Original Works

Primary Demyelination in Experimental Canine Distemper Virus Induced Encephalomyelitis in Gnotobiotic Dogs

Sequential Immunologic and Morphologic Findings*

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Summary. Experimental infection of gnotobiotic Beagle dogs at 21 days of age with neurovirulent R252 strain of canine distemper virus (R252-CDV) resulted in a non-suppurative encephalomyelitis. Segmental internodal primary demyelination was found in almost 90% of the dogs from 27 days post inoculation (DPI). Ultrastructurally demyelination was initiated by the insertion of CDV-infected astrocytic processes at nodes of Ranvier with subsequent cleavage of well-preserved myelin from the axolemma. CDV-infected macrophages were consistently involved in myelin phagocytosis. Some remyelination of denuded axons occurred after 35 DPI. Persistent productive infection of the choroid plexus and ependyma in the fourth ventricle was consistently associated with subependymal foci of demyelination.

Primary demyelination occurred without detectable CDV-specific virus-neutralizing (CDV-VN) antibody in either serum or cerebrospinal fluid (CSF). There were no immunoglobulin deposits or inflammatory cells within the lesions. These findings indicate that both direct CDV antibody-dependent and CDV antibody-dependent cell-mediated immune mechanisms of cytolysis or myelin destruction are not involved in the genesis of initial primary demyelination. The sequential morphologic and serologic findings in this model of demyelinating encephalomyelitis indicate that direct virus-induced injury has a major role in both the initiation and early progression of primary demyelination.

Key words: Canine — Canine distemper virus — Demyelination — Encephalomyelitis — Antibodies

Introduction

Despite recent ultrastructural studies on natural [23, 32] and experimental [20, 26] CDV-induced demyelinating encephalomyelitis, the actual mechanism of primary demyelination remains controversial [23]. Some investigators favor direct virus-induced oligodendroglial or myelin injury [23] while others [10, 31] postulate primary humoral or cell-mediated immune mechanisms of demyelination. Recently, antibody and complement dependent [6], and lymphocyte-mediated anti-CDV directed cellular cytotoxic immune mechanisms have been demonstrated in vitro [24]. Similar events could occur in vivo.

The age-dependent variation in both the immunologic and neuropathologic host response to CDV is well-known [13]. In our laboratory manipulation of the canine model for CDV-induced demyelinating encephalomyelitis by inoculation at 21 days of age rather than at 4—8 weeks [9] has resulted in a much higher incidence of consistent demyelinating lesions in the rostral medullary velum (RMV) (R. J. Higgins et al., unpublished data 1980). This finding has thus enhanced the potential usefulness of this model for sequential morphologic and immunologic studies designed to critically evaluate the temporal role of both viral and immune factors in vivo. The first objective of this study therefore was to examine the sequential histologic and ultrastructural morphogenesis of the earliest lesions leading to primary demyelination in the RMV and to correlate these changes with the presence of any viral antigen or immunoglobulin.
deposits within these sites. The second aim was to interpret these findings in relation to parallel sequential studies to detect any CDV-associated humoral immune responses.

Material and Methods

Animals

A total of 20 gnotobiotic dogs from 6 litters were derived by caesarian section from date-mated CDV sero-negative pregnant Beagle bitches. They were raised in flexible plastic isolators using conventional techniques by methods described elsewhere [4, 14]. The animals were examined clinically twice daily.

Virus Inoculum

At 21 days of age, 17 dogs each received intraperitoneally 0.2 ml of freshly thawed 20% spleen-thymus homogenate, containing 10^6.5 canine pulmonary macrophage culture infective doses of neuroviral R252-CDV per ml (PMCIDs0/ml). The origin and in vivo properties [20, 21] of this virus have been reported. Three dogs were maintained separately in isolators as uninoculated controls. At intervals of 8, 10, 14, 18, 21, 24, 27, 31, 35 and 42 DPI dogs were killed with 2, 1, 1, 2, 1, 4, 1, 1 and 2 dogs on each day, respectively. Uninoculated controls were killed at 28, 43 and 70 days of age.

Virologic and Immunologic Studies

Both clotted and unculted blood samples were collected once prior to inoculation and at weekly intervals from control and CDV-infected dogs and evaluated for total and differential white blood cell counts. In addition, an aliquot from each was evaluated for leukocyte-associated virusemia using a direct immunofluorescence technique as described previously [13]. Dextran-saline sedimented peripheral blood lymphocytes were tested for in vitro lymphocyte blast transformation (LBT) using plant mitogens phytohemagglutinin-P and pokeweed [11]. Cell-free preparations of cerebrospinal fluid (CSF) were assayed from 2 dogs for infectious CDV using canine pulmonary macrophage cultures [12]. Cells recovered from the CSF samples from 12 dogs without clinical signs were evaluated for the presence of viral antigen by direct immunofluorescence. Serum and CSF antibody titers to R252-CDV were determined using a microtiter virus neutralisation (VN) system [2].

Histopathology and Immunofluorescence (IF)

Under deep anesthesia, a CSF sample was taken from the cisterna magna, the left half of the brain of the 20 dogs was removed aseptically, followed by perfusion fixation of each dog for subsequent morphologic examination. Six selected blocks of fresh CNS tissue from all the CDV-infected dogs and the 3 uninoculated dogs were processed by routine techniques for direct IF [27]. These included direct IF staining for both CDV antigens and immunoglobulins, using fluorescein-isothiocyanate-labelled rabbit anti-CDV immunoglobulin G and rabbit anti-dog polyvalent immunoglobulin (Miles Research Labs., Inc.) respectively. Sites routinely examined for the presence of virus and immunoglobulins were the olfactory bulb and tract, frontal and temporal cortex, basal ganglia, hippocampus, thalamus, midbrain, cerebellar cortex, choroid plexus of the fourth ventricle, cerebellar peduncles and medulla oblongata. Similarly processed tonsillar or lymph node tissue from conventional animals, followed by perfusion fixation of each dog for subsequent morphologic examination. Sections 1 μm thick cut from the longitudinally or transversely oriented blocks were stained with alkaline toluidine blue and then examined by light microscopy. Thin sections were cut from selected sites of the relevant blocks, mounted and stained with uranyl acetate and then lead citrate before ultrastructural examination.

Results

Hematology, Virology and Immunology

From 7 DPI, all infected dogs had a sustained leukopenia mainly due to a relative or absolute lymphopenia. There was a cell-associated viremia as determined by IF in all dogs to 21 DPI. Four of the dogs were IF positive at 28 DPI and 1 dog was viremic throughout the course of infection. Free infectious virus was not detected in the CSF from two dogs examined terminally at 31 and 42 DPI. However, CDV antigen was consistently demonstrated in cells recovered from the CSF in all the 12 inoculated dogs which were tested. One dog had a terminal serum CDV-VN antibody titer of 1:16 when killed 27 DPI, but previous weekly samples from this dog and serum and CSF samples from all the other dogs tested had no antiviral antibodies (see Table 1). Phytohemagglutinin-induced LBT assays for both T- and B-cell-responsive lymphocyte populations remained consistently depressed compared to the controls throughout the course of infection [16].

Clinical Findings

No clinical neurologic signs were observed in 13 dogs killed between 8 and 42 DPI. In the remaining four dogs the earliest common clinical signs, starting between 24 and 31 DPI, were depression, anorexia and occasional respiratory distress followed within 12–24 h by isolated irregular myoclonus of facial or limb muscles, mild truncal ataxia and intermittent, unintentional fine head tremors. Intermittent facial myoclonus with salivation progressed within 24–72 h to grand-mal seizures of increasing frequency with decreasing intervals between their onset. Dogs became prostrate once continuous seizures developed and were then killed. One dog had no other premonitory clinical signs prior to the sudden onset of grand-mal convulsions. Rectal temperatures of all dogs remained within normal levels (38.3–38.5°C) throughout the course of infection with elevation to 40.1°C only in dogs at terminus.

Immunofluorescent Findings

Earliest detectable CDV-IF antigen was restricted to the cytoplasm of isolated mononuclear cells randomly
Table 1. Significant sequential clinical, morphologic and immunologic data from CDV-infected gnotobiotic dogs

| Days post inoculation (DPI) | 8  | 10 | 14 | 18 | 21 | 24 | 27 | 31 | 35 | 42 |
|-----------------------------|----|----|----|----|----|----|----|----|----|----|
| No. of dogs in group        | 2  | 1  | 1  | 2  | 1  | 1  | 1  | 1  | 2  |
| Dogs with neurologic signs  | neg| neg| neg| neg| neg| (2) | neg| (1) | neg| neg|
| Light microscopic lesions   | neg| neg| neg| neg| neg| ABC| ABC| ABC| AB | ABC|
| Ischaemic neuronal necrosis | neg| neg| neg| neg| neg| +  | (2)+| +  | neg| (1)+|
| Demyelination in RMV        | neg| neg| neg| neg| neg| neg| +  | +  | neg| +  |
| VN antibody titres          | neg| neg| neg| neg| neg| neg| neg| neg| neg| neg|
| (CSF)                       | neg| neg| neg| neg| neg| neg| neg| neg| neg| neg|
| (Serum)                     | neg| neg| neg| neg| neg| neg| neg| neg| neg| neg|
| Immunoglobulin in lesions   | neg| neg| neg| neg| neg| neg| neg| neg| neg| neg|
| CDV antigen in lesions      | neg| neg| neg| neg| neg| neg| neg| neg| neg| neg|

a Numbers in parenthesis indicate no. affected in group
b A Non suppurative meningitis, focal microgliosis
B Astrocytic hypertrophy, microglial cells
C Spongy vacuolation and loss myelin staining
c CDV-VN antibody titre expressed as serum dilution
+ Positive
neg Negative
distributed in the meninges of the spinal cord and brain at 10 DPI.

By 14 DPI, antigen was detected in single isolated neurons within the grey matter of the frontal, temporal and cerebellar cortex together with occasional fluorescence within the parenchyma adjacent to blood vessels in grey matter. Single infected ependymal cells were found in the ependymal lining of lateral, third and fourth ventricles and in the choroid plexus epithelium of the fourth ventricle. At 18 DPI, multiple clusters of infected epithelial cells were seen in the choroid plexus and ependyma of the fourth ventricle (Fig. 1). Within the CNS parenchyma, IF positive single neurons contained granular or diffuse cytoplasmic fluorescence and sometimes intranuclear inclusions. From the perikaryon there was extension of
immunoglobulin deposits as determined by IF were
the five dogs killed beginning at 24 DPI had micro-
neuritic astrocytes. Throughout the course of this study,
neutrophilic inclusion bodies were found within morphologically intact
vessels. Occasionally structurally intact axons had segments of myelin sheath thinning or vacuolation without phagocytosis.

Light Microscopy
Coronal Sections of the CNS. Of the 13 dogs without
neurologic signs killed between 8 and 42 DPI, only
the distribution though widespread, was restricted to single cells in the grey matter, although occasional fluorescent cells presumably astrocytes, were noted
in the white matter of the cerebellar folia. There was
the earliest lesion was an increased number of reactive astrocytes, either singly or in clusters, randomly dis-
tributed between intact myelinated axons at 24 DPI. Some astrocytes were bi- or multinucleate. Many astrocytes had single intranuclear or multiple intracytoplasmic CDV inclusion bodies. Single myelinated axons had sharply demarcated segments of the axo-
lemma devoid of myelin adjacent to normally my-
elinated internodes (Fig. 1). Many individual demy-
elinated axons were found in areas without cell in-
filtration and often abutted normal myelinated axons. Conversely, the cytoplasmic membrane of many in-
fected reactive astrocytes bordered intact myelinated
internodes of demyelinated axons. Adjacent blood
vessels were normal (Fig. 2).

By 24 DPI small multifocal subpial or subependym-
al foci of increased cellularity, including hypertrophic astrocytes, were consistently found around the fourth
ventricle in the medulla oblongata and in the pons
and spinal cord. In these areas from 27 to 31 DPI
other alterations consisted of vacuolar spongy change
with loss of myelin staining as demonstrated by LFB-
CEV staining. There were also microglial and active
gitter cell accumulations. By 35 DPI additionally there
was some confluent malacia. Between 35 and 42 DPI,
three dogs also had concurrent multifocal malacia
with cyst formation and similar associated cell types
in foci of the parietal and frontal cerebral cortex.

Perivascular cuffing was not found in any of these
lesions. One dog killed at 27 DPI with a terminal
serum VN antibody titer of 1:16 and negative CSF
titre had widespread unique individual axonal necrosis
with demyelination in cerebral white matter of the
frontal, parietal and occipital lobes. No lesions were
found in the overlying grey matter.

One dog without observed neurologic signs killed
at 42 DPI had striking acute ischaemic neuronal necrosis without associated inflammatory cell re-
response. These multifocal, bilateral and symmetrical
lesions were found mainly in the temporal cortex, in-
cluding the amygdala and hippocampus. In the four
dogs killed after sustained terminal seizures there were
varying degrees of similarly distributed bilaterally sym-
metrical ischaemic neuronal necrosis (Table 1).

Rostral Medullary Velum. Lesions in the RMV were
similar in temporal sequence and severity to those
demyelinating lesions in the other sites in the CNS. The earliest lesion was an increased number of reactive astrocytes, either singly or in clusters, randomly dis-
tributed between intact myelinated axons at 24 DPI. Some astrocytes were bi- or multinucleate. Many astrocytes had single intranuclear or multiple intracytoplasmic CDV inclusion bodies. Single myelinated axons had sharply demarcated segments of the axo-
lemma devoid of myelin adjacent to normally my-
elinated internodes (Fig. 2). Many individual demy-
elinated axons were found in areas without cell in-
filtration and often abutted normal myelinated axons. Conversely, the cytoplasmic membrane of many in-
fected reactive astrocytes bordered intact myelinated
internodes of demyelinated axons. Adjacent blood
vessels were normal (Fig. 2).

By 27 DPI other partially or completely demy-
elinated axons were surrounded by actively phagocytic
cells containing CDV nucleocapsids, myelin and
granular debris and multiple empty vesicles (Fig. 3).
There were also microglial and active gitter cell accumulations. By 35 DPI additionally there
was some confluent malacia. Between 35 and 42 DPI,
three dogs also had concurrent multifocal malacia
with cyst formation and similar associated cell types
in foci of the parietal and frontal cerebral cortex.

By 42 DPI there was almost total demyelination
of all axons which lay within a network of astrocytic
Fig. 4. A structurally intact myelinated axon with multiple cytoplasmic processes containing nucleocapsids (arrows) and glial filaments () adjacent to the axolemma at the node of Ranvier. Normal exposed axolemma (double arrows). 24 DPI, × 6,000

processes. These processes surrounded many of the intact naked axons in the loose edematous matrix. Numerous phagocytic cells were distended with myelin debris.

Blood vessels were prominent but had no perivascular inflammatory cell cuffing. Swollen degenerating axons had either partially intact or vacuolar ballooning of myelin sheaths. Similar lesions were consistently found in sub-ependymal sites of the medulla close to the origin of the RMV.

Ultrastructural Lesions

Rostral Medullary Vellum. The earliest ultrastructural change in morphologically intact myelinated axons was the insertion of nucleocapsid-bearing astrocytic cell processes onto nodes of Ranvier between intact paranodal areas and their subsequent attachment to the exposed axolemma at 24 DPI (Fig. 4). This attachment zone did not always completely encircle the node. At 27 DPI in segmentally demyelinated axons, these cell processes initially inserted under the myelin paranodal-axonal junction and then extended between the axolemma and overlying myelin lamellae, with relative preservation of the separated myelin sheath. Vacular intramyelinc lamellar separation did not occur around axons undergoing cell-mediated segmental internodal stripping of the myelin sheath. Tight junctions between astrocytic cell processes were often found. Also by 27 DPI nucleocapsid-containing actively phagocytic cells surrounded many demyelinated but intact axons. Their elongate nuclei often had multiple indentations and uniformly dispersed abundant heterochromatin. The dense, dark staining cytoplasm contained abundant rough endoplasmic reticulum, multiple clusters of lamellar myelin-like profiles, lipid inclusions, granular debris and prominent lysosomes but no filaments or microtubules. These cells surrounded single intact denuded axonal segments while neighboring adjacent myelinated axons were intact. Sometimes these cells were between other naked axons (Fig. 5). Although infected hypertrophic astrocytes containing glial filaments were adjacent to myelinated or demyelinated axons, these cells did not have evidence of phagocytosis of myelin before 35 DPI. By 35 DPI some axons had thin attenuated myelin sheaths of uniform thickness but with intact paranodal junctions suggesting remyelination (Fig. 7). Other axons had multiple compacted myelin-like lamellae membranes. By 42 DPI cytoplasmic processes of reactive CDV-containing astrocytes were often found wrapped around groups of naked axons (Fig. 6).

Choroid Plexus. Between 18 and 42 DPI, nucleocapsid containing choroid plexus and ependymal epithelium of the fourth ventricle had parallel dense intramembranous spikes along the luminal cytoplasmic border. Budding to form mature intact virions occurred along most of the plasmalemma between existing microvilli and cilia (Fig. 8). Although some mononuclear cells in the meninges did have similar membrane spiking, viral budding suggestive of productive viral replication was not seen in these or any other CNS cells.

Discussion

This study demonstrated that experimental infection with neurovirulent R252-CDV in gnotobiotic dogs at 21 days of age resulted in a non-suppurative encephalomyelitis with primary demyelination occurring in almost 90% of the dogs from 27 DPI. Compared to other experimental studies [1, 20] this high incidence of primary demyelination in the RMV in our dogs thus significantly enhances the potential usefulness of this model [9] for the investigation of proposed mechanisms of CDV-induced demyelinating encephalomyelitis in vivo. Initial segmental internodal demyelination was always associated with CDV in the lesions but occurred without either detectable CDV-VN specific antibody response in the serum or CSF, perivascular mononuclear cell cuffing or immunoglobulin deposits within lesions.

In the known predilection sites of CDV induced primary demyelination, the earliest change was increased numbers of hypertrophic CDV-infected astrocytes. Ultrastructurally, astrocytic cell processes containing glial filaments and nucleocapsid were closely applied to the axolemma at the nodes of Ranvier. Subsequent disruption of paranodal junctions of relatively intact myelin by these underrunning processes
suggests a primary role for infected astrocytes in the initial demyelinating processes. Although these early changes have not been reported, phagocytosis of myelin lamellae by astrocytes in CDV-induced demyelination has been recognised previously in both spontaneous [23] and experimental studies [26]. In our series myelin phagocytosis was mediated by CDV-infected macrophages several days before similar participation by astrocytes.

Although a scavenger role for CDV-infected macrophages around denuded axons [26] has been confirmed in this study, their role in initiating primary demyelination remains speculative. The highly selective pattern of internodal myelin destruction appears incompatible with that from extracellular release of myelinolytic proteases from CDV-infected macrophages, as demonstrated in vitro with non-specifically activated macrophages [3]. Our morphologic interpretation however would be consistent with a mechanism of intracellular protease-induced myelinolysis following CDV-induced macrophage fusion with individual oligodendroglia or their membranes. In our
study, such a non-specific bystander mechanism of myelin injury would not be dependent on any antiviral humoral response [31]. Unequivocal identification of these phagocytic cells could be best resolved by the application of specific cell marker labelling techniques [8].

The segmental internodal uniform myelin loss over denuded axons, sharply demarcated from adjacent intact myelin paranodal attachments, suggests a primary and highly selective insult to oligodendroglia or their myelin lamellae. Although CDV infection of oligodendroglia is rare [23, 27] and was not recognized in this study, a non-cytolytic infection of these cells could result in this pattern of demyelination. This pattern is similar to that of experimental coronavirus-induced demyelinating encephalomyelitis in mice where virus-induced cytolysis of oligodendroglia results in primary segmental demyelination [29]. It contrasts sharply with the multifocal, irregular lysis of myelin lamellae and primary demyelination with perivenular mononuclear cell infiltrates characteristic [18] of experimentally-induced autoimmune-mediated acute [17] and chronic [25] experimental allergic encephalitis. The finding in our dogs of inappropriately thin internodal myelin sheaths of uniform thickness that terminated in normal paranodal complexes fulfill the established criteria for remyelination within the CNS [22]. Thus, the presence of remyelination after 35 DPI implies a capacity for functional restoration of injured oligodendroglia or remyelination from other intact oligodendroglia as in corona virus infection in mice where myelin injury would not be dependent on any antiviral humoral response [31]. Unequivocal identification of these phagocytic cells could be best resolved by the application of specific cell marker labelling techniques [8].

In conclusion, the high incidence of demyelination in the RMV and the lack of a detectable CDV-specific humoral response, features of infection at 21 days of age, allowed a critical in vivo evaluation of certain postulated humoral immune-mediated mechanisms of demyelination. Direct CDV-induced injury appears to play a major role in initiation of the primary demyelination. The absence of specific CDV antibody in these dogs with multifocal demyelinating lesions suggests that the genesis of initial primary demyelination in CDV-induced encephalomyelitis at this age occurs independently of both direct and indirect humoral antiviral immune mechanisms.

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