Immunohistochemical comparison of mononuclear cell populations in diseases of the canine central nervous system

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Immunohistochemical comparison of mononuclear cell populations in diseases of the canine central nervous system

Karen Lynne Kline

A thesis submitted to the graduate faculty in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

Major: Veterinary Pathology
Major Professor: Dr. Claire Andreasen

Iowa State University
Ames, Iowa
2001

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Graduate College
Iowa State University

This is to certify that the Master's thesis of

Karen Lynne Kline

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy
DEDICATION

This thesis and research is dedicated to those individuals in my life whose guidance, inspiration, and patience have allowed me to follow my dreams and, ultimately, my bliss. First, my parents, Herman and Lois Kline, who told me to always “do the best you can” and expected nothing more. Second, my husband, Karl Kersting, whose patience and understanding would rival Job’s. Without his steadfast love, none of this would be possible. Third, my friends, colleagues, and mentors who made me into the veterinarian and person I am today. Fourth, Dr. William Fenner, who sparked my interest in veterinary neurology and made me realize the timeless importance of teaching and its virtues. Fifth, Dr. Claire Andreasen, who has been an inspiration and source of infinite knowledge for me. She has been a fantastic mentor and friend. And finally, my patients and students whose lives touch mine on a daily basis. What I give to them is merely a fraction of what they give back to me in return. And Chip, although you’re gone, I think about you every day.
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CHAPTER 1. GENERAL INTRODUCTION

Inflammatory central nervous system (CNS) disease in the canine can be associated with a number of infectious and non-infectious etiologies that include canine distemper virus (CDV), granulomatous meningoencephalomyelitis (GME), lymphoma, the protozoal agents, *Toxoplasma gondii* and *Neospora caninum*, and rickettsial diseases, *Ehrlichia canis* (ehrlichiosis) and *Rickettsia rickettsii* (Rocky Mountain Spotted Fever, (RMSF)). It is currently unknown if the diseases we diagnose as GME are a single disease syndrome or a group of diseases. Diagnosis of these CNS diseases is most commonly accomplished through cerebrospinal fluid (CSF) analysis and titers to differentiate infectious agents. Each disorder is characterized in the CSF by primarily a mononuclear pleocytosis consisting of lymphocytes, macrophages, monocytoid cells, and lesser numbers of neutrophils. The increase in mononuclear cell counts, primarily lymphocytes, is similar in these diseases.\(^1\)\(^-\)\(^3\), \(^5\)\(^-\)\(^11\) Lymphoblast and prolymphocyte-like cells have been noted in CSF from canine distemper cases and malignant lymphoma. Total protein content is usually elevated. Measuring titers for infectious diseases may be complicated by peripheral blood and serum contamination of the samples. In many cases, the cellular composition of the CSF associated with these diseases is quite similar, making antemortem diagnosis, treatment and prognostication difficult. We hypothesize that diseases producing mononuclear pleocytosis are distinct and can be distinguished from each other by using immunohistochemical typing of lymphocytes and monocyte/macrophages. This retrospective study will correlate immunohistochemical staining of T and B lymphocytes and
monocytes/macrophages in CNS tissues to postmortem disease diagnosis using light microscopy. The objectives of this study are to help differentiate these inflammatory, infectious and neoplastic diseases immunohistochemically and to aid in treatment protocols and prognostication for each disease. This research will aid investigations into the etiology, primary cell type and possible mechanisms of GME. Also, some of these canine diseases may serve as a model for other human CNS disorders such as multiple sclerosis and Lyme neuroborreliosis where cytologic findings are similar to the inflammatory diseases discussed above.

Thesis Organization

This thesis contains four chapters: Chapter 1, A General Introduction which outlines the problem being researched and the objectives of the study, and the thesis organization; Chapter 2, a separate Literature Review of the previous work on the subject to be submitted to the Journal of Veterinary Internal Medicine; Chapter 3, a manuscript that is being submitted to the journal "Veterinary Pathology"; and Chapter 4, a final chapter including General Conclusions.

Chapter 1 - The first chapter consists of a general introduction to the problem of differentiating CNS inflammatory and infectious disease in the canine and how immunohistochemistry plays a role in the differentiation of these disorders. Included also is the thesis organization.

Chapter 2 - The second chapter is the literature review that details the many uses of immunohistochemistry, the different methods used and their use to differentiate those canine CNS inflammatory and infectious disorders that produce a mononuclear pleocytosis. These diseases, their pathophysiology, histologic and
immunohistochemical characteristics are discussed. This manuscript is prepared for submission to the *Journal of Veterinary Internal Medicine*.  

**Chapter 3** - In this manuscript, we hypothesize that canine central nervous system (CNS) inflammatory and infectious diseases producing mononuclear pleocytosis can be differentiated using immunohistochemistry. Disease categories include granulomatous meningoencephalomyelitis (GME), canine distemper virus, toxoplasmosis, neosporosis, malignant spinal cord lymphoma, and unclassified CNS inflammation. Immunohistochemical staining (CD3, lysozyme, anti-canine IgG, M, A) was performed. Results were compared and contrasted to the literature. Considerations for future research include the use of immunohistochemical methods for CSF samples to aid in antemortem diagnosis of these diseases. This manuscript is prepared for submission to the journal, *Veterinary Pathology*.  

**Chapter 4** - Includes a general discussion of the problems in differentiating canine CNS inflammatory and infectious diseases that produce mononuclear pleocytosis and recommendations for future research into the role of immunohistochemistry in antemortem diagnosis of these diseases. References will be presented at the end of each chapter. Figures and tables with legends will be presented at the end of Chapters 2 and 3.
References


CHAPTER 2. LITERATURE REVIEW - THE USE OF IMMUNOHISTOCHEMICAL TECHNIQUES TO DIFFERENTIATE CANINE CNS INFLAMMATORY AND INFECTIOUS DISEASES PRODUCING MONONUCLEAR PLEOCYTOSIS

A paper to be submitted to the Journal of Veterinary Internal Medicine

Karen L. Kline, Claire Andreasen

Abstract

Central nervous system (CNS) inflammatory diseases are common in the canine population, and the collection of cerebrospinal fluid (CSF) is used to correlate cytologic findings with disease etiology. The most common of these diseases is granulomatous meningoencephalomyelitis (GME). The etiology of GME is still unknown, although immune-mediated, neoplastic and infectious pathogeneses are proposed. Differentiation of GME from other inflammatory and neoplastic CNS diseases, including canine distemper virus (CDV), encephalitis, rickettsial (Ehrlichia canis and Rickettsia rickettsii) meningitis, and protozoal (Neospora caninum and Toxoplasma gondii) CNS disease can be difficult. Clinical neurologic signs may be quite similar among cases. Each disorder is characterized by a primarily mononuclear CSF pleocytosis consisting of lymphocytes, macrophages, and lesser numbers of neutrophils. Immunohistochemical techniques have been used in some of these diseases (GME and CDV) to identify B and T cell and macrophage subpopulations and to correlate cell population to disease diagnosis. This review focuses on a discussion of canine CNS inflammatory and
infectious diseases, proposed pathogeneses, neurologic signs, diagnostic findings, histopathologic lesions, and how current immunohistochemical techniques may aid in their characterization and differentiation.

Key words: Granulomatous meningoencephalomyelitis; canine distemper; protozoal infection, rickettsial infection

Discussion

Inflammatory and infectious diseases of the CNS in the canine have been discussed in the literature for a number of years. Examples of these include granulomatous meningoencephalomyelitis (GME), pug encephalitis, and necrotizing meningitis of the Yorkshire terrier and Maltese breeds. Infectious agents include canine distemper virus, the rickettsial agents *Ehrlichia canis* and *Rickettsia rickettsii* (RMSF), and protozoal agents (*Toxoplasma gondii* and *Neospora caninum*). Clinical neurologic signs can vary as focal or multifocal, with all portions of the central nervous system being affected. Some of these diseases also manifest more systemic signs, especially infectious agents such as rickettsial agents.

Diagnostic evaluation of dogs suspected to have inflammatory or infectious CNS disease relies primarily on patient signalment, an accurate history, recognition of abnormal clinical and neurologic signs, minimum data base results, and ancillary testing, such as CSF analysis and diagnostic imaging such as CT scan or MRI. The antemortem differentiation based upon CSF analysis, can be quite difficult, in many cases, since the CSF contains a mixed population of inflammatory cells that are primarily mononuclear with lesser numbers of neutrophils (Fig 1). In addition,
CSF findings can vary with timing of sampling and chronicity of the disease process. Because of this, differentiation of these diseases can be challenging, especially, since treatment protocols used for one disease may not be the best for another. The purpose of this review is to discuss CNS diseases that produce mononuclear pleocytosis in regard to clinical signs, proposed etiology, diagnosis, and histopathologic findings. Also, immunohistochemistry is being used to help differentiate these diseases that produce a mononuclear pleocytosis. Diseases discussed are granulomatous meningoencephalomyelitis (GME), canine distemper virus encephalitis, toxoplasmosis, neosporosis, ehrlichiosis and Rocky Mountain Spotted Fever.

**Immunohistochemistry**

In order to acquaint the reader with the potential use of immunohistochemical staining in the diagnosis of CNS diseases, a brief description will be given. Immunohistochemical staining is the detection of antigens in tissue sections and other cytologic preparations by using labeled specific antibodies so that sites of attachment become visible microscopically. Immunostaining relies on the ability of the primary antibody to bind to the antigens in the tissue specimen. Uses include diagnosis of diseases associated with autoantibodies, neoplasia, immune complex deposition, and detection of infectious microorganisms. Multiple techniques and methods used in immunohistochemistry have been described.

One of the most commonly used techniques is the indirect immunostaining method in which an enzyme-conjugated anti-immunoglobulin second antibody is
used to detect binding of the primarily antibody to a paraffinized tissue section (Fig. 2). The advantage to this technique is enhanced sensitivity of antigen detection (immunoreactivity) because several secondary antibodies will bind to each primary antibody and thus intensify the visible color produced via a chromagen. Also, indirect stains do not require conjugation of each primary antisera. An even more sensitive indirect immunoenzyme technique that amplifies the immunoreactivity is the avidin-biotin complex (ABC) method. Briefly, this technique relies upon the affinity of the B-vitamin, biotin, for avidin, an egg-white glycoprotein. Antigens bind an unlabelled primary antibody followed by a secondary antibody to an immunoglobulin labeled with biotin. After the secondary antibody is applied, the tissue is exposed to preformed avidin and biotin complexes and these complexes bind to tissue-associated biotinylated secondary antibody. The biotin molecules are labeled with an enzyme substrate (usually peroxidase) and a color reaction product forms after application of a chromagen, at the site of antibody-enzyme complex binding. This is visualized using light microscopy. The amplification obtained from this staining technique is helpful in detecting scarce antigens or antigens that have been affected by formalin fixation. Examples of these include stains for the T cell antigens [CD3, CD4 and CD8], lysozyme, (macrophage/histocyte antigens), and B cells (anti-canine IgG, A and M).

In summary, immunohistochemical techniques can be helpful in detection of cellular antigens. These antigens can be detected not only on neoplastic and infectious cells themselves, but can be normal constituents of white blood cells, glial cells and other neural cells found in the CNS in healthy and affected animals. Thus,
the use of immunohistochemical staining has been described in the identification and characterization of mononuclear cell populations associated with such inflammatory diseases of the CNS as GME and canine distemper virus.\textsuperscript{24-32} It is hoped that the characterization of these different mononuclear subpopulations, specifically in GME, will aid in discovering the true pathogenesis and proper treatment of these disorders, as well as learning about a possible animal (canine) model for human immune-linked neurologic diseases such as multiple sclerosis.\textsuperscript{3,34}

**CNS Diseases**

Granulomatous meningoencephalomyelitis (GME) is an inflammatory disease of the central nervous system that occurs in the canine (Table 1). This syndrome has been discussed for years in the veterinary literature and its underlying etiology has yet to be determined.\textsuperscript{1-3,6,9,11,13,32,35} Theories include an infectious cause (viral or bacterial), an immune-mediated phenomenon that may or may not be triggered by an infectious agent, or an underlying neoplastic etiology. In older literature, some cases of GME were referred to as “neoplastic reticulosis”. To date, none of these theories has been proven, although an underlying immune pathophysiology is suspected.\textsuperscript{35} Signalment of these dogs is variable, but the disease is reported to affect young to middle aged toy breeds with a greater female than male gender predilection. Most dogs are otherwise healthy, have current vaccinations, unremarkable physical examinations, and no known toxin exposure. Neurological clinical signs reflect the focal or multifocal nature of the CNS lesions. Signs can be acute or chronically progressive, and have been described as focal, disseminated and/or ocular.\textsuperscript{1,2,8,11,13,35} The ocular form is most commonly observed with the
disseminated (multifocal) form. The GME focal form mimics a focal mass that can cause forebrain (cerebral), brain stem or spinal cord signs. Forebrain clinical signs can include seizures, circling, behavior changes, proprioceptive ataxia, and hemiparesis. Neurologic examination shows contralateral partial cranial nerve deficits, conscious proprioceptive loss, vision loss, hemiparesis and hemisensory loss. Focal brainstem disease signs vary according to lesion location, but can include consciousness changes, central vestibular signs, such as ipsilateral or contralateral head tilt, complete ipsilateral cranial nerve deficits, hemiparesis, and conscious proprioceptive loss. If the medulla (myelencephalon) is involved, ventilatory changes and cardiac arrhythmias may occur. If pathologic nystagmus is present, it may be especially pronounced with a change in body position. Focal cerebellar signs may include intention tremor, dysmetria and ipsilateral menace loss. The cervical spinal cord is most often affected with focal GME, and signs can range from neck pain to concurrent conscious proprioceptive loss, tetraparesis/plegia, and loss of conscious pain perception. Focal GME has been described as slowly progressive, much like a focal mass, and is due to a focal whorling accumulation of mononuclear cells in the brain parenchyma.

Disseminated GME has been described as more acute and insidious in onset and having a poorer prognosis. Neurologic signs, as the name implies, can be multifocal with a combination of forebrain, brainstem, central vestibular, and spinal cord abnormalities. Concurrent ocular changes, include optic disk bulging and optic neuritis, can be observed in any form of GME.
Even though toy breeds are commonly affected in GME, the literature cites other dog breed-related granulomatous or mononuclear meningitis and encephalitis. Breeds include Yorkshire terriers, Maltese, and pugs (Table 1). Clinical neurologic signs observed in these breeds are similar to other dogs diagnosed with GME and it is unknown whether these are merely subsets of the same disease process or separate entities. Some speculate there is evidence of a predisposing genetic factor. A breed-specific tissue antigen could act as a receptor for an unknown virus or may have a breed-specific composition of the immune response genes that leads to an aberrant immunologic reaction toward a known pathogen. The only difference in these diseases has been the variability in histopathologic lesions observed.

The diagnostic test of choice for GME, and the pug, Yorkshire terrier and Maltese encephalitides is CSF analysis, obtained either from the cisterna magna or lumbar subarachnoid space. CSF analysis in these dogs contains a mixed population of inflammatory cells that are primarily mononuclear (lymphocytes/macrophages) with equal or lesser numbers of neutrophils (Fig. 3). CSF total protein can be normal to extremely elevated, and protein content and inflammatory cell numbers can vary due to the focal or disseminated lesions. In a few cases, CSF analysis can be within normal limits.

Histopathologic findings in GME have been described. Lesions can be focal or multifocal and consist of mild to severe perivascular mononuclear infiltrates comprised of lymphocytes, plasma cells, and macrophages involving the brain parenchyma and/or meninges (Fig 4). This can be observed alone or in
combination with mild to severe granulomatous infiltrates composed primarily of lymphocytes, plasma cells, macrophages and lesser numbers of neutrophils either in the brain parenchyma or meninges (Fig. 5). One report cites the formation of eccentrically situated nodular foci of macrophages within perivascular infiltrates as a characteristic finding. 

The histopathologic features of pug encephalitis, and necrotizing encephalitis of Maltese dogs are similar, and are different from findings in other known canine CNS infections. The predominantly mononuclear inflammation is suggestive of a viral cause, but none has been isolated. In both cases, there is a predilection for the cerebrum. A disseminated necrotizing meningitis, choroiditis and encephalitis is present in the cerebrum, with perivascular and meningeal infiltrates composed of lymphocytes, plasma cells and macrophages invading the parenchyma. Extensive focal subpial and subventricular lesions may extend deep into the parenchyma with severe microglial malformation and tissue destruction leading to cerebrocortical necrosis. Cerebral white matter can be affected, as well, with perivascular infiltrates and gliosis. Leukomalacia with liquefaction and cavitation also is observed.

The histopathologic lesions observed in Yorkshire terrier encephalitis are quite different from those in the pug and Maltese breeds. Again, a viral cause is suspected. The lesions consist of a malacic center surrounded by blood vessels with perivascular infiltrates of cells, involve the cerebral cortex and brainstem white matter, and are multifocal and destructive. Older lesions are cystic with intense astrocytic sclerosis. No causative agents have been observed microscopically nor
detected immunohistochemically in cases of GME and the encephalidites of the pug, Yorkshire terrier, and Maltese breeds.

Immunohistochemical (IHC) staining of the brain tissue classified as GME has been performed to characterize the subpopulation of lymphocytes, macrophages, and other inflammatory cells. The subpopulations of these immunohistochemically reactive cells in dogs with GME have been described in the literature. In cases of GME, IHC for canine distemper, toxoplasmosis and neosporosis has been negative. In one paper, the predominant cell populations observed in the brains of dogs with GME were CD3 antigen positive T cells with lesser numbers of IgM and IgG B cells (Figs. 6, 7). The majority of macrophages have both lysozyme and DH82 antigen, while MHC II antigen major histocompatibility complex II antigen (MHC II), was found in all inflammatory cells, pericytes and microglia within and associated with lesions. In the above study of 11 dogs, the pathogenesis of GME is theorized to be a T-cell mediated delayed-type hypersensitivity of an organ specific (brain) auto-immune disease. More studies are needed to substantiate these results. In another study of 17 dogs, IgA, IgG and IgM B cells were found to be numerous, although the comparative T cell population was not studied. The etiology of GME is still unknown, but further investigation using immunohistochemical techniques may lead to a more thorough understanding of the underlying etiologies and, thus, more specific and efficacious treatment options. To date, little has been done immunohistochemically to characterize the cellular infiltrates in pug, Maltese, and Yorkshire terrier encephalitis.
Canine distemper virus (CDV) is a contagious viral disease of the family Paramyxoviridae, genus Morbillivirus (Table 1). This virus is infectious for a number of carnivores including dogs, coyotes, wolves, as well as ferrets, minks, skunks, and weasels. Tissue tropism varies among strains and neural tissue is commonly affected. Viral transmission is more common through the aerosol route. Factors influencing virulence include viral strain, age, breed, and immunocompetence of the dog. Pathogenesis is well known and 7% of dogs that develop serum virus-neutralizing antibody titers (>1:100) within 9 to 14 days of exposure develop neurologic signs 6 to 9 weeks after initial exposure despite adequate neutralizing antibody titers. Systemic clinical signs are non-specific and include fever, diarrhea, cough, nasal discharge, decreased appetite, and weight loss. CDV has a tropism for neural tissue, particularly the white matter of the CNS with a few exceptions. Antiviral antibody and immune complex deposition may facilitate spread of the virus throughout the CNS vascular endothelium. Forebrain, brainstem, cerebellum and spinal cord may be affected and this is dependent upon the animal’s immunocompetence, age and concurrent disease. Neurologic signs commonly follow 2 to 3 weeks after the initial systemic signs and are age-dependent. Presentation varies from forebrain signs including generalized to “chewing gum type” seizures, behavior changes, and blindness to central vestibular signs, to spinal cord signs such as proprioceptive ataxia, tetra or para-paresis/plegia and generalized hyperesthesia. Myoclonus also has been described as a pathognomonic feature of CDV. One retrospective study of 38 dogs on necropsy categorized them as having either polioencephalomyelopathy (PEM),
leukoencephalomyelopathy (LEM) or equal PEM and LEM. Dogs with PEM were immature and had an acute onset of seizures (within 12 days), while dogs with PEM/LEM or LEM were mature with a longer duration (mean 23 days) of gait and central vestibular dysfunction. Other studies have classified CDV as having a definite temporal relationship to clinical signs and histopathologic findings.

Diagnosis of neurologic CDV is dependent upon history, vaccination status, and signalment of the dog, as well as, findings on physical and neurologic examination. Other key diagnostic findings are chorioretinitis (either active or inactive), enamel hypoplasia, and a predominantly lymphocytic mononuclear pleocytosis on CSF analysis, with or without an elevated CSF total protein. An elevated CSF CDV Ab titer (IgG, IgM), especially in a dog with a questionable vaccination history, is also helpful. Inclusion bodies in blood cells and/or conjunctival scrapings may or may not be seen. Histopathologic lesions of CDV vary with disease process and range from mild (acute stage) to severe (chronic stage). The primary lesions, especially in the more subacute to chronic stages, are moderate to severe mononuclear perivascular infiltrates of mononuclear cells, microglial proliferation, mononuclear pial and arachnoid meningial infiltrates, and intranuclear and/or intracytoplasmic inclusion bodies. In the acute phase of infection, demyelination may be the only lesion observed with minimal to no mononuclear cellular infiltration. This may be accompanied by gliosis, astrocytosis, vascular endothelial cell hypertrophy, and mild macrophage infiltration. Demyelination has been described as most prominent in the cerebellum, cerebellar peduncles, optic nerves, optic tracts, medullary velum
and spinal cord. It is theorized that minimal inflammatory changes observed in acute CDV infection may be due to the physiologic immaturity of the puppy’s immune system.\textsuperscript{12,17,36,38} The demyelination observed in early lesions is thought to be direct virus-induced oligodendroglial damage.\textsuperscript{40}

In mature dogs, more chronic multifocal encephalitis or encephalomyelitis may occur and may not be preceded by systemic signs of disease. As the disease becomes more subacute to chronic, mononuclear perivascular infiltrates and glial lesions become more severe and prominent. Subacute to chronic CDV infections have been termed “old dog encephalitis”. These dogs usually are older and have primarily forebrain signs such as seizures, blindness, and behavior changes, such as circling and head pressing. Histopathologic changes consist of diffuse sclerosis, increased numbers of astrocytes and microglial proliferation, and cerebrocortical neuronal degeneration. It has been theorized that CDV sequesters itself in the older dog’s CNS, even in the face of an effective immune response, and induces the secretion of inflammatory mediators by perivascular macrophages. Such mediators include O\textsubscript{2} radicals, and cytokines, which may lead to further demyelination and inflammation. This response has been called the “bystander mechanism”\textsuperscript{26,35,37,39,40-42}. In the sclerotic stage, inclusion bodies are rarely found.

Post-vaccinal CDV infection has been reported and can occur 1 to 2 weeks post-vaccination with a modified-live, attenuated vaccine. Seizures, aimless circling, blindness and aggression can be seen. Histopathologic lesions affect more gray matter, than white matter and consist of diffuse malacia of the ventral pontine region, neuronal inclusions, and mononuclear perivascular infiltrates.\textsuperscript{43,44}
The immunohistochemical characterization of CDV has demonstrated there is a definite temporal association between onset of infection, type of cell, and lesion produced (Fig. 8). As stated previously, lesions can be acute (non-inflammatory), subacute (inflammatory) chronic, or sclerosing. In the acute stage, there tends to be a diffuse up-regulation of T cells throughout the CNS and T cell invasion in early demyelinating lesions, even though there is minimal inflammation. T cell invasion has been correlated with sites of viral replication and coincides with the demonstration of an early immune response against the nucleocapsid protein of CDV. Microglial cell activation is theorized to elicit T cell migration to the CNS by secretion of cytokines, namely IL-8. In the more subacute to chronic stages of the disease, the perivascular infiltrates were composed of 60% CD3+ lymphocytes, and 40% B cells. In another study, the numbers of IgG, IgA, and IgM B cells were variable in CDV dogs with IgG and IgA increased with chronic to sclerotic lesions and mononuclear perivascular infiltrates. Another study recognized B cells in chronic lesions and their importance for intrathecal antibody production. In chronic CDV lesions, MHC II was markedly up-regulated throughout the white matter. CDV immunohistochemistry has been used to identify CDV antigen, and in one study, CDV antigen was identified in brain tissue, haired skin, nasal mucosa and footpad epithelium. The immunohistochemical detection of CDV antigen is more prominent within astrocytes in acute stages of disease than in the chronic stages.

The protozoal diseases caused by Toxoplasma gondii and Neospora caninum have been recognized as causes of central and peripheral nervous system
disease in the canine.\textsuperscript{13,51-57} Areas of the affected nervous system can include the forebrain, brainstem, cerebellum, spinal cord, nerve roots, peripheral nerve and muscle, alone or in combination (Table 1). Affected dogs can be any age or gender. The pathogenesis of these 2 diseases is dependent upon the host's immunocompetence and presence of current disease. Clinical signs are similar and can involve multiple organ systems, in addition to the nervous system. Systemic signs include fever, icterus, cough, dyspnea, gastroenteritis, and sudden death. Neurologic clinical signs are dependent upon the area of the nervous system affected, can be focal or disseminated (multifocal), and can include seizures, circling, behavior changes, central vestibular signs, spinal ataxia, hemiparesis/plegia, and generalized or focal hyperesthesia. Lower motor neuron signs and muscle atrophy, muscle pain and contracture, and hyperextension of the hind limb musculature also can be observed.

Diagnostic evaluation of a dog suspected of having neurologic protozoal disease includes signalment, thorough history, documentation of concurrent disease(s), physical and neurologic examinations, ophthalmic exams, CBC and blood chemistry, diagnostic imaging, and CSF analysis. CSF analysis can be quite variable, but commonly consists of a pleocytosis with a predominance of mononuclear cells, with lesser numbers of neutrophils and, on occasion, eosinophils. Total protein can be mildly to severely elevated. Etiologic agents are rarely seen in CSF cytology. Serologic titers in serum or CSF for protozoal agents are often negative.
The key histologic feature of CSF protozoal disease is necrosis followed by inflammation in multiple organs including the brain, spinal cord, and muscle. Nonsuppurative necrotizing encephalomyelitis, myocarditis, hepatitis and myositis have been reported.\textsuperscript{54} Encephalomyelitis can be characterized by polyradiculonuritis, ganglionitis, axonal degeneration, perivascular infiltrates, necrosis, granuloma formation, and glial nodule formation. Both white and gray matter can be affected and organisms (tachyzoites) may be seen in areas of necrosis.

Immunohistochemical differentiation of \textit{Toxoplasma gondii} from \textit{Neospora caninum} using an indirect fluorescent antibody test and peroxidase-antiperoxidase techniques have been described.\textsuperscript{52-57} This can be helpful in cases of non-specific meningoencephalitis where no organisms are found. Currently, there are no publications of immunohistochemical characterization of inflammatory cell populations found in the brains of dogs infected with these protozoal agents (Fig. 9).

The rickettsial agents \textit{Ehrlichia canis}, the ehrlichial granulocytic strains, and \textit{Rickettsia rickettsi} (the causative agent of Rocky Mountain Spotted Fever) have been associated with central nervous system signs in the canine (Table 1). The pathogenesis of these agents has been described and the common vector is the tick.\textsuperscript{13,32,58-61}

Clinical signs are multisystemic with acute or chronic progression. Fever, depression, weight loss, polyarthropathies, and bleeding tendencies, due to thrombocytopenia, have been described along with other signs of multi-organ involvement. The neurologic signs observed with both diseases are similar and can
affect any portion of the nervous system (brain, spinal cord, or neuromuscular).

Signs can be focal or multifocal and are primarily due to meningitis from inflammation and/or petechiation or hemorrhage, and include seizures, stupor, ataxia, tetra, para or hemiparesis, central vestibular signs, upper or lower motor neuron dysfunction, tremor, dysmetria, generalized or localized hyperesthesia and polymyositis.

Diagnosis of neurologic disease caused by *E. canis* or *R. rickettsii* is based upon history, hematologic results, joint fluid cytology, titers, and concurrent signs of non-neurological systemic disease. CSF analysis differs slightly between these agents. *E. canis* can cause a primarily mild to moderate mononuclear inflammatory meningitis comprised of lymphocytes, plasma cells and macrophages; total protein may be elevated. Organisms (morulae) may or may not be seen. The CSF analysis observed in RMSF is primarily a mixed pleocytosis with higher numbers of neutrophils admixed with mononuclear cells and an occasional eosinophil. Total protein may or may not be elevated, and organisms usually are not seen. Titers in CSF have been found to be equivocal depending on the acute or chronic nature of the disease and the severity of breakdown in the blood brain barrier due to inflammation.⁶⁰

Histopathologic lesions observed with ehrlichial infections range from multifocal nonsuppurative meningoencephalitis involving the cerebrum, brainstem, (ventrally and around the periventricular gray and white matter) spinal cord, and rarely the cerebellum. Concurrent ocular lesions also are described.⁶² A lymphoplasmycytic perivascular meningeal infiltrate may be observed, even in
neurologically normal dogs. Brain lesions with RMSF are similar, and consist of a necrotizing vasculitis with perivascular and meningeal polymorphonuclear and lymphocytic infiltration. In acute cases, meningoencephalitis with vasculitis and focal nodular gliosis in the parenchyma can be observed. Immunohistochemical staining on brain tissue for *Ehrlichia spp.* and *Rickettsia rickettsii* has not been described, nor has immunohistochemical characterization of the inflammatory cell populations.

In conclusion, immunohistochemistry is a useful tool in the differentiation of canine CNS inflammatory and infectious diseases. Its use on paraffin embedded CNS tissue and CSF may aid in more accurate diagnoses, and in the implementation of more useful and accurate therapeutic options and prognostication in dogs.

**Acknowledgments**

The author would like to thank Shawna Green, Elise Huffman, and the entire histopathology lab at Iowa State University Department of Veterinary Pathology for technical assistance; Jim Fosse and the Iowa State University Biomedical Communications Department for photographic and computer assistance; and Sandy Popelka for the manuscript preparation.
References


Table 1: Disease, Histologic localization, CSF Cell Type and Immunohistochemical findings in Dogs with Inflammatory/Infectious CNS Disease

<table>
<thead>
<tr>
<th>Disease</th>
<th>Histologic Location</th>
<th>CSF Cell Type</th>
<th>Described Immunohistochemical Findings</th>
</tr>
</thead>
</table>
| GME                              | Multifocal brain and cervical spinal cord | Mononuclear pleocytosis, lesser neutrophils | T cells: CD3, CD43, CD45R
B cells: Anti-canine IgA, M, G
Macrophages: Lysozyme
MHCII, DH 82
(-) CDV Ag, Toxo, Neospora |
| Pug Encephalitis                 | Multifocal brain
Primarily cerebral cortex           | Mononuclear pleocytosis           | T cells: CD3
B cells: Anticanine IgG
GFAP (Human Glial Fibrillary Acidic Protein)
(-) CDV, Toxo, Neospora Ag |
| Necrotizing Meningitis of Yorkshire Terriers | Primarily brainstem
Cerebral cortex                        | Mononuclear pleocytosis           | (-) CDV, Toxo, Neospora Ag |
| Necrotizing Meningitis of Maltese Dogs | Multifocal brain
Primarily cerebral cortex             | Mononuclear pleocytosis, neutrophils | (-) CDV, Toxo, Neospora Ag |
Table 1. (continued)

<table>
<thead>
<tr>
<th>Disease</th>
<th>Lesion</th>
<th>Cellular Infiltrate</th>
<th>Immunologic Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Canine Distemper Virus (CDV)</strong></td>
<td>Multifocal brain spinal cord</td>
<td>Mononuclear pleocytosis, Primarily lymphocytes, lymphoblasts</td>
<td>T cell: CD3, CD4, CD8, CD45RA, CD18, B cell: Anti IgG, A, M, Ki67, (+) CDV Ag (Brain, hair footpad, nasal mucosa), (-) Toxo, Neospora Ag</td>
</tr>
<tr>
<td>Rickettsials: <em>Ehrlichia canis</em></td>
<td>Multifocal CNS +/- PNS</td>
<td>Mononuclear pleocytosis +/- morulae</td>
<td>Not described</td>
</tr>
<tr>
<td><em>Rickettsia rickettsii</em></td>
<td>Multifocal CNS +/- PNS</td>
<td>Mononuclear cells neutrophils, eosinophils</td>
<td>Not described</td>
</tr>
<tr>
<td>Protozoans: <em>Toxoplasma gondii</em></td>
<td>Multifocal CNS +/- PNS</td>
<td>Mononuclear cells neutrophils eosinophils</td>
<td>(+) Toxo Ag, others not described</td>
</tr>
<tr>
<td><em>Neospora caninum</em></td>
<td>Multifocal CNS +/- PNS</td>
<td>Mononuclear cells neutrophils, eosinophils</td>
<td>(+) Neospora Ag, others not described</td>
</tr>
</tbody>
</table>

CNS Central Nervous System  
PNS Peripheral Nervous System  
Ag Antigen  
CDV Canine Distemper Virus  
Toxo Toxoplasma gondii  
GME Granulomatous Meningoencephalomyelitis
Figure 1. Cerebrospinal fluid containing a mixed population of inflammatory cells including lymphocytes, macrophages (monocytes) and lesser numbers of neutrophils. Antemortem differentiation of canine CNS diseases producing this type of inflammation presents a difficult challenge. Bar = 180 µm

Figure 2. Illustration of immunohistochemical staining using the streptavidin-biotin immunoperoxidase technique. (DAKO LSAB2 HRP systems). This technique allows more sensitive visualization of tissue antigens.

Figure 3. Cerebrospinal fluid sample from a dog diagnosed with granulomatous meningoencephalomyelitis (GME). Note the mixed population of lymphocytes and monocytes (macrophages) (arrows) with lesser numbers of neutrophils. Bar = 14 µm (100 oil mag.)
Immunohistochemical Staining

1.

2.

3.
Figure 4. Histopathologic lesion with marked multifocal perivascular infiltrates (arrow) and granuloma formation containing lymphocytes, macrophages, plasma cells and lesser number of neutrophils in a dog with granulomatous meningoencephalomyelitis. Brainstem. HE (6.3X). Bar = 100µm.

Figure 5. Magnification of Fig. 4 showing marked perivascular infiltration of inflammatory cells that extend into the surrounding neuropil. Brainstem. HE (16X) Bar = 100 µm.

Figure 6. Perivascular lesion containing CD3 antigen positive lymphocytes (arrow) in the cerebral cortex of a dog with GME. Streptavidin-biotin-immunoperoxidase technique. Hematoxylin counterstain. 16X Bar = 100 µm.

Figure 7. Cerebral cortex perivascular infiltrates containing positive immunoreactivity for lysozyme in a dog with GME. Streptavidin-biotin immunoperoxidase technique. Hematoxylin counterstain (6.3 x mag.) Bar = 100µm.

Figure 8. Cerebral cortex; canine distemper encephalitis. Diffuse infiltrate of lysozyme positive cells. Streptavidin-biotin immunoperoxidase technique. Hematoxylin counterstain (16X) Bar = 100 µm.

Figure 9. Marked perivascular infiltrates in the cerebral cortex of a dog with disseminated Toxoplasma gondii. Note the lymphocytes with positive reactivity to anticanine IgA, M, and G antigen (arrows). Streptavidin biotin immunoperoxidase technique. Hematoxylin counterstain. (16X). Bar = 100 µm.
CHAPTER 3 IMMUNOHISTOCHEMICAL COMPARISON OF MONONUCLEAR CELL POPULATIONS IN DISEASES OF THE CANINE CENTRAL NERVOUS SYSTEM

A paper to be submitted to the journal, *Veterinary Pathology*

Karen L. Kline, Claire Andreasen

Abstract

A total of 39 dogs with central nervous system (CNS) mononuclear pleocytosis, confirmed histologically were characterized immunohistochemically using antibodies against CD3, lysozyme, canine immunoglobulin (IgG, IgM, IgA), canine distemper virus, *Toxoplasma gondii*, and *Neospora caninum*. Twenty-six dogs had lesions compatible with granulomatous meningoencephalomyelitis (GME), 6 canine distemper virus (CDV), 1 toxoplasmosis, 1 neosporosis, 2 had CNS lesions of unknown cause, and 3 had malignant CNS lymphoma involving the spinal cord. All dogs were evaluated immunohistochemically for antigens to CDV, *Toxoplasma gondii* and *Neospora caninum*. Dogs ranged in age from 2 months to 14 years, and the ratio of females to males was 2:1. There was no breed predilection. CNS lesions consisted primarily of lymphocytes and macrophages in perivascular and granulomatous parenchymal and meningeal infiltrates. Macrophages and lymphocytes comprised the majority of the infiltrates, in each disease category. Dogs with GME had primarily CD3 antigen positive T cells, as well as, lysozyme positive cells. The distemper subgroup had less numerous cellular infiltrates overall, with lysozyme reactivity barely predominating over anti-canine Ig reactivity. CD3+
reactivity was minimal. The 2 dogs with toxoplasmosis and neosporosis had marked lymphocytic perivascular infiltrates that were predominantly B-cell origin, and lysozyme staining was less common, but predominated in the neosporosis case. CD 3 antigen-positive T cells were rare in this subgroup. The 2 unclassified cases had equal lysozyme, CD 3, and B cell staining. The 3 malignant spinal lymphoma cases were B-cell origin. These results demonstrate that: 1) subpopulations of mononuclear cells can be determined by assessing antigen expression and 2) different populations of mononuclear cells are present in GME, CDV, *Toxoplasma gondii* and *Neospora caninum*.

**Key Words**

CNS. Canine; Granulomatous meningoencephalomyelitis; Canine Distemper; Protozoal Diseases; Immunohistochemistry.

**Introduction**

Canine inflammatory and infectious diseases of the CNS have been difficult to diagnose and differentiate. The most unique disease is granulomatous meningoencephalomyelitis (GME), but others include canine distemper virus (CDV), protozoal diseases such as *Toxoplasma gondii* and *Neospora caninum*, and the rickettsial agents, *Erhlichia canis* and *Rickettsia rickettsi*. Theories surrounding the etiology of GME have been numerous, ranging from an immune-mediated to an infectious or neoplastic cause, although, none have been proven. As a result, the term granulomatous meningoencephalomyelitis has primarily been a descriptive morphologic term to describe the type of CNS lesions observed
histopathologically, or on antemortem diagnostics, such as cerebrospinal fluid (CSF) analysis. The difficulty is whether GME encompasses a plethora of CNS inflammatory and infectious diseases such as pug encephalitis, necrotizing encephalitis of Maltese dogs, necrotizing encephalitis of Yorkshire terriers, toxoplasmosis, or neosporosis. The challenge has been to devise more sensitive antemortem and postmortem diagnostic techniques that aid in the differentiation of these diseases. On CSF analysis, these diseases are similar resulting in a mononuclear pleocytosis with lesser numbers of neutrophils. Causative agents often are difficult to demonstrate.

The purpose of this retrospective study was to use immunohistochemical techniques to help differentiate and characterize T and B lymphocyte and monocyte/macrophage cell populations in perivascular-associated and granulomatous cellular infiltrates, to identify infectious causes, and to correlate the cell populations to disease etiology. The following disease categories were used: GME, canine distemper virus, toxoplasmosis, neosporosis, undefined CNS inflammation, and primary CNS lymphoma. The purpose of this study was to 1) assess commercially available immunohistochemical methods to differentiate the aforementioned diseases, 2) to promote more accurate and beneficial diagnostic and treatment protocols, 3) to help define the underlying pathophysiologic mechanisms of these diseases (primarily GME), and 4) to serve as a possible animal model for human diseases such as multiple sclerosis and subacute sclerosing panencephalitis.
Materials and Methods

Animal and tissue processing

A retrospective study was done on the brain and spinal cord tissue from 39 dogs. The study group consisted of 26 dogs with histopathologically confirmed granulomatus meningoencephalomyelitis (GME), 6 dogs with canine distemper virus (CDV), 1 dog with disseminated toxoplasmosis, 1 dog with disseminated neosporosis, 2 dogs with uncharacterized CNS inflammation, and 3 dogs with CNS (spinal cord) lymphoma. The spinal cord lymphoma cases were included for comparison, since CSF fluid can appear very similar in these CNS diseases. Histopathology is usually able to distinguish CNS lymphoma from other inflammatory CNS diseases. The medical records of all of the dogs were examined and tissue blocks containing brain and spinal cord sections were retrieved. All animals had histopathologic evaluation at Iowa State University College of Veterinary Medicine. Brain tissues were fixed in 10% nonbuffered formalin and embedded in paraffin. Four µm sections were stained with hematoxylin and eosin for histologic assessment and additional sections were used for immunohistochemistry (IHC). Brain tissue from one dog with no clinical or histopathologic evidence of CNS inflammation was used as a control.

Immunohistochemistry

Immunohistochemistry was done on 10% neutral buffered formalin-fixed paraffin-embedded brain or spinal cord tissue. Tissue sections were immunohistochemically stained using a streptavidin-biotin immunoperoxidase technique. Rabbit anti-serum against canine immunoglobulins (IgA, IgG and IgM,
Immunovision, diluted 1:60,000), commercial rabbit antiserum against human T cell antigen CD3+ (DAKO, diluted 1:100), and rabbit antiserum against human lysozyme (DAKO, diluted 1:800) were used (Tables 1 and 2). Controls consisted of canine jejunum and lymph node for immunoglobulins, canine lymph node for CD3+ T-cells, and canine spleen for lysozyme. Slides with 3 µm tissue sections were placed in a 57° C oven for at least 30 minutes, then deparaffinized and rehydrated by sequential immersion in xylene, graded concentrations of ethanol (100% alcohol, 95% alcohol, and 70% alcohol), then ultrapure water. Antigen retrieval was accomplished either using microwave heating or enzyme digestion (Table 2). For the microwave technique, slides were placed in a Coplin jar containing Tris (pH 10) and microwaved on full power, then 180 Watts for 5 minutes, slides cooled for 20 minutes in a -20° C freezer, and transferred to Tris buffer for 5 minutes. For enzyme digestion, slides were placed in Tris for 5 minutes at room temperature, transferred into a 0.05% protease (Protease, Sigma P5147, Type XIV) solution for 6 minutes at room temperature followed by a rinse in Tris for 5 minutes. After the microwave or enzyme digestion procedures, slides were blocked with a 10% solution of normal goat serum diluted in Tris/PBS/BSA, for 20 minutes at room temperature, incubated in a humidified chamber either for 20 minutes at room temperature for CD3 or overnight for canine immunoglobulin or lysozyme. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide (H₂O₂) twice for 10 minutes. After rinsing with Tris, the slides were then incubated at room temperature for 15 minutes with the appropriate biotinylated secondary antibody (Bio Genex’s Multilink (HK 268-UK) diluted 1:80 in Tris/PBS/BSA, rinsed, followed by horseradish peroxidase-
streptavidin (Zymed's HRP-Streptavidin (43-4323) diluted 1:200 in Tris/PBS/BSA) at room temperature for 15 minutes. Slides were rinsed with Tris for 5 minutes, and the substrate was then developed with chromagens: 3-amino-9 ethylcarbazole (AEC) for CD 3 and lysozyme or Nova Red Substrate Solution for canine immunoglobulins. Chromagen development occurred over 5 to 20 minutes. Slides were then counterstained with 1/4 strength hematoxylin. AEC slides were coverslipped using a permanent aqueous mounting medium (Crystal/Mount, Biomedia Corp. cat no. MO3). Nova Red slides, were dehydrated in 95% ethanol, absolute ethanol, and cleared with xylene, and coverslipped with a non-aqueous mounting media (Acrytol Mounting Medium (Surgipath)).

*Toxoplasma gondii* IHC

The sections were cut at 4 microns, mounted on slides, and dried in a 60°C oven for 15 minutes. All buffers and trypsin were pre-warmed to 37°C before using. Positive and negative controls were *Toxoplasma gondii* positive and negative feline brain tissue, respectively. A microprobe system was used (Fisher Scientific-catalog #15-188-20). Briefly, tissues were deparaffinized in xylene or Pro-par for a total of 10 minutes, rinsed 3 times in alcohol, placed in a 3% H₂O₂ bath for 5 minutes, then rinsed well in deionized water, and placed in a 0.1% Trypsin bath (50 ml Tris, pH 7.6 + 0.05 gm trypsin) for 30 minutes at 37°C. Then slides were washed in deionized water 5 minutes, rinsed in PBS/Tx (0.13 M), and cover plated for 30 minutes at room temperature. *T. gondii* primary antibody (DAKO 1:3000) was incubated for 2 hours. Then linking reagent (Dako II) was incubated for 20 minutes at room temperature, and then slides were rinsed and the labeling reagent (Dako II) was
placed on the slides and incubated 20 minutes at room temperature. Slides were counterstained with Gill's Hematoxylin for 10 seconds, washed for 5 minutes, dehydrated, and coverslipped. Positive slides contained brown staining of trophoblasts, scattered tissue cysts, and tachyzoites.

**Neospora caninum IHC**

Tissues were fixed in 10% neutral buffered formalin, 4 micron sections were cut, and mounted on poly-L-lysine-coated slides. Sections were dried in 60° C oven for 15 minutes, tissues were deparaffinized twice at 15 minutes, hydrated through graded ethanol and deionized water, quenched in 3% H₂O₂ for 15 minutes, and rinsed in deionized water. 0.05% protease (Sigma P-5147) for 2 minutes was used for antigen retrieval and then slides were rinsed in Tris/PBS for 5 minutes 5% rabbit serum in Tris/PBS was used for blocking. DAKO *N. caninum* antibody (1:1 dilution) was applied for 1 hour at room temperature. Slides were rinsed in Tris/PBS, and a secondary DAKO LSAB2 antibody (rabbit antigoat) applied for 10 minutes, slides rinsed again in Tris/PBS, a tertiary DAKO LSAB2 antibody was applied for 10 minutes, and the slides rinsed in Tris/PBS. 3,3 diaminobenzidine tetrahydrochloride (DAB) (DAKO) was incubated for 5 minutes, and slides rinsed with deionized water. Slides were counterstained with Gill's hematoxylin for 10-30 seconds, rinsed, dehydrated, cleared and coverslipped. Positive slides contained scattered brown chromagen-stained zoites and cysts.

**Canine Distemper Virus-IHC**

Five-µm sections of each tissue block were cut and submitted for CDV immunohistochemistry. Sections were mounted on slides coated with 0.1% poly D-
lysine stained using an avidin-biotin complex technique adapted for a robotic slide stainer using a rabbit polyclonal antiserum to measles virus. Two sections of each tissue block were stained with 1:4,000 and 1:8,000 dilutions of anti-CDV antiserum, and the third section was stained with a 1:4,000 dilution of an unrelated rabbit antiserum (negative control). A section of brain tissue from a CDV-infected dog was stained at the same time (positive control). Immunohistochemical staining for CDV was graded as either positive or negative.

**Lesion Scoring System**

The quantification of positive immunoreactive mononuclear cells was performed using light microscopy and the lesion reaction scoring system used by Kipar, et al. The numbers of immunohistochemically reactive cells (CD3+, anti-canine Ig, and lysozyme) were evaluated on each slide as follows: - (0-5% cells; negative) + (6-25% cells, single cells), ++ (26-50% cells, few cells), +++ (51-75% cells moderate number of cells), ++++ (76-100% cells, marked number of cells). A minimum of 10 perivascular or granuloma cell infiltrates from each animal were evaluated. The magnification used was 10 x.

**Results**

**Clinical Presentation (Table 3)**

**Granulomatous Meningoencephalomyelitis (GME) (n=26.)**

Dogs ranged in age from 2 months to 14 years. The ratio of females to males was approximately 2:1 (Table 3) and there was no breed predilection. Neurologic clinical signs were variable and ranged in duration from hours to weeks. Acute or chronic forebrain (cerebral) signs, such as behavior changes, seizures,
hemianopsia, contralateral partial cranial nerve deficits, proprioceptive loss and hemiparesis were observed in 11 cases. Central vestibular signs, such as an altered level of consciousness from dullness to coma, head tilt, vertical down beat and changing nystagmus, ipsilateral complete cranial nerve deficits, conscious proprioceptive loss and paresis, were observed in 3 cases. Cervical, thoracolumbar or lumbosacral spinal cord signs, such as spinal hyperpathia, proprioceptive loss and ataxia, tetra or paraparesis/plegia, were seen in 6 cases. Six dogs in this group presented with multifocal (brain and spinal cord) neurologic deficits. Fourteen dogs had cerebrospinal fluid (CSF) analysis performed (Table 3). CSF white blood cell (WBC) counts ranged from 0 to 1600/µl and the percent mononuclear cells ranged from < 10% to < 100%. After presentation to the Veterinary Teaching Hospital (VTH) 19 cases were euthanized and 7 died upon or shortly after presentation.

Canine Distemper Virus (n=6)

Six cases of canine distemper were evaluated. Dogs ranged in age from 4 weeks to 6 years (Table 3). Neurological signs consisted of behavior changes and focal to generalized seizures in 5 dogs, and myoclonus in 1 dog. One dog (case 30) exhibited blindness as a presenting complaint. This dog had been vaccinated 10 days prior to the onset of its clinical signs with a modified-live-5-way vaccine. Another dog (case 32), had not been vaccinated since puppyhood (8 weeks of age) and had localized right forebrain signs. Five of the 6 dogs were refractory to seizure management. CSF analysis was performed on cases 30 and 32 (Table 3). CSF WBC counts ranged from 64 to 318 µl, and the percent mononuclear cells ranged
from 98% to 100%. In these 2 cases, lymphocytes were the primary cell type. All 6 dogs were euthanized at the VTH.

**Uncharacterized CNS Disease (n=2)**

These two dogs presented with more protracted, insidious neurologic signs than those in the other groups. Dog 33 demonstrated abnormal conscious proprioception at the time it presented for an orthopedic evaluation. Signs progressed to circling, hypermetria and pawing at the face, consistent with multifocal CNS involvement over a period of 10 days. MRI was performed and revealed severe, diffuse forebrain edema and no focal lesions. A CSF analysis was within normal limits (WBC 4 µl, RBC 75 µl). Dog 34 was the oldest dog (14 years) and exhibited progressive left forebrain disease for several months. MRI revealed no abnormalities. The CSF was within normal limits (WBC 3µl, RBC 3350µl).

**Malignant Spinal Cord Lymphoma (n=3)**

Three dogs presented with neurological signs consistent with extradural spinal cord impingement including severe cervical neck and thoracolumbar pain. Dog 35 presented with tetraparesis and signs of a left C6-T2 myelopathy. Chest radiographs revealed a large thoracic mass, confirmed at necropsy, to invade the cervical skeletal musculature and the brachial plexus. Dog 36 presented with an acute onset of hind limb paresis. A myelogram revealed an extradural spinal cord compression at L₂-L₃. The lesion was partially resected at surgery during a hemilaminectomy. Dog 37 presented with signs consistent with a T3-L3 myelopathy (hind limb paralysis, thoracolumbar hyperpathia and bilateral proprioceptive loss). Myelography revealed an L1-2 extradural compression which was partially debulked.
surgically during a hemilaminectomy. CSF analyses on both cases 36 and 37 were acellular.

Neosporosis (n=1)

Dog 38 presented with a progressive history of hind limb weakness of 1 week duration. A T3-L3 myelopathy was suspected, and a lumbar CSF analysis and myelogram were performed. The myelogram was found to be within normal limits, but the CSF analysis had a mixed pleocytosis with 397 leukocytes/µl and 3 RBC/µl (See Table 3). The dog was placed on corticosteroids for a presumptive diagnosis of spinal GME (in the absence of observing any infectious agents, negative CSF toxoplasmosis and rickettsial titers). Three months later, the dog represented in fulminant liver failure and immunohistochemical staining confirmed *Neospora caninum*.

Toxoplasmosis (n=1)

Dog 39 presented for a week’s duration of progressive circling and falling to the right. The dog exhibited multifocal CNS signs and was euthanized by the referring veterinarian due to rapid progression of signs. No CSF analysis was performed. Immunohistochemical staining confirmed *Toxoplasma gondii*.

Histopathologic Findings

GME (Figs 1 and 2)

CNS lesions in this subgroup were multifocal in character and affected, in the majority of cases, all areas of the brain (cerebrum, thalamus, brainstem, cerebellum) and spinal cord. Specifically, 10 dogs had multifocal lesions involving brain and spinal cord; 6 had lesions affecting the brain only; 3 had lesions affecting the
cerebral cortex alone, 2 had lesions affecting the spinal cord alone; 2 the cerebral cortex and brainstem alone; and one each: the brainstem only; cerebrum and spinal cord only; and the brainstem, cerebellum, and spinal cord only. Lesions affected both the white matter (predominantly) and grey matter. The most common lesion was perivascular infiltrates that varied from mild to severe with aggregates of lymphocytes, plasma cells and macrophages. The numbers of lymphocytes, plasma cells, and macrophages were variable, but no specific mononuclear cell predominated in lesions around the vessels or in Virchow-Robin spaces. The perivascular infiltrates extended out to the leptomeninges in 9 cases. Distinct granulomas were observed in 7 cases. Other lesions included focal gliosis, (3 cases), small foci of malacia (5 cases), neuronal ischemia (1 case), hemorrhage (3 cases), evidence of foramen magnum herniation (2 cases), hydrocephalus (4 cases), cavitation (2 cases), demyelination (1 case) and axonal swelling (1 case). One case with spinal cord involvement (multifocal), showed an infiltrate of plump, round to spindle-shaped cells with an abundant foamy cytoplasm and 1 mitotic figure/per high-power field.

**Canine Distemper Virus** (Fig. 3)

Of the six cases observed, 4 had multifocal lesions in the brain only, 1 had multifocal lesions in the brain and spinal cord, and 1 had lesions in the brainstem and cerebellum only. Lesions in these 6 cases were mild, multi-focal, and occasionally coalescing with perivascular infiltrates of equal numbers of lymphocytes and plasma cells, with lesser numbers of macrophages, primarily involving the white matter neuropil, and less often the leptomeninges. White matter lesions included
the anterior medullary velum, the cerebellar folia, and the cerebellar peduncles. In addition, focal areas of mild to severe demyelination, dilated axonal sheaths, and gliosis were observed (cases 27-30 and 32). The six cases had intranuclear (case 27 and 28) and intracytoplasmic (cases 29-32) inclusion bodies.

Uncharacterized CNS Diseases

Both cases were unusual in that the histopathologic lesions were vastly different from the other cases. Case 33 contained severe, multifocal malacia of the cortical white matter with large areas of dilated axonal sheaths, and neuropil degeneration, described as primary demyelination. In addition, focal accumulations of glial cells and perivascular infiltrates composed primarily of lymphocytes, were found in the white matter (Fig. 4). There also was moderate multifocal lymphocytic and plasma cell infiltration of the leptomeninges. Case 34 had predominately white matter vacuolation in the neuropil unaccompanied by glial changes, as well as astrocyte hypertrophy described as spongiform myelinopathy.

Malignant Spinal Cord Lymphoma

Cases 35-37 had characteristic lesions of spinal lymphoma; one cervical (case 35) and the other 2 thoracolumbar (cases 36 and 37). In each case, the extradural mass was composed of sheets of round cells with irregular ovoid to slightly cleaved nuclei with prominent nucleoli (lymphoblasts). The mitotic index ranged from low (case 36) to high (cases 35 and 37).

Neosporosis. (Fig. 5)

Case 38 had a moderate to severe meningeal infiltrate of lymphocytes and macrophages with smaller foci of neutrophils and macrophages in the neuropil,
especially in those areas associated with organisms. Within the neuropil, dilated axonal sheaths and necrosis were observed associated with macrophages. Although these lesions diffusely involved the brain, the brainstem was most severely affected. White matter vacuolation was observed in the spinal cord.

**Toxoplasmosis** (Fig. 6)

In case 39, both the brain and spinal cord had multifocal lesions. The cerebral cortex and brainstem had a mononuclear meningeal infiltration consisting of lymphocytes, plasma cells, and macrophages. The brain parenchyma contained zones of nodular to diffuse gliosis, thick perivascular areas of mononuclear inflammatory cells, and foci of malacia in which occasional neurons contained protozoal tachyzoites or rosettes. In the spinal cord, there were multiple foci of perivascular inflammation, gliosis, and malacia.

**Immunohistochemistry**

Negative control brain and spinal cord tissue revealed no T, B cell or lysozyme activity. In addition, stains for canine distemper virus, *T. gondii* and *N. caninum* were all negative in control tissue. Immunohistochemical findings will be discussed according to disease category.

**Granulomatous Meningoencephalomyelitis (GME)**

Twenty-five GME cases had a majority of anti-canine IgG, IgM and IgA reactive cells in the perivascular regions (Fig. 7). Lesser numbers of positive cells were in parenchymal granulomas. Perivascular infiltrates were present in 20 cases and classified as: few (++, 26-50%) in 13 cases and moderate (+++, 51-75%) in 7 cases. Cases 1, 3, 14, and 17 had single positive (+, 6-25%) cells. No marked
(+++, 76-100%) cellular infiltrates were observed. Case 11 was negative. Anti-canine Ig reactivity in granulomas was few (+++, 26-50%) in 9 cases, moderate (+++, 51-75%) in 5 cases, marked (+++, 76-100%) in 2 cases, single (+, 6-25%) in 3 cases, and negative (-, 0-5%) in 6 cases.

In 26 GME cases, there was a more even distribution of CD3+ T-cells (Fig. 9) between perivascular infiltrates and granulomas. Marked (+++, 76-100%) T cell numbers were observed in 5 cases, moderate (+++, 51-75%) in 15 cases, few (+++, 26-50%) in 4 cases, and single (+, 6-25%) in 2 cases. Granulomas containing T cells were classified as: marked (+++, 76-100%) in 1 case, moderate (+++, 51-75%) in 16 cases, few (+++, 26-50%) in 5 cases, and negative in 4 cases.

Perivascular infiltrates and granulomas had immunoreactivity for lysozyme (Fig 12). Perivascular infiltrates had marked reactivity (+++, 76-100%) in 3 cases, moderate (+++, 51-75%) in 10 cases, few (+++, 26-50%) in 10 cases, single (+, 6-25%) in 1 case, and negative (-, 0%) in 2 cases. Lysozyme reactivity within granulomas ranged from marked (+++, 76-100%) in 3 cases, moderate (+++, 51-75%) in 9 cases, few (+++, 26-50%) in 9 cases, and negative in 5 cases. All 26 dogs had negative reactivity for Toxoplasma gondii, Neospora caninum and canine distemper IHC results.

**Canine Distemper Virus**

Of the 6 dogs with CDV evaluated for anti-canine IgM, IgG, and IgA reactivity, the majority (all 6) demonstrated positive staining in perivascular areas. Few granulomatous lesions were observed. None of the perivascular infiltrates showed marked reactivity, 2 showed moderate numbers of cells (+++, 51-75%), and 4
demonstrated few cells (++, 26-50%). Case 30 demonstrated a more pronounced granulomatous infiltrate with, few reactive cells (++, 26-50%). The remaining 4 cases were negative. T cell CD3 reactivity in the CDV dogs was minimal, both perivascularly and in granulomas. Cases 27 and 32 had single (+, 6-25%) T cell infiltrates perivascularly, while case 28 was moderate (+++, 51-75%). The remaining cases were negative. Case 28 was the only one with T cell positive staining within a granulomatous region that was moderate (+++, 51-75%). No other granulomatous regions (though minimal in numbers) had T cell reactivity.

Lysozyme staining in CDV dogs was predominantly perivascular. Moderate cell numbers (+++, 51-75%) were observed in 3 cases (Cases 27-29) (Fig. 11) and few (++, 26-50%) in 2 cases (Case 30 and 32). Case 31 was negative. Granulomatous infiltrates were rare, and only one case (case 29) had moderate (+++, 51-75%) reactivity. IHC for Toxoplasma gondii and Neospora caninum was negative in all 6 dogs. Of these 6 dogs, four had a positive CDV IHC in CNS tissues.

Uncharacterized CNS Cases

The 2 unclassified cases (cases 33 and 34) had variable results. Case 33 showed moderate (+++, 51-75%) perivascular anti-canine Ig reactivity, with negative granuloma reactivity. Case 34 had few reactive cells (++, 26-50%) in perivascular and granulomatous areas. Perivascular T cell reactivity in both cases was few cells (++, 26-50%). Lysozyme reactivity was perivascular only and ranged from few cells (++, 26-50%) in case 33 to single cells (+, 6-25%) in case 34. No granulomas were
observed. Both cases were IHC negative for *Neospora caninum*, *Toxoplasma gondii*, and CDV antigen.

**Malignant Spinal Cord Lymphoma**

The 3 spinal lymphoma cases were confirmed as B cell in origin. All had negative IHC for *Toxoplasma gondii*, *Neospora caninum* and CDV antigen.

**Neosporosis**

Case 38 had immunoglobulin reactivity both perivascularly (+++, 51-75%) with moderate numbers of cells and in numerous granulomas (+++, 51-75%). T cell reactivity was negative or absent, both perivascularly and within granulomas. Lysozyme reactivity was moderate (+++, 51-75%) both perivascularly and within granulomas (Fig. 10). IHC for *Toxoplasma gondii* and CDV antigen was negative.

**Toxoplasmosis**

Toxoplasmosis (case 39), had moderate (+++, 51-75%) canine anti-Ig reactivity both perivascularly (+++, 51-75%) and within granulomas (+++, 51-75%) (Fig. 8). T cell reactivity was minimal (+, 6-25% cells) both perivascularly (17%) and within granulomas (6%). Lysozyme reactivity was minimal perivascularly (+, 21%) and was negative in granulomatous lesions. IHC was negative for *Neospora caninum* and CDV antigen. A summary of the immunohistochemical findings is presented (See Figs. 13, 14).

**Discussion**

The inflammatory cell populations of dogs with histopathologically confirmed GME (granulomatous meningoencephalomyelitis), canine distemper virus (CDV), toxoplasmosis, and neosporosis, were characterized by immunohistochemistry.
Two other cases of unknown CNS inflammation and 3 cases of spinal cord lymphoma also were characterized.

**Granulomatous Meningoencephalomyelitis (GME)**

The lesions observed histopathologically in 26 dogs with GME were consistent with those described in the literature.\(^{5,8-10,29,31,42,55,59,61,64,67,69}\) GME has a definite tropism not only for the brain parenchyma, but for the vessels that nourish the parenchyma and meninges, leading to a breakdown in the protective blood-brain barrier. Only a few studies have immunohistochemically characterized the lesions and cellular infiltrates seen with GME.\(^{31,64,69}\) The distributions of mononuclear cells were consistent with those reported in the literature, but there were some distinct differences.

In the 26 dogs with GME, (CD3+) T cell staining was observed most often in both perivascular infiltrates and granulomas. This was followed by lysozyme and then anti-canine Ig in lesser numbers. The presence of CD3 positive T cells in GME has been associated with a possible T cell-mediated delayed-type hypersensitivity autoimmune dysfunction.\(^{31}\) The results of this study indicate this theory may be correct, based upon the high representation of CD3 positive T cells. Further research is still necessary to elucidate the sub-components involved in the possible immune-related dysfunction, i.e. specific cytokines, and other T-cell subpopulations such as CD4+, CD8+ cells. Several studies have suggested that GME is initiated by an underlying infectious mechanism (bacterial, viral, protozoal agents) that stimulates an aberrant immune-mediated inappropriate T cell response like the response seen in multiple sclerosis.\(^{5,8-10,31,42,55,59,61,64,67,69}\)
The second most observed antigen in GME was lysozyme. Lysozyme has been used as an immunohistochemical marker of histiocytic and monocytic/macrophage differentiation in human proliferative and granulomatous disorders and also has been used to study canine GME. It is a marker for monocytes, tissue macrophages, and their precursors; the percentage of positive cells increases with maturation. Some studies suggest that lysozyme may be a marker for recent blood-derived macrophages. The presence of lysozyme positive cells in GME perivascular infiltrates and granulomas may help support theories of hematogeneous and temporal origin, respectively. These cells can potentially be observed with more chronic, ongoing lesions.

Anti-canine Ig reactivity although less than CD3 and lysozyme reactivity was observed more than in other previous studies. The granulomas observed in GME had more Ig reactivity than perivascular infiltrates. The significance of this finding has to be determined and may be limited to this subpopulation of 26 cases. It is interesting to note the lesions and cell populations did not change in spite of treatment with anti-inflammatory drugs, such as corticosteroids. The significance of altered or lack of altered cell populations, due to treatment, is currently unknown.

Of the 26 GME cases, none were determined to be neoplastic origin. One study of primary reticulosis of the canine brain indicated a possible reclassification of reticulosis to primary histiocytic lymphosarcoma, in a small number of cases. In our study, there was less convincing evidence of neoplasia based upon the immunohistochemical results since the cell populations were mixed in origin.

Canine Distemper Virus
The six dogs observed with CDV-related histopathologic lesions had immunohistochemical staining that differed from dogs with GME. Overall, there were less perivascular and granulomatous cellular infiltrates than the predominantly perivascular lesions discussed in the literature.\textsuperscript{1,16,22,24,28,33,48,50,53,60-62,64,67,74} In all but two cases, granulomatous lesions were not observed. Lysozyme reactivity predominated when compared to CD3 and anti-canine Ig reactivity. One possible explanation of this would be due to the time course of infection and cellular maturation; although, some studies have identified diffuse macrophage infiltration in the later stages of CDV.\textsuperscript{60} No studies on CDV have looked at lysozyme reactivity and 3 cases had prominent perivascular reactivity. It appears that immunoglobulins play a minimal role in the pathogenesis\textsuperscript{68} and reactivity in CDV was minimal. One case demonstrated moderate perivascular Ig reactivity. T cell (CD3) reactivity also was minimal. This finding differs from other studies that describe CD3+ expression as being a key feature of CDV, dependent upon the time of infection and its clinical course. Many reports in the literature describe a definite temporal relationship in canine distemper as to type, extent and severity of lesions observed.\textsuperscript{1,28,33,48,58,65,70-72} Acute demyelination is thought to occur first with the absence of cellular infiltrates due to a period of severe immunosuppression in the absence of inflammatory changes. This is followed by the subacute phase characterized by continued demyelination accompanied by reactive changes, including astrocyte and microglial proliferation, with moderate mononuclear perivascular infiltration. The chronic phase follows which is characterized by thick mononuclear perivascular infiltrates (in one study, 60% CD3+, 40% B cells) and a severe vacuolated appearance in the
white matter (severe demyelination). Inclusion bodies may be seen in 80% of these lesions. Finally, the sclerotic phase may be observed which is composed of well-delineated marked myelin loss, astroglial proliferation, perivascular infiltrates (50% of cases), and very few viral inclusions. Immunohistochemical identification of CDV antigen is quite prominent in the acute and subacute phases, but is much less evident in the chronic and sclerotic phases. The early demyelinating stage in acute lesions has been theorized to be due to direct virus-induced oligodendioglial changes. One study describes a diffuse up-regulation of T-cells and diffuse T-cell invasion in the acute demyelinating lesions near sites of viral replication due to activation of microglia and secretion of chemokines, such as IL8. Cytokines also have been theorized as promoting mononuclear (lymphocyte and macrophage) chemotaxis, which in the more chronic stages, can lead to further cytokine release, demyelination and white matter necrosis. Some authors view this as an important role of the humoral immune system in the pathogenesis of CDV and called the "bystander effect." Theories about the acute demyelinating lesions in CDV infection are numerous and include a direct interaction of CDV with oligodendroglial cells, followed by T cell invasion. This is not thought to be a direct consequence of apoptosis or necrosis, but may be directly due to viral-induced metabolic dysfunction of oligodendiocytes or supporting cells in the acute phase of illness. Others theorize a down-regulation of myelin gene transcription in the oligodendrogliocyte that leads to decreased specific enzyme activity. The chronic stage is thought to occur due to more profound immunologic complications, although active studies are still ongoing. Another theory states, during chronic CDV infection, microglia and
astrocytes up-regulate class II MHC expression through cytokines secreted by infiltrating T cells and macrophages. This has been described as an "epiphenomenon" resulting from mononuclear cell infiltration of the CNS or a direct effect of CDV on infected neural cells, since there is limited expression of class II MHC in acute or subacute CDV cases.¹

Due to the minimal inflammatory changes observed in the 6 dogs with CDV, it is possible that the temporal theory behind the inflammatory and demyelinating lesions observed in CDV may be justified. These dogs may die in the more acute phase of CDV infection and have less profound perivascular and granulomatous infiltrates, but still have variable degrees of demyelination. Only one case (28) had moderate CD3+ expression both perivascularly and in granulomas. It is possible that this dog had a more subacute to chronic disease course. All dogs had a few cells (++, 26-50%) expressing anti-canine Ig perivascularly which differs from the literature, since B-cells were found in more subacute to chronic lesions, and tended to be associated with CD3+ positive cells.⁷² Perivascular lysozyme reactivity was moderate in 3 cases, and in one case (29), was moderate in granulomas. This is somewhat intriguing since lysozyme reactivity is associated with cellular maturation and macrophage lineage often reflecting the chronicity of a lesion. Macrophage infiltration is associated with chronic CDV. Further studies will need to be conducted on other CDV cases to observe if this is a significant finding or trend. In the one dog thought to have post-vaccinal distemper, T-cell (CD3+) reactivity was negative, lysozyme reactivity was few (++) perivascular cells, and canine anti-Ig was (++) both perivascular and in granulomas. In one study, grey matter involvement was thought
to be more prevalent in post-vaccinal CDV. In this study, lesions were most prominent in the cerebral cortex and brainstem consisting of neuronal necrosis and mononuclear perivascular infiltrates with an occasional neuronal intranuclear inclusion.

**Uncharacterized CNS Cases**

The 2 dogs with uncharacterized inflammation were negative on IHC for canine distemper virus, *Toxoplasma gondii* and *Neospora caninum*. It is known that CDV IHC is less reliable in lesions of chronic CDV and antigen can be undetected. Case 33 had both moderate perivascular infiltrates and primary demyelination, but was negative on CDV IHC in a well-vaccinated dog. Anti-canine Ig, T cell CD3, and lysozyme expression was (++, 26-50%) to (+++, 51-75%). Anti-canine Ig expression was exclusively perivascular. In light of the negative CDV IHC, this could be subacute to chronic CDV, although no viral inclusions were found. Case 34 had mild inflammatory changes, and had profound white matter vacuolation and astrocyte hypertrophy. Any similarities to GME or CDV in the literature are vague at best, although this case may fit best with the Type 4 (sclerotic) lesions of CDV.

**Malignant Spinal Cord Lymphoma**

The 3 spinal lymphoma cases supported the literature in regard to the frequency of B cell over T cell related neural lymphoma. B cell tumors are far more common.
Toxoplasmosis/Neosporosis

No reports have identified the immunohistologic characteristics of the mononuclear cell populations of toxoplasmosis and neosporosis. Outcomes of immunohistochemical staining for *Toxoplasma gondii* and *Neospora caninum* organisms have been described in the literature. There were differences between the cell populations in the 2 cases. In Case 38 (neosporosis), canine anti-Ig and lysozyme reactivity were moderate (+++, 51-76%), and no CD3+ lymphocytes were identified. Since only one case was examined, it is difficult to make any conclusions. In contrast, Case 39 (toxoplasmosis) had moderate expression of anti-canine Ig and minimal (+, 6-25%) to no CD3+ or lysozyme reactivity. In case 38, perivascular infiltrates and granulomatous mononuclear infiltrates were equally represented and marked in number. The disproportionate cell types observed in both cases could be due to enhanced humoral immunity (anti-canine Ig, Case 39), and chronicity of the inflammatory process. (lysozyme, Case 38). The absence of T cells differs from cases of GME and CDV cited in the literature. In this one case, it would appear that cell-mediated immunologic mechanisms play a lesser role in the pathogenesis of these protozoal diseases. Macrophages may, in this case, be involved in cytokine and inflammatory mediator release. More studies should be conducted to determine the roles of Class II MHC, astrocytes and microglia, as well as other immune cells. Case 39 had both perivascular and granulomatous infiltrates that could play a role in the pathogenesis of toxoplasmosis through humoral mechanisms.
The dogs with GME had a population of CD3+ cells that predominated, possibly inciting an immune response that is primarily cell-mediated. The 6 dogs with canine distemper in this study had more lysozyme and anticanine Ig reactivity with less profound perivascular infiltrates and granulomas. The significance of the lysozyme production has yet to be determined, but may be due to microglial chemotaxis in the early stages of infection. Five of the 6 dogs with CDV demonstrated minimal cellular infiltrates that were consistent with acute infection at the time of necropsy with the accompanying demyelination, but had little CD3+ expression. It would be helpful to document CD4+ and CD8+ expression in these cases to further evaluate the role of T lymphocytes in the pathogenesis of acute CDV. From our study, timing of euthanasia of these dogs probably had an effect on the lesions observed.

The 2 uncharacterized cases showed lesions that were consistent with chronic to sclerotic CDV infection, but this cannot be proven due to the absence of inclusion bodies and demonstratable CDV antigen on immunohistochemistry, and low CD3+ expression. These cases bear little resemblance immunohistochemically to cases in our study with GME that were predominantly CD3+.

The 2 dogs with neospora and toxoplasmosis had different immunohistochemical findings when compared, although each are protozoans. The key feature of each is enhanced B cell reactivity with minimal to no T cell reactivity. Lysozyme reactivity was marked in the case with neosporosis versus toxoplasmosis. The role of humoral immunity in cases of neosporosis and toxoplasmosis should be evaluated more thoroughly to understand the pathogenesis.
In conclusion, this study demonstrates that immunohistochemistry is useful in distinguishing the cell populations in dogs with GME, canine distemper virus, *Neospora caninum*, *Toxoplasma gondii*, and malignant spinal cord lymphoma. This study may stimulate further evaluation of these diseases immunohistochemically using different antibodies, and allow a potential way to differentiate these diseases antemortem using immunocytochemical techniques on CSF. Also, this study may result in an enhanced ability to utilize appropriate therapies and afford better prognostication.

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This work was supported by funds from a Small Animal Research Grant obtained from Iowa State University College of Veterinary Medicine. This work is dedicated to the patients in this study who are gone now, whose diseases we research in hopes to find a treatment or cure to end further suffering.
References


Request reprints from Dr. Karen L. Kline, Iowa State University College of Veterinary Medicine, Veterinary Clinical Sciences, South16th Street, Ames, IA 50011-1250 USA. e-mail: kkline@iastate.edu.
Table 1. Immunohistochemical markers for the characterization of canine lymphocytes and macrophages

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cell Specificity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal Antibodies (Rabbit-anti-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human CD3</td>
<td>T cells</td>
<td>DAKO</td>
</tr>
<tr>
<td>Dog IgG, IgM, IgA Whole Molecules</td>
<td>B cells, plasma cells</td>
<td>Immunovision</td>
</tr>
<tr>
<td>Human Lysozyme</td>
<td>Monocytes/Macrophages Granulocytes</td>
<td>DAKO</td>
</tr>
<tr>
<td>Toxoplasma gondii</td>
<td>Toxoplasma gondii</td>
<td>DAKO</td>
</tr>
<tr>
<td>Neospora</td>
<td>Neospora caninum</td>
<td>DAKO</td>
</tr>
<tr>
<td>Canine Distemper</td>
<td>CDV Antigen</td>
<td>Prairie Diagnostics</td>
</tr>
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Table 2. Antibodies and their dilutions used for the streptavidin-biotin immunoperoxidase technique

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Ab</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal (Rabbit - Anti)</td>
<td>Primary</td>
<td>1:100, 20 min RT</td>
<td>0.05% Protease</td>
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<tr>
<td></td>
<td>Secondary</td>
<td>1:80, 15 min RT</td>
<td></td>
</tr>
<tr>
<td>Human CD3</td>
<td>1° DAKO T cell, CD3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2° Biogenex Multilink</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog IgG, A and M</td>
<td>1° Immunoglobulins (G,M,A,H,&amp;L)</td>
<td>1:60,000 overnight</td>
<td>microwave</td>
</tr>
<tr>
<td></td>
<td>2° Biogenex Multilink</td>
<td>1:80, 15 min RT</td>
<td></td>
</tr>
<tr>
<td>Human Lysozyme</td>
<td>1° Lysozyme (Muramidase)</td>
<td>1:800, overnight</td>
<td>microwave</td>
</tr>
<tr>
<td></td>
<td>2° Biogenex Multilink</td>
<td>1:80, 15 min RT</td>
<td></td>
</tr>
<tr>
<td>Toxoplasma</td>
<td>1° DAKO Toxo</td>
<td>1:3000, 2 hours</td>
<td>0.1 % Trypsin</td>
</tr>
<tr>
<td></td>
<td>2° Linking agents (DAKO II)</td>
<td>1:200, 20 min, RT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3° Labeling agent (DAKO II)</td>
<td>1:200, 20 min, RT</td>
<td></td>
</tr>
<tr>
<td>Neospora (Monoclonal)</td>
<td>1° Neospora DAKO</td>
<td>1:1 1 hour</td>
<td>0.05% Protease</td>
</tr>
<tr>
<td></td>
<td>2° DAKO LSAB2</td>
<td>1:200, 10 min RT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3° DAKO LSAB2</td>
<td>1:200, 10 min RT</td>
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</tr>
<tr>
<td>Canine Distemper</td>
<td>1° CDV</td>
<td>1:4000, 1:8000</td>
<td>Protease XIV, (Sigma)</td>
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<td></td>
<td>2° Biotinyltated Goat antirabbit</td>
<td>1:400</td>
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</table>

RT = Room temperature
Table 3. Disease category, neurolocalization, breed, age, sex, cerebrospinal fluid (CSF) results and outcome of 39 affected dogs.

<table>
<thead>
<tr>
<th>Case</th>
<th>Disease category</th>
<th>Breed</th>
<th>Age</th>
<th>Sex</th>
<th>CSF Tap</th>
<th>Findings</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Disseminated Brain and SC</td>
<td>Chihuahua</td>
<td>3 yrs</td>
<td>FS</td>
<td>WBC 130 RBC 6600 TP 47</td>
<td>Mixed Pleocytosis Even number of cells 22% Neutrophils 35% Lymphocytes 43% Macrophages</td>
<td>Euthanasia</td>
</tr>
<tr>
<td>2</td>
<td>Disseminated Brain and SC</td>
<td>Rottweiler</td>
<td>9 yrs</td>
<td>FS</td>
<td>WBC 50 RBC 33 TP 119</td>
<td>Mixed Pleocytosis 50% Lymphocytes 48 Monocytes 2% Neutrophils</td>
<td>Euthanasia</td>
</tr>
<tr>
<td>3</td>
<td>Disseminated Brain</td>
<td>Springer Spaniel</td>
<td>5 yrs</td>
<td>FS</td>
<td>WBC 7 RBC 328 TP 31</td>
<td>Mixed Pleocytosis 81% Lymphocytes 18% Macrophages 1% Neutrophils</td>
<td>Euthanasia</td>
</tr>
<tr>
<td>4</td>
<td>Disseminated Brain</td>
<td>Dalmatian</td>
<td>6 yrs</td>
<td>MI</td>
<td>No</td>
<td></td>
<td>Euthanasia</td>
</tr>
<tr>
<td>5</td>
<td>Disseminated Brain</td>
<td>Pomeranian</td>
<td>10 mos</td>
<td>MI</td>
<td>WBC 125 RBC 8 TP 92</td>
<td>Mixed Pleocytosis Primary lymphocytes 70% Lymphocytes 25% Macrophages 5% Neutrophils</td>
<td>Euthanasia</td>
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Table 3. (continued)

<table>
<thead>
<tr>
<th>Case</th>
<th>Disease</th>
<th>Breed/Location</th>
<th>Age</th>
<th>MI</th>
<th>MCL</th>
<th>FL</th>
<th>WBC</th>
<th>RBC</th>
<th>TP</th>
<th>FS</th>
<th>WBC</th>
<th>RBC</th>
<th>TP</th>
<th>Diagnosis</th>
<th>Outcome</th>
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<tr>
<td>6</td>
<td>BS/Cerebellum</td>
<td>Maltese</td>
<td>7 yrs</td>
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<td>Euthanasia</td>
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<tr>
<td></td>
<td>Multifocal</td>
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<td>MI</td>
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<tr>
<td>7</td>
<td>Spinal Cord</td>
<td>Maltese</td>
<td>3 yrs</td>
<td>MC</td>
<td>No</td>
<td>-</td>
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<td></td>
<td></td>
<td>Euthanasia</td>
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<td>8</td>
<td>Disseminated Brain + Cervical SC</td>
<td>Brittany</td>
<td>5 yrs</td>
<td>FL</td>
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<tr>
<td>9</td>
<td>Disseminated BS &amp; SC</td>
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<td>Euthanasia</td>
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<tr>
<td>10</td>
<td>Disseminated Brain</td>
<td>Labrador</td>
<td>6 yrs</td>
<td>FS</td>
<td>WBC 576</td>
<td>RBC 26</td>
<td>TP 59</td>
<td>Primarily mononuclear Small lymphs, monocytesactivmos</td>
<td>Euthanasia</td>
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<td>RBC 4</td>
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<tr>
<td>11</td>
<td>SC</td>
<td>Boston Terrier</td>
<td>10 yrs</td>
<td>FS</td>
<td>WBC 30</td>
<td>RBC 360</td>
<td>TP 144</td>
<td>Mixed Mononuclear 70% Macrophages 30% Lymphocytes occ. Erythrophagocytosis</td>
<td>Euthanasia</td>
<td></td>
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<tr>
<td>12</td>
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<td>Shih Tzu</td>
<td>3 yrs</td>
<td>MC</td>
<td>No</td>
<td>-</td>
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<tr>
<td>13</td>
<td>Brainstem &amp; Spinal Cord</td>
<td>Poodle</td>
<td>3 yrs</td>
<td>FI</td>
<td>No</td>
<td>-</td>
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<td></td>
<td>Euthanasia</td>
</tr>
<tr>
<td>Case 14</td>
<td>Disseminated Brain &amp; SC</td>
<td>Boston Terrier</td>
<td>2 yrs</td>
<td>MI</td>
<td>WBC 360 RBC 186 TP 285</td>
<td>Mixed 85% Mononuclear 15% Neutrophils</td>
<td>Euthanasia</td>
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<tr>
<td>Case 15</td>
<td>Disseminated Brain</td>
<td>Yorkshire Terrier</td>
<td>5 yrs</td>
<td>FS</td>
<td>WBC 9 RBC 233 TP 45</td>
<td>Mononuclear 60% Macrophages 40% Lymphocytes</td>
<td>Euthanasia</td>
<td></td>
<td></td>
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<tr>
<td>Case 16</td>
<td>Brain &amp; SC</td>
<td>Shih Tzu</td>
<td>3 yrs</td>
<td>FS</td>
<td>TP 24</td>
<td>Unknown</td>
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<td>-</td>
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Table 3. (continued)

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<td>Neosporosis</td>
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<td>Case 38</td>
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<td>Bichon Frise</td>
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Figure 1  Brainstem; Case No. 23. Perivascular (arrow) and granulomatous (arrow) infiltrates composed of lymphocytes and macrophages with lesser numbers of neutrophils in a dog. HE. 6.3X Bar = 100 µm.

Figure 2  Brainstem; Case No. 23. Magnification of the perivascular infiltrate composed of lymphocytes, macrophages and lesser numbers of neutrophils in a dog with GME. HE 16X. Bar = 100 µm.

Figure 3  Cerebral Cortex; Case No. 28. Severe white matter demyelination and dilated axon sheaths that are empty (arrow) in a dog with canine distemper encephalitis. HE 16X. Bar = 100 µm.

Figure 4  Cerebral Cortex; Case No. 33. Severely dilated axon sheaths that are empty, lymphocytic perivascular infiltrates, and demyelination in a dog with uncharacterized CNS disease. HE 16X. Bar = 100 µm.

Figure 5  Cerebral cortex; Case No. 38. Dilated axon sheaths that are empty and perivascular infiltrates composed of lymphocytes, macrophages, and neutrophils in a dog with disseminated neosporosis. HE 16X. Bar = 100 µm.

Figure 6  Cerebral cortex; Case No. 39. Perivascular infiltrate composed of lymphocytes, macrophages and neutrophils in a dog with Toxoplasma encephalitis. HE 16X. Bar = 100 µm.
Figure 7. Brainstem; GME. Case No. 24. Perivascular infiltrates with lymphocytes staining positive for anti-canine IgG, M, A. Streptavidin-biotin, immunoperoxidase technique. Hematoxylin counterstain. 16X Bar = 100 µm.

Figure 8. Cerebrum; Toxoplasma encephalitis, Case No. 39. Perivascular infiltrate comprised of positive anti-canine Ig, M and A staining lymphocytes. Streptavidin-biotin immunoperoxidase technique. Hematoxylin counterstain 16 X Bar = 100 µm.

Figure 9. Cerebral cortex; GME; Case No. 25. Perivascular infiltrate with CD3+ staining lymphocytes (arrow). Streptavidin-biotin immunoperoxidase technique. Hematoxylin counterstain. 16X Bar = 100 µm.

Figure 10. Cerebral cortex; Neosporosis, Case No. 38. Granulomatous and perivascular macrophages with positive lysozyme staining. Streptavidin-biotin immunoperoxidase technique. Hematoxylin counterstain. 16X Bar = 100 µm.

Figure 11. Cerebral cortex; canine distemper encephalitis. Case No. 28. Diffuse infiltrate of lysozyme positive macrophages. Streptavidin-biotin immunoperoxidase technique. Hematoxylin counterstain. 16X Bar = 100 µm.

Figure 12. Brainstem; GME Case No. 23. Perivascular cuff and granulomas comprised of lysozyme positive macrophages. Streptavidin-biotin immunoperoxidase technique. Hematoxylin counterstain. 16 X Bar = 100 µm.
Figure 13. Absolute numbers of dogs per category with B cell (Anti-canine IgG, A and M), T cell (CD3) and lysozyme staining in perivascular infiltrates and granulomas using Kipar’s Lesion scoring method.\textsuperscript{31}
Anti-Canine IgG, A, M:
Perivascular infiltrates

Anti-Canine IgG, A, M:
Granulomas

T lymphocyte (CD3):
Perivascular infiltrates

T lymphocyte (CD3):
Granulomas

Lysozyme: Perivascular infiltrates

Lysozyme: Granulomas

Figure 13
Figure 14  Percentage of dogs per category showing B cell (Anti-canine IgG, A and M), T cell (CD3) and Lysozyme staining in perivascular infiltrates and granulomas using Kipar’s lesion scoring method.\textsuperscript{31}
Figure 14
CHAPTER 4. GENERAL CONCLUSIONS

This study used immunohistochemistry to identify CNS cell populations in inflammatory diseases that produce a mononuclear pleocytosis in CSF. These findings were compared to those animals who had CNS spinal lymphoma and uncharacterized CNS disease. Inflammatory diseases included GME, canine distemper virus, toxoplasmosis and neosporosis. Antemortem differentiation of these diseases can be difficult and can make treatment choices and prognostication difficult. This study does support the utility of immunohistochemical techniques to differentiate CNS inflammatory diseases. The antibodies used were anti-human CD3+, anti-dog IgG, M, and IgA, and anti-human lysozyme.

The 26 dogs with GME had CD3+ reactivity most often in perivascular infiltrates and granulomas, followed by lysozyme reactivity. The results correlate with other studies on the immunohistochemical characterization of GME and may help to define the role of a T cell-mediated delayed-type hypersensitivity of a brain-specific autoimmune disease. Evaluation of other T cell subpopulations such as CD4+ and CD8+, as well as, the role of inflammatory mediators, such as the cytokines, may aid in the discovery of new treatment protocols, such as immunomodulators, that are currently being investigated in the treatment of multiple sclerosis. Lysozyme reactivity in our study correlated with that observed in the literature, although MHC Class II staining was not done. These findings suggest that activated macrophages (MCH II positive), may play a role in antigen preservation and processing. IHC for CDV, toxoplasmosis and neosporosis in 26 GME cases were negative. No causative agent has been documented in GME. It is
known that the cell-mediated immune-response in this disease leads to devastating
effects in the CNS and can be refractory to current drug treatments, such as
corticosteroids. Other immunosuppressive agents and immunomodulators such as
Azathioprine (Imuran®), Cyclophosphamide (Cytoxan®), Cyclosporin and Interferon
may be of benefit.

The 6 dogs diagnosed with canine distemper had findings on
immunohistochemistry that were different from dogs with GME and dogs in the
literature with CDV. The inflammatory changes, both perivascular and
granuloma, were minimal in all cases. Granulomas were rare, and CD3+, lysozyme
and canine Ig reactivity varied, with moderate lysozyme reactivity in 3 cases and
moderate canine Ig reactivity in 2 cases. Demyelination was present in all dogs on
histopathology. In one case, T cell reactivity was moderate and was perivascular.
Inflammatory changes were minimal in the five of the 6 cases studied, although
demyelination was already occurring. Literature on neurologic CDV cites that in the
acute phases of CDV infection, minimal perivascular cell infiltrates occur and
demyelination is due to a direct viral effect (marked down-regulation of myelination
on the oligodendrocyte’s supporting cells). As the disease progresses
through the subacute, chronic, and sclerosing stages, perivascular infiltrates
becomes more prominent. Some studies indicate the acute stages have minimal to
moderate up-regulation of T cells due to the release of chemokines, such as IL8
from the neuroglial cells. MHC II expression on microglial cells, in chronic lesions, is
prominent and more evident than in the acute stages. An explanation for the
paucity of CD3+ cells in our study could have been due to the time course of
infection. Other studies suggest that CD8+ lymphocytes are more prominent in the acute/subacute lesions, and CD4+ cells are more common in the subacute to chronic lesions. The CD8+ cells are theorized to function as cytotoxic effector cells, and the CD4+ cells are thought to initiate a delayed-type hypersensitivity reaction. The presence of moderate numbers of canine Ig reactivity in 2 cases differs from another study that correlates B cell expression with more chronic lesions. Further immunohistochemical evaluation of CDV lesions on a temporal basis will need to be done to determine if cell populations differ over the disease course.

The 2 uncharacterized cases had severe demyelination (case 33) and vacuolation (Case 34), with no evidence of CDV antigen, *Neospora caninum* or *Toxoplasma gondii*. Inflammatory cells were minimal and in 1 case (33), were perivascular with canine Ig reactivity. Whether or not these two animals had CDV cannot be differentiated by immunohistochemical results, since in more chronic lesions, IHC for CDV antigen is less reliable. Also, in chronic or sclerotic cases, inclusion bodies are rarely detected. The demyelinating lesions observed were quite striking and were similar to those observed in dogs with chronic and sclerosing CDV infection.⁴,⁵

The 2 dogs with neosporosis and toxoplasmosis had immunohistochemical results that were different from those dogs with GME and CDV. Canine Ig and lysozyme reactivity were moderate in neosporosis, while CD3+ reactivity was negative. Canine Ig reactivity was moderate in the dog with toxoplasmosis, while CD3+ and lysozyme reactivity was minimal. It is possible that humoral immunity
may play a more important role in the pathogenesis of protozoal diseases than cell-mediated immunity, although, a larger number of cases would need to be studied.

This study has identified the immunohistochemical characteristics of canine CNS diseases that produce a mononuclear pleocytosis in CSF. There are similarities between these diseases immunohistochemically, but enough differences exist that further study of these diseases and their immunohistochemical properties is warranted. A continuation of this study would entail the immunocytochemical analysis of the cerebrospinal fluid (CSF) saved from the aforementioned patients compared to healthy dogs, in an attempt to correlate antemortem with postmortem disease processes. In addition, titers on CSF samples for CDV, *Toxoplasma gondii*, and *Neospora caninum* can be submitted to determine whether or not these findings correlate with the immunohistochemical and cytochemical findings. Other diagnostic tools such as polymerase chain reaction (PCR) are currently being validated to make the diagnosis of infectious agents more accurate. In addition, further research into these diseases is warranted since their pathogeneses are very similar to and can serve as animal models for such human inflammatory CNS disorders as multiple sclerosis, subacute sclerosing panencephalitis, and Lyme borreliosis.

References


