless of its size or shape. In old infected cultures, however, many cytoplasmic inclusions failed to fluoresce. The morphologic features of the inclusions were identical to those detected in May-Grunwald-Giemsa stained preparations.

Intranuclear inclusions fluoresced as uniform green bodies (Fig. 31). The time of their detection, which followed the appearance of intracytoplasmic viral antigen, also coincided with their
occurrence in the May-Grünwald-Giemsa preparations. The uniformity of the viral antigen in these inclusions was comparable to that described for intracytoplasmic inclusions.

Discussion

The first objective of this study was to determine the value of explant cultures of canine cerebellum as a model for studying the virus associated with demyelinating canine distemper encephalitis. To prove valuable such a method should permit the evaluation of the viral effect on all cultural components. These requirements were mainly fulfilled.

In order to properly evaluate the results, however, two factors had to be given consideration. These involved the nature of the culture growth and the relatively long incubation period of the distemper virus in tissue culture. The characteristics of the explant tissue cultures continuously changed with increasing age. For example, several cells, including oligodendroglia, granule cells and macrophages, decreased in the outgrowth as the culture became older, whereas astroglial growth became more abundant. In comparison, the large neurons (Purkinje cells and neurons from the medullary nuclei) and myelin sheaths required a relatively long time (three to four weeks) for maturation and development. Therefore, in order to evaluate the effects of the distemper virus on as many components of the culture as possible in this study, cultures were inoculated shortly after explantation.

The slow CPE exhibited by the distemper virus also affected the evaluation of infected cultures, particularly regarding
oligodendroglia and, to a lesser extent, macrophages. These two cell types were usually scarce or absent in the outgrowth by the time the viral effect was detected. For future investigation it appeared that two things might be done to obviate this limitation. First, to inoculate cultures earlier, possibly at the time of explantation, and second, to further reduce the time required for CPE by additional virus passage, as had been demonstrated with distemper virus in other tissue culture systems (3, 13, 58).

One of the most characteristic features of explant culture infection was the manner in which the CPE developed. The lesion first was detected in the peripheral outgrowth and then progressively involved the inner outgrowth and explant. A similar pattern has been observed in monolayer cultures infected with distemper (3) and rinderpest (56) viruses in that cells at the edge of the cell sheet were initially affected. In the present study the CPE consisted of inclusion body formation, syncytial development and the production of a reticulated pattern in the outgrowth. These lesions in the outgrowth were comparable to those described for strains of distemper virus in monolayer cultures (3, 12, 13, 14, 21, 58, 59, 61). The reported CPE varied, however, with the strains of virus employed, passage history of the virus and the type of culture used (13, 14, 35, 36, 59). The viruses of measles and rinderpest, which are related to the canine distemper virus (15, 24, 32, 42, 48, 57, 64), also produced a CPE in tissue culture with similar characteristics. In addition, syncytial formation and intracytoplasmic inclusion body development have been reported.
to follow tissue culture inoculation with certain strains of mumps, newcastle disease and para influenza 2 viruses (10, 11).

In trypsinized cultures the viruses of distemper, measles and rinderpest produced a CPE characterized by syncytial giant cells, inclusion bodies and often stellate or spindle cell formation (3, 12, 13, 14, 21, 35, 40, 50, 54, 55, 56, 58, 59, 61, 64). These lesions did not involve the whole cell sheet simultaneously but occurred in isolated foci which progressively enlarged (3, 14, 55, 58). These were comparable to the early changes in the peripheral outgrowth of affected explant cultures in the present study. The stellate cell forms that occurred in monolayer cultures also compared with the changes in the outgrowth surrounding explants. In this latter instance, however, the transformation was from protoplasmic astrocytes with short, delicate processes to fibrous astrocytes with large, prominent processes. In the later stages of culture infection these cells had a high percentage of intranuclear inclusions, often without cytoplasmic involvement. The reason for the nuclear predisposition could not be explained.

Various types of syncytial giant cell formations have been described in monolayer cultures infected with either of the three above mentioned viruses (56, 58, 59). These formations in explant cultures of canine cerebellum were most numerous in the periphery of the outgrowth but also occurred in the inner outgrowth and explant proper. All variations mentioned by other investigators were detected but the majority of the giant cells contained 10 to 30 nuclei. Some with as many as 50 nuclei were
also seen. The nuclei were arranged in a circular formation or clumped together. Contracted, degenerated–appearing, giant cells, as described by Shaver et al. (59) were also detected. These were usually intensely basophilic and often had pyknotic nuclei containing inclusions. Vacuolation, which has been reported in syncytia and in individual cells (58, 61), was detected rarely in infected explant cultures.

The effect of distemper virus on oligodendroglia could not be properly evaluated since they had mainly disappeared from the outgrowth by the time of CPE. The glial cell concerned with myelination, however, apparently persisted indefinitely in the explants of control cultures since myelinated axons were often maintained for several weeks. A separate electron microscopic study of normal myelinated cultures of canine cerebellum has shown that the glial cells participating in myelin formation have characteristics of oligodendroglia (38). It is this cell that should be examined in infected cultures but the density of the explant growth largely prevented this with routine light microscopy of intact cultures.

The developmental and morphologic characteristics of intracytoplasmic inclusion bodies in infected cells during the present investigation were comparable to reports involving distemper (3, 13, 14, 21, 59, 61), measles (13, 14, 50, 59), and rinderpest infected cultures (40, 50, 54, 55). This included the general observation that cytoplasmic inclusions were the first to occur (3, 13, 59, 61). The interpretation by Bittle et al. (3),
that these cytoplasmic inclusions were first seen as small granules that later coalesced into masses of various sizes and shapes, coincided with the process in affected astrocytes.

No mention was made by the above investigators of the early developmental characteristics of intranuclear inclusion bodies which were detected during the present study. This may have been because stained preparations usually were inadequate to demonstrate the internal structure. Also, Black et al. (4) pointed out that inclusions of measles varied in appearance depending on the fixative used. Various sizes and shapes of intranuclear inclusions were detected in affected canine cerebellar explants. Black et al. stated that measles inclusions were initially small and often multiple within a nucleus. Later they increased in size and fused until the whole nucleus was occupied. Plowright and Ferris (56) described a vaculation in intranuclear inclusions which was not detected in the present investigation.

Several factors had to be considered in interpreting neuronal degeneration in infected cultures. Some of the neurons (granule cells and stellate cells) gradually decreased in number with increasing culture age. There were, therefore, usually a few degenerated cells present in uninfected cultures. Normal cells, however, could always be detected as long as any cells were present. In infected cultures, when the entire outgrowth had signs of CPE, practically all of the granule cells were undergoing degeneration. Intranuclear inclusions were also frequent.
The evolution of the large neurons (Purkinje cells and neurons of medullary nuclei) within the explant had to be given the same consideration as the above mentioned cells since there was always some degree of neuronal degeneration occurring in uninfected cultures. The interpretation of the viral effect in these cells was, therefore, based upon both the quality and quantity of neuronal degeneration in infected cultures.

The differences between control and infected cultures was definite. Three to four weeks post-inoculation infected cultures usually contained no neuronal perikarya or processes, whereas the same components were routinely present in the controls. Whether this was due to a specific viral action or to a secondary effect related to general culture deterioration could not be determined. No definite signs of viral effect, such as the inclusion body formation illustrated by Fernandes and Pomerat (18) in rabies infected explants, could be detected in these neurons, but the occurrence of such infectivity could not be excluded.

Two factors had to be taken into account when the viral effects in explant tissue cultures were compared with the lesions of the brain in canine distemper. First, the relative quantity and distribution of the cell population in the two tissues is different, and second, the virus in culture has equal access to all cellular components, while this exposure is modified in the brain by the blood-brain barrier.

There were, however, several similarities of the lesions in the two tissue systems. In tissue culture the astrocytes were
prominently affected which was comparable with the findings reported for distemper encephalitis (20, 33, 34, 39, 62, 63). The occurrence of neuronal degeneration in tissue culture also was compatible with the lesion in vivo (20, 22, 23, 33, 34, 39). The existence of inclusion bodies in large motor neurons, which are detected in brain sections (20, 33, 34), were not demonstrated in tissue culture. The occurrence of intranuclear inclusions in granule cells, however, was compatible with descriptions of the distemper lesion (20). Intracytoplasmic, and particularly intranuclear inclusions of astrocytes, also are a prominent part of the lesion in both tissue systems (20, 33, 34, 39).

Another change detected in tissue culture should be compared with a morphologically similar counterpart in the brains of dogs infected with distemper; mice and hamsters experimentally infected with measles virus; and mice experimentally infected with the virus of distemper, measles and rinderpest. In dogs, cell aggregates (giant cells) have been described as involving the ependymal cells and the granular cells of the cerebellar cortex (20, 63). Fischer (20) referred to these structures as conglomerates of pyknotic granule cells. Multinucleated astrocytes have also been reported in the brains of infected dogs (20, 33, 39). Matumoto et al. (41) described the formation of multinucleated giant cells as being formed by fusion of nerve cells following measles infection in mice. They were most frequent in Ammon's horn. Waksman et al. (63) described giant cells in measles-infected hamsters. They were detected in the germinal
plate adjacent to the lateral ventricles, in the ependyma, the fascia dentata of the hippocampal gyrus, granular cell layer of the cerebellar cortex and in the neocortex. These workers concluded that the giant cells were clearly derived from either the ependymal or nerve cells. There was no evidence of the formation originating from glial elements. Cytoplasmic (41) or cytoplasmic and nuclear (63) inclusions were also detected in mice (41) and hamsters (63) following infection with measles virus. In addition, Imagawa (31) detected giant cells in the brains of mice infected with distemper, measles and rinderpest viruses. He did not specify the cell type involved.

One of the most significant similarities that infected cultures had with the brain lesion concerned the effect on myelin. In infected cultures myelin either failed to develop or was destroyed shortly after being formed. Whether this was the result of a direct viral activity on cells, as has been proposed for the lesion in the dog (30, 62, 63), or due to influences secondary to viral activity was not determined. The fact that the neurons and their processes persisted longer than the myelin sheaths was not surprising since this is compatible with several diseases, including canine distemper, that affect the central nervous system. Neuronal destruction, however, does occur with distemper in areas of severe involvement. Further study of the mechanism involved in myelin destruction of infected cultures is warranted and could prove quite fruitful in the elucidation of the demyelinating process associated with canine distemper.
The existence of viral antigen in cellular inclusion bodies (intracytoplasmic and intranuclear) was confirmed by employing the immunofluorescent method. The fluorescent material corresponded in size, shape and time of occurrence to the inclusions detected following staining. The appearance of antigen first in the cytoplasm followed by nuclear involvement corresponded to the findings by Black et al. (4) with measles virus in tissue culture but differed from the results of Palacios (49) who detected viral antigen sequentially at the cell membrane, nucleus and cytoplasm. In contrast to Leiss and Plowright (40), who examined the effect of rinderpest virus on Hela cells, the appearance of fluorescent and stainable inclusions occurred simultaneously in the present study. These authors detected viral antigen three to five days before identifiable inclusions could be demonstrated. Also, Brandt (11) concluded that the cytoplasmic inclusions occurring in cultures infected with mumps or newcastle disease viruses did not represent concentrated viral antigen since the fluorescence was more widely and evenly distributed in the cytoplasm of infected cells than were the inclusions. Karzon (35) stated that with measles virus there was no morphologic correspondence between inclusion bodies and collections of antigen or nucleic acid.

In the brain lesions of canine distemper, Moulton (44) detected viral antigen in astrocytic and possibly other glial nuclei. Myelin sheaths, neurons and axis cylinders had no fluorescence. Coffin and Liu (16) demonstrated viral antigen in infected dog brains in several cells including ependymal cells,
astrocytes, neurons and their processes. Cells containing viral antigen in canine cerebellar explants of the present study were predominantly limited to the astroglia. The reduction of demonstrable antigen in intracytoplasmic inclusions with increasing age was comparable to the behavior of other viral inclusions in tissue culture (45). This manifestation has been explained as possibly due to a cessation of viral synthesis.

Summary

Canine cerebellar explant cultures were employed as a model for studying the effects of the virus associated with demyelinating canine distemper encephalitis. Cultures were inoculated four to seven days following preparation with distemper virus (Lederle strain) and had beginning cytopathic effect (CPE) in the peripheral outgrowth on the eighth post-inoculation day. The CPE consisted of inclusion body development (intracytoplasmic and intranuclear), syncytial giant cell formation and production of a reticulated type of growth, mainly composed of fibrous astroglia, in the outgrowth. The CPE, which predominantly involved astrocytes, first occurred in the peripheral outgrowth and then progressively developed in the inner outgrowth and explant. Cellular degeneration was common but one prominent characteristic of infected cultures was the high degree with which the virus (inclusion bodies) and cells appeared to co-exist.

Neuronal degeneration followed either a lack of myelin development or demyelination in infected cultures. Four weeks post-inoculation, infected cultures had few or no large neurons
(Purkinje cells or neurons of the cerebellar nuclei), whereas these cells were numerous in the controls. Smaller neurons (granule and stellate cells), often located in the outgrowth, also became degenerated and frequently contained intranuclear inclusions. These were the only neuronal cell types in which inclusions were demonstrated.

Immunofluorescent studies established that cytoplasmic, as well as nuclear, inclusion material contained viral antigen. The areas of viral antigen concentration in cells were comparable in shape, size and time of occurrence with inclusions demonstrated in stained preparations.
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