GERMAN SHEPHERD DOG DEGENERATIVE MYELOPATHY: CEREBROSPINAL FLUID ANALYSIS IN A SPONTANEOUS CANINE MODEL OF DEMYELINATING DISEASE

By

TAKASHI OJI

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2006

I dedicated this work to my parents, Mr. Toshiki Oji and Mrs. Sayoko Oji.

ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. Roger M. Clemmons, the chair-man of my supervisory committee, for his guidance, advice, and support throughout my master's project and giving me the opportunity to study under his instruction. I also would like to acknowledge my other committee members, Drs. Cheryl L. Chrisman and Rick A. Alleman, for their enthusiastic instructions and valuable discussions. I wish to thank Dr. Hiroaki Kamishina and Mrs. Jennifer A. Cheeseman for their technical assistance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

GERMAN SHEPHERD DOG DEGENERATIVE MYELOPATHY:
CEREBROSPINAL FLUID ANALYSIS IN A SPONTANEOUS CANINE MODEL OF
DEMYELINATING DISEASE

By

Takashi Oji

August 2006

Chair: Roger M. Clemmons
Major Department: Veterinary Medicine

To evaluate the pathological significance of the cerebrospinal fluid (CSF) in degenerative myelopathy (DM) of the German shepherd dog (GSD), Myelin Basic Protein (MBP) levels, anti-MBP antibody, oligoclonal band pattern, and IgG index were investigated.

The neurodegenerative diseases, primary progressive multiple sclerosis (PPMS) and German shepherd degenerative myelopathy (GSDM), appear to be similar in nature. Both are related to an immune dysfunction, both occur later in life and both are progressive spinal cord diseases once they begin. Based upon the hypothesis that GSDM and PPMS are closely related, the purpose of this thesis is to further our understanding of the relationship between these diseases by evaluating possible CSF protein changes. MBP levels were elevated in the CSF of DM patients [3.43 ± 0.45 ng/ml (sem)]. In contrast, the anti-MBP antibody was not detected in the ELISA. Oligoclonal band pattern in the CSF was demonstrated with isoelectric focusing-immunofixation in three of six GSDM (50%).

The IgG index was calculated by comparing serum and CSF IgG to albumin ratios. In GSDM, although the IgG index was normal [0.42 ± 0.17 (sem)], the detection of the oligoclonal band in the CSF suggested the intrathecal IgG synthesis.

These facts suggest the presence of active demyelinative lesions in the spinal cord of GSDM and indicate the immune-mediate etiology of GSDM. The age of onset, the time course, the location of neurologic damage, the type of neurologic pathology and the CSF change demonstrated in this study the fact that GSDM is analogous to PPMS.

CHAPTER 1
INTRODUCTION

Neurodegenerative diseases affect both man and animals leading to prolonged disability, lack of productive life and eventually death. Of these diseases, one in man, primary progressive multiple sclerosis (PPMS), and one in dogs, German shepherd degenerative myelopathy (GSDM), appear to be similar in nature. Both are related to an immune dysfunction, both occur later in life and both are progressive spinal cord diseases once they begin. In order to better understand the relationship between these 2 diseases, the following studies on cerebral spinal fluid (CSF) have been undertaken.

Clinical Significance of PPMS

Primary progressive multiple sclerosis occurs in approximately 10% of all multiple sclerosis (MS) cases (Montalban 2005; Confavreux and Vukusic 2006). It is estimated that the prevalence of MS in the US is approximately 13/10,000 people. With a current population of 295,734,134 people, then approximately 394,312 people have MS and, of those, approximately 39,431 have PPMS (13/100,000).

Clinical Significance of GSDM

Degenerative myelopathy of German shepherd was first described by Averill in 1973 as a progressive degenerative neurological disorder (Averill 1973). In contrast to the low incidence (0.19%) of DM in dogs, a high incidence (2.01%) in German shepherd dog was reported by the recent epidemiologic study. There are currently around 63,916,000 dogs in the US and of those approximately 3,124,568 are German Shepherd dogs, who represent the forth most popular dog breed of those recognized by the

American Kennel Club. If the incidence of GSDM is 2.01 percent, then there are currently around 62,804 GSDM patients in the US which is higher than PPMS in human beings (10/10,000).

Clinical Signs of PPMS

Primary Progressive MS is characterized by a gradual progression of spinal cord disease that may exacerbate but has no remissions (Bashir and Whitaker 1999; Montalban 2005). There may be periods of a leveling off of disease activity and there may be good and bad days or weeks, as with secondary progressive MS (SPMS). PPMS differs from Relapsing/Remitting MS (RRMS) and SPMS in that onset is typically in the late thirties or early forties, there is no sex preference (men are as likely women to develop PPMS), and initial disease activity is in the spinal cord and not in the brain. Primary Progressive MS may eventually progress to involve the brain, but brain damage is much less likely than RRMS or SPMS (Montalban 2005). People with PPMS do not usually develop cognitive problems (Thompson et al., 2000). Primary Progressive MS is characterized by a progressive onset of walking difficulties; steadily worsening motor dysfunctions and increased disability, but with a total lack of distinct inflammatory attacks. Fewer and smaller cerebral lesions, diffuse spinal cord damage, and axonal loss are the hallmarks of this form of PPMS. There is continuous progression of deficits and disabilities, which may quickly level off, or continue over many months and years (Ebers 2004).

As a result of the inflammatory, demyelinating process in the central nervous system, people with MS can experience a wide variety of symptoms. The most common symptoms of MS include: fatigue (also called MS lassitude to differentiate it from tiredness resulting from other causes); problems with walking; bowel and or bladder disturbances; visual problems; changes in cognitive function, including problems with

memory, attention, and problem-solving; abnormal sensations such as numbness or "pins and needles"; changes in sexual function; pain; and depression and/or mood swings. Less common symptoms include: tremor; incoordination; speech and swallowing problems; and impaired hearing. In addition to the primary symptoms caused by demyelination, there are other types of problems or complications that can occur as indirect results of the primary symptoms or the experience of having a chronic illness. Primary Progressive MS patients show those signs which are related to spinal cord involvement and less from the effects for brain involvement, including difficulty walking, urinary and fecal incontinence, pain, and paresthesia (Coyle 2001).

It is important to remember that not every person with MS experiences all of these symptoms. Some people may experience only one or two of them over the course of the disease, while others experience quite a few. Symptoms can come and go quite unpredictably, and no two people experience them in exactly the same way.

Other symptoms of MS in people are the social, vocational and emotional complications associated with the primary and secondary symptoms (Zabad et al., 2005). The diagnosis of a chronic illness can be damaging to self-esteem and self-image. A person who becomes unable to walk or drive may lose his or her livelihood. The strain of dealing with a chronic neurologic illness may disrupt personal relationships. People with MS frequently experience emotional changes as well, but it is important to note that mood swings and depression can occur as primary, secondary, or tertiary symptoms of the disease (Warren et al., 1982; Pozzilli et al., 2004).

Clinical Signs of GSDM

The age of onset GSDM were reported between 5 and 14 years (Averill 1973; Braund and Vandevelde 1978; Romatowski 1984; Barclay and Haines 1994; Johnston et

al., 2000). The clinical signs of this disease primary present an ataxia of pelvic limbs including a proprioceptive function deficit and a signs of hypermetria. The clinical signs of dogs affected with GSDM were detailed by Averill (Averill 1973). In twenty two dogs, two dogs (9.0%) presented a conscious propriocaptive deficit in thoracic limbs, while non-ambulatory status in pelvic limbs were reported in the sixteen dogs (77%); the patellar reflex was exalted in the all dogs examined. The clinical symptoms lead waxing and waning course or steadily progressive (Braund and Vandevelde 1978; Clemmons 1992). Pain sensation and urinary, fecal continent are spared until the late phase of the disease. The severe muscle atrophy of the pelvic limbs is observed simultaneously. The patient eventually develop forelimb dysfunction and brain stem involvement (Clemmons 1992). However, no cranial nerve deficit was reported in GSDM (Averill 1973).

Pathologic Findings of PPMS

The pathology of MS is thought to be secondary to an immune process directed at parts of the central nervous system. There are elevations of circulating immune complexes in MS patients and this appears to result in damage to vascular structures of the nervous system, presumably due to the concentration of antigens to which the immune complexes are directed in nervous tissue. It is not know what specific antigens are involved, but reactivity to myelin basic protein is speculated (Dasgupta et al., 1983; Dasgupta et al., 1984). Peripherally, the immune response is altered due to the presence of circulating suppressor cells which seem to be present following exacerbations of RRMS, but which tend to increase and persist in PPMS (Antel et al., 1979). The typical response to the immune dysfunction is to develop plaques of demyelination in the nervous system with increased perivascular lymphocytes in the periphery of the plaques. However, in PPMS, the onset of changes is much slower and there is an increase in

axonal loss in conjunction with the demyelination, there are fewer reactive cells in the regions of damage, and inflammatory plaques typical of RRMS are absent (Revesz et al., 1994).

Primary progressive MS is particularly difficult to diagnose, because people do not experience relapses. The standard criteria for diagnosing MS requires that there are at least two separate relapses involving different parts of the central nervous system at different times. MRI scans of people with primary progressive MS are often hard to interpret because: there are fewer lesions on the brain; it is sometimes difficult to distinguish MS scars on an MRI scan from other damage that might have been caused by normal aging; and other neurological conditions can appear similar on scan results (Montalban 2005). Therefore, a neurologist may recommend a lumbar puncture, which can help confirm the diagnosis, based upon finding elevated IgG (immunoglobulin G) in the CSF (Freedman 2004; Freedman et al., 2005).

Pathologic Findings of GSDM

Pathological change of GSDM was previously described. The lesions were recognized as a vacuolar change with astrogliosis and an oil red O-positive macrophage in the marginal zone of the white matter, including lateral corticospinal tract, vestibulospinal tract, and dorsal columns (Averill 1973; Braund and Vandevelde 1978; Johnston et al., 2000). Although the lesions were disseminated through entire segment in the spinal cord, thoracolumbar segment was mostly affected. The histological changes in the spinal cord were not related to osseous dural metaplasia and vertebral spondylosis grossly observed in aged large breed dog. The distribution and intensity of lesions are not symmetric. The pathological changes of the neural cells following to Wallian degeneration were reported by Johnston as a brain lesion of GSDM (Johnston et al.,

2000).The destruction of the dorsal root and nerve cell loss observed in the Clark's column and inter-neuron regions of the gray matter were reported by Averill (Averill 1973). As a morphologic feature of the axonal loss in the demyelinative lesion, the dying-back pathology were proposed by Griffiths et al. in other breed dogs with degenerative myelopathy (Griffiths and Duncan 1975). On the other hand, Braund et al. refuted this pathological process in dogs with GSDM based on the morphometric study of spinal cord and peripheral nerve (Braund and Vandevelde 1978).

Clinical Diagnosis of PPMS

There are no laboratory tests, symptoms, or physical findings that can determine if a person has MS. Furthermore, there are many symptoms of MS that can also be caused by other diseases. Therefore, the MS diagnosis can only be made by carefully ruling out all other possibilities.

The long-established criteria for diagnosing MS are: 1) there must be evidence of two exacerbations, flare ups, or relapses defined clinically as the sudden appearance of an MS symptoms, which lasts more than 24 hours; 2) the exacerbations must be separated in temporally and spatially; and 3) there must be no other explanation for these exacerbations (Rolak 1996). Of course, in PPMS, it is the development of chronic progressive spinal cord dysfunction without other explanation and the presence of elevated IgG in CSF.

Over the past 20 years, tests such as magnetic resonance imaging (MRI), examination of CSF, and evoked response (EP) testing have played an important role in the diagnostic process (Bashir and Whitaker 1999; Thompson et al., 2000). In 2001, the International Panel on the Diagnosis of Multiple Sclerosis issued a revised set of diagnostic criteria that have become the world wide standard (McDonald et al., 2001). In

PPMS, the MRI is less helpful and normal MRI cannot rule out a diagnosis of MS. There are also spots found in healthy individuals, particularly in older persons, which are not related to any ongoing disease process. A persistent negative MRI study in suspected RRMS patients is a reason to look for other causes (Dujmovic et al., 2004).

Clinical examinations can look for evidence of the neurologic deficits present during exacerbations or as part of the progressive disease like PPMS. Tests to evaluate mental, emotional, and language functions, movement and coordination, vision, balance, and the functions of the five senses are performed depending upon the type of MS suspected (Rot and Mesec 2006). History including sex, birthplace, family history, and age of the person when symptoms first began is also taken into consideration. It is not usually necessary to do all diagnostic tests for every patient. If, however, a clear-cut diagnosis cannot be made based on the tests above, additional tests may be ordered. These include tests of evoked potentials, cerebrospinal fluid, and blood.

Evoked potential tests are recordings of the nervous system's electrical response to the stimulation of specific sensory pathways (e.g., visual, auditory, general sensory). Because demyelination results in a slowing of response time, EPs can sometimes provide evidence of scarring along nerve pathways that is not apparent on a neurologic exam. Visual evoked potentials are considered the most useful for confirming the RRMS diagnosis, whereas spinal EPs are more helpful in PPMS (Dujmovic et al., 2004).

Cerebrospinal fluid, sampled by a spinal tap, is tested for levels of certain immune system proteins (elevated IgG levels) and for the presence of oligoclonal bands of IgG. Occasionally there are also certain proteins that are the breakdown products of myelin (myelin basic protein). These findings indicate an abnormal autoimmune response within

the central nervous system, meaning that the body is producing an immune response against itself. Oligoclonal bands are found in the spinal fluid of about 90-95% of people with MS, but less in patients with PPMS than in those with RRMS (Freedman 2004). Oligoclonal bands are present in other diseases as well, however.

Clinical Diagnosis of GSDM

No significance of the clinical diagnosis has been reported. The criteria for the clinical diagnosis of GSDM were previously described by Clemmons in 1992 (Clemmons 1992). 1) The elevation of cerebral spinal fluid (CSF) protein in the lumbar cistern. 2) The electrophysiological diagnosis is required ruling out the peripheral neuropathy and muscle abnormality. 3) Spinal cord evoked potential recording may show slight delay of the conductive velocity of thoracolumbar spinal segment caused by the demyelination. 4) Significant spinal cord compression and segmental disease, including intervertebral disk disease and vertebra tumor, are ruled out by myelography (Romatowski 1984; Clemmons 1992). In asdition, a minor disk protrusion, vertebral spondylosis, and osseous dural metaplasia are seen as non-clinically related abnormalities. In this decade, advanced imaging modalities including computer tomography (CT) and magnetic resonance imaging (MRI) are applied for the diagnosis of spinal cord disease. The characteristic aspects of GSDM were described by Jones et al (Jones et al., 2005) in CT-myelography. In this study, images were analyzed qualitatively and quantitatively. Spinal stenosis, focal attenuation of the subarachnoid space, spinal cord deformity, and paraspinal muscle atrophy were observed in GSDM significantly (Jones et al., 2005). No study of GSDM based on the MRI diagnosis has been reported at this time because of a small size of the spinal cord and an artifact related with cardiac, respiratory motion of the anesthetized animal.

Etiology of PPSM

Even though the exact cause of MS remains unknown, a combination of several factors appears to be involved (Fischman 1982). The scientific theories about the causes of MS involve immunologic, environmental, genetic, and possibly infectious factors. The latter appears less likely, but the first 3 factors due appear to work together to create individual MS risk.

It is now generally accepted that MS involves an autoimmune process directed parts of the central nervous system (Bitsch et al., 2004). The exact antigen remains unknown; however, researchers have been able to identify which immune cells are mounting the attack, some of the factors that cause them to attack, and some of the sites on which the attacking cells that appear to be attracted to the myelin to begin the destructive process (Brokstad et al., 1994; O'Connor et al., 2003; Mantegazza et al., 2004; Grigoriadis and Hadjigeorgiou 2006). The destruction of myelin as well as damage to the nerve fibers themselves, cause the nerve impulses to be slowed or halted and produce the symptoms of MS.

Migration patterns and epidemiologic studies have shown that the location at puberty seems to set the risk of developing MS, suggesting that exposure to some environmental agent around puberty may predispose a person to develop MS later on. Multiple Sclerosis is known to occur more frequently in areas that are farther from the equator. Some scientists think the reason may have something to do with vitamin D, which is thought to have a beneficial impact on immune function and may help protect against autoimmune diseases like MS.

A number of childhood viruses, bacteria and other microbes are known to cause demyelination and inflammation. It is possible that a virus or other infectious agent is the

triggering factor in MS. More than a dozen viruses and bacteria, including measles, canine distemper, human herpes virus-6, Epstein-Barr, and Chlamydia pneumonia have been or are being investigated to determine if they are involved in the development of MS, but as yet none has been definitively proven to trigger MS (Franciotta et al., 2005; Grigoriadis and Hadjigeorgiou 2006; Rima and Duprex 2006).

Multiple sclerosis is not an inherited disease in the strict sense, but there are certain genetic markers that appear to be common in MS patients, including PPMS patients. Having a relative such as a parent or sibling with MS increases an individual's risk of developing the disease several-fold above the risk for the general population. Common genetic factors have also been found in some families where there is more than one person with MS. It appears that MS develops when a person is born with a genetic predisposition and reacts to some environmental agent that triggers an autoimmune response eventually leading to MS (Haegert and Marrosu 1994).

Etiology of GSDM

The etiology and pathogenesis of GSDM are certainly unknown. The deficit of the nutritional factor including Vitamin B12 and Vitamin E were suggested by Williams et al. In this study, the small intestinal mal-absorption of the vitamins were suspected as a cause of lower concentration of these vitamins in serum (Harding et al., 1989; Salvadori et al., 2003). Besides, an abnormal vitamin E transport resulting from an impaired function of the hepatic tocopherol binding protein were suggested by Traber et al. in GSDM (Traber et al., 1993). However, a high concentration of the alpha-tocopherol in serum of GSDM were reported by Johnston et al in later study (Johnston et al., 2001). Furthermore, no significant difference were observed between GSDM and healthy control group, based on the sequence comparison of the nucleotide, amino acids, and the

mRNA expression levels of canine alpha-tocopherol (Fechner et al., 2003). Therefore, the nutritional pathogenesis of GSDM was refuted. On the other hand, the immune-mediated pathogenesis of GSDM was suggested by Waxman et al. (Waxman et al., 1980; Waxman et al., 1980). In this study, a depression of T cell response to the mitogen, including concanavalin A and phytohemagglutinin P, were reported in peripheral blood of GSDM; and a presence of the activated suppressor cell in the peripheral blood was proposing to result in a depression of T cell response (Waxman et al., 1980; Waxman, et al., 1980). In addition, the deposition of IgG and C3 to the demyelinative lesions of the spinal cord was described by Barclay et al (Barclay and Haines 1994). Thus, immune-mediated pathogenesis of GSDM was also supported by immunohisto-chemical findings.

Genetic Significance of GSDM Related to PPMS

In the recent research, the genetic similarity of GSDM with primary progressive multiple sclerosis (PPMS) were suggested based on DNA evidence. PPMS has been found to be genetic in nature and linked to the Human Leukocyte Antigen (HLA) at the DRB1 region (McDonnell et al., 1999). Analysis of the DLA-DRB1 was performed using polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and direct sequencing. RFLP analysis of the 285bp PCR product produced identical results in all dogs tested suggesting them to be homozygous for DLA-DRB1 allele*1101. Further analysis of the PCR product by sequencing confirmed the presence of DLA-DRB1 *1101, and revealed a homozygous point located at hypervariable region 2 (HVR2) of the DLA-DRB1 allele*1101 of GSD with DM (Clemmons 2006). Healthy GSD were found to be heterozygous at this point suggesting the homozygous point to be unique in DM. The myelin basic protein (MBP) allele consisting of a 70 bp tandem repeat (TGGA) deletion was found in 88% of DM dogs tested and only in 57% of healthy GSD

(Clemmons 2006). This deletion correlates to the same deletion found in a population of multiple sclerosis (MS) patients in Finland (Tienari et al., 1994; Tienari et al., 1998).We do not find changes in the HFE, TGFβ1 region, but there are alternation in the apoE and IL4R regions. The former is thought to be related to RRMS, while the later 2 are associated with PPMS (McDonnell et al., 2000; Ramsaransing et al., 2005; Ristic et al., 2005). Clearly, these areas in people are only associated with, but not necessarily diagnostic of the disease; but we do find similar changes in the GSDM patients. Using random repeat primers from Amersham, a number of changes in DNA of dogs with GSDM have been found which have not completely characterized. These changes are reproducible and do fit the patients who we can diagnose clinically as having DM by available neurologic tests. So, looking for genetic changes which have been found in MS patients in dogs with GSDM leads to a number of findings that support the hypothesis that GSDM is analogous to PPMS.

Purpose of Thesis Research

Based upon the hypothesis that GSDM and PPMS are closely related, the purpose of this thesis is to further our understanding of the relationship between these diseases by evaluating possible CSF protein changes. To that end, the project will specifically evaluate levels and nature of IgG in CSF of normal and GSDM patients and evaluate MBP concentrations in CSF of normal and GSDM patients. We expect that changes in GSDM will parallel PPMS patients and further advance GSDM as an animal model of PPMS.

CHAPTER 2
MEASUREMENT OF MYELIN BASIC PROTEIN IN THE CEREBROSPINAL FLUID OF DOGS WITH DEGENERATIVE MYELOPATHY

Introduction

CSF analysis has been established as a diagnostic method in the case of neurological disorders. Total protein determination, cell count, leukocyte differentiation, and antigen or antibody of the infectious disease are routinely used for the clinical diagnosis in veterinary medicine (Vandevelde and Spano 1977; Chrisman 1992; Tipold et al., 1993). The evaluation of CSF gives information of blood-brain barrier (BBB) integrity and the existence of inflammation/infection in the central nervous system (CNS). In human medicine, in Alzheimer disease, a number of protein biomarkers of the CSF have been used to confirm the clinical diagnosis (Blennow 2004).

Myelin basic protein (MBP) is a protein restricted to the nervous system. This protein composes 30 % of total protein in the myelin sheath and is encoded by a single gene normally expressed by oligodendrocytes. The isoforms of molecular weight 21.5kD, 18.5 kD and 14.5kD were reported in mammals; and the 170 amino acid residue dominate is contained in adult human CNS myelin (Whitaker 1978) . The presence of MBP in CSF was reported in several investigations of active demyelinative disorders and CNS injury accompanied by myelin damage (Whitaker et al., 1980; Whitaker 1998). The elevation of MBP in CSF is not disease specific, but signifies the existence of demyelinative lesions in CNS. Therefore, MBP has been used for a disease marker of demyelinative CNS disorders (Ohta et al., 2000; Lim et al., 2005).

A chronic demyelinative disorder, Degenerative Myelopathy (DM), was previously described in German shepherd dogs (GSDM) (Averill 1973; Clemmons 1992). The clinical signs of GSDM commonly arise at the age of 5 to 7 years with a slowly progressive course of six month to one year. Although histological findings reveal the loss of myelin sheath and axon in white matter, the etiology and pathogenesis of GSDM have been unknown. Clinical pathologic findings are usually normal except for an elevated CSF protein in the lumbar cistern. Therefore, the diagnosis of GSDM is usually made by ruling out other diseases affecting the spinal cord (Clemmons 1992).

The objective of this study was to evaluate a method for the determination of MBP concentrations in the CSF of dogs with German shepherd degenerative myelopathy using a human MBP ELISA based assay.

Materials and Methods

Nine German Shepherd Dogs, ranging from 5 years 3 months to 12 years in age (median 8 years 10 months) were included in this study. These dogs were presented to the Neurology service at the Veterinary Medical Center of the College of Veterinary Medicine, University of Florida and clinically diagnosed as GSDM based on the criteria previously described (Clemmons 1992). Normal CSF samples were collected from 8 mongrel canine cadaver euthanized at the local animal shelter. These samples were collected immediately after euthanasia. 9 DM dogs were administrated general anesthesia during the CSF collection. CSF was sampled from the cisterna magna and lumbar cistern by 18G spinal needle. All CSF samples were centrifuged at 14,000 rpm for 10 minutes and frozen at -20°C until used. Brain tissues were obtained from canine cadavers. The use of these animals was approved by the Institutional Animal Care and Use Committee of the University of Florida (IACUC protocol number E335).

Canine MBP was extracted by organic concentration (Maatta et al., 1997). Frozen canine brain was homogenized in chloroform and separated by centrifugation. Collected chloroform was washed and methanol was added. Following brief mixture, the acidic aqueous phase was collected by adding 1M HCl and desalted by Sepharose column (PD-10 desalting column, Amersham Biosciences Corporation, Piscataway, NJ) The concentration of 50µg/ml of MBP was isolated.

In order to assess the cross-reactivity of ELISA assay, the reactivity of anti-human MBP antibody to canine MBP was first tested with Western blotting. Bovine MBP (Myelin basic protein from bovine brain, Sigma, St. Louis, MS) was used as a molecular weight control. Isolated canine MBP was separated in 15 % SDS- polyacrylamide gels and transferred to nitrocellulose membranes (Nitrocellulose membrane, Bio-Rad Laboratories, Inc. Hercules, CA) followed by the blocking with TBS containing 3% BSA and 0.1% Tween20 at 4°C overnight. Membranes were, then, incubated with biotin conjugated anti-human MBP antibody (Goat anti-human MBP polyclonal antibody, Diagnostic Systems Laboratories, Inc, Webster, TX) (1:15,000) overnight at 4°C. After washing with TTBS (0.05% Tween20 in TBS), the membranes were incubated with horse-radish peroxidase (HPR) conjugated streptavidin for 1 hour at RT. The membranes were washed three times in TTBS and developed in 4CN substrate.

The concentration of MBP in CSF was determined with human MBP ELISA (Active® MBP ELISA, Diagnostic Systems Laboratories, Inc, Webster, TX) following manufacture's instruction. Human MBP was employed to produce the standard curve and served as a detection control. The absorbance at 450nm was recorded by a microplate

reader (EL340 Biokinetics Reader, Bio-Tek Instruments, Winooski, VT). Coefficient variance (CV) was calculated to evaluate the reproducibility of the assay.

The amounts of protein in the CSF were determined by Bradford protein assay kit (Bio-Rad Protein assay, Bio-Rad Laboratories, Inc. Hercules, CA,).

The reference range of total protein in the CSF was described as the mean values of the normal group with 95% confidence intervals. Turkey-Kramer HSD was used for all pair's comparison. $P<0.05$ was considered statistically significant. The results are given as mean and ± SEM.

Results

The age, sex, duration of the clinical signs, nuclear cell counts, and total protein concentrations in the CSF were summarized in table 2-1. The age of dogs with GSDM were ranged between 5 years 3 months and 12 years (median 8 years 10 month) and male dogs were likely more affected than female in the current study. The duration of the clinical symptoms was between 5 weeks and 1 year 3 months. The numbers of nuclear cell in the CSF were within the reference range (>5 cells/µl) except for the CSF obtained from lumbar cistern of dog No. 7. The mean value of total protein concentrations of the CSF obtained from lumber cistern were significantly elevated in GSDM (Figure 2-1).

A result of Western blotting was shown in figure. 2-2. Cross reactivity of anti-human MBP antibody to isolated canine MBP was proven. A band visible in Coomassie staining reacted with the polyclonal anti-human MBP anti-body used in the human MBP ELISA, showing an expected molecular site of around 18.5kDa.

MBP concentration of canine CSF samples was estimated based on the standard curve. CSF samples obtained from the cisterna magna of GSDM were higher (1.38±2.06) than that of normal dogs (0.47±0.06), although no significance was observed in these two

groups. CSF from lumber cistern of GSDM dogs presented significantly higher concentration of MBP (3.43±1.54) than that of normal control groups (0.58±0.11). (Figure 2-4) The reproducibility was confirmed by a detection control in each assay (CV=8.68).

Discussion

The application of the commercial human MBP ELISA for canine CSF was first evaluated in this study. MBP isolated from canine brain tissue sufficiently reacted with the polyclonal anti-human MBP antibody used in the MBP ELISA. A dose dependent reaction was observed in the ELISA as well (Figure 2-3). Therefore, we suggest that the human MBP ELISA based assay is sufficiently sensitive and specific enough to determine the concentration of MBP in canine CSF. Immunochemical cross-reactivity of MBP among various species was also previously described (Whitaker 1978).

Nine German shepherd dog patients with chronic symptoms of upper-motor neuron hind limbs ataxia were subjected in this study. Spinal cord compression was ruled out by myelography in all cases. In addition, no abnormal muscle discharge and no peripheral nerve conductive delay were observed in the electrophysiology. In the routine CSF examination, one dog had pleocytosis in the CSF collected from the lumbar cistern (17cells/μl; reference range <5 cells/μl). From the red blood cell count and cell differentiation, however, slight blood contamination was suspected in this case. An elevation of total protein was observed in three CSF samples obtained from the lumbar cistern (49.5, 56.6, and 52.3mg/ml; reference range <45mg/ml). No other abnormality was observed in the CSF examination. Hence, all dogs were exclusively diagnosed with a degenerative myelopathy without histological confirmation. Eight canine cadavers were

employed as a normal group. The CSF samples were collected immediately after euthanasia. No elevation of the total protein was observed in normal group CSF.

Demyelination has been recognized as one of the most characteristic features in GSDM. However, the diagnosis is usually made by ruling out other diseases affecting spinal cord and no direct information concerning the myelin sheath has been reported.

In human, MBP has been used as a biochemical marker of myelin damage (Alling et al., 1980; Whitaker et al., 1980; Whitaker 1998). The elevation of MBP in the CSF is observed following the damage of the myelin sheath in various neurological diseases and becomes undetectable within 10 to 14 days after myelin damage (Ohta et al., 2000; Lim et al., 2005). As we hypothesized, significantly increased concentration of MBP were observed in CSF collected from the lumbar cistern of GSDM, but not in the cisterna magna. It suggests the presence of an active demyelinative lesion restricted to the spinal column. Therefore, we propose that the elevation of MBP in CSF is direct evidence of active demyelination in GSDM. An immune-mediated reaction was previously reported as an etiologic factor of demyelinative lesions in GSDM (Waxman et al., 1980; Waxman et al., 1980; Barclay and Haines 1994). It may cause a chronic active demyelination in this disease. Although no other diseases were tested in this study, this finding may be different with chronic non-active spinal cord disorders such as intervertebral disk protrusion.

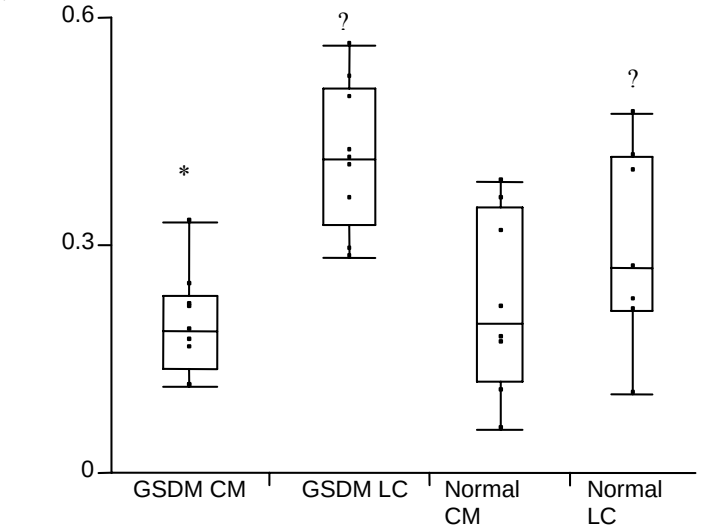
In small animal medicine, the concentrations of MBP in CSF have been measured by a canine MBP coated ELISA assay using experimental canine distemper virus (CDV) infection; and the correlation between MBP levels and histological findings was reported (Summers et al., 1987). In the clinical diagnosis, however, it is not pragmatic to develop

canine derived ELISA for the laboratory test. Moreover, no other clinical studies describe MBP concentration in CSF within canine disease groups. In the current study, we presented a validation of the human MBP ELISA to determine the MBP concentration in canine CSF and suggested the presence of an active demyelinative lesion in GSDM. In order to elucidate the diagnostic significance of MBP further studies such as the analysis in other differential neurological disorders would be recommended.

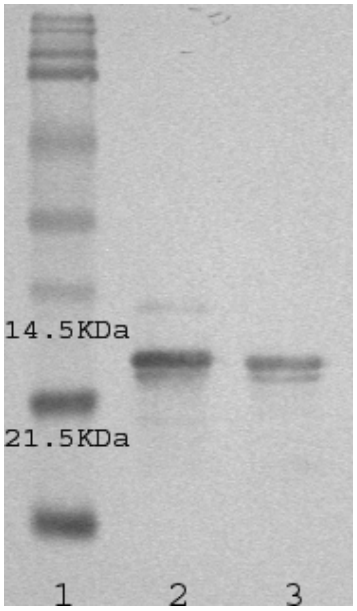
Table 2-1. Clinical observations and CSF appearances

Dog	age	sex	CM NC	CM* protein	LC NC	LC# protein	Duration of clinical sign	CM MBP	LC MBP
1	8y10m	M	1	33.3	0	42.6	5 weeks	6.86	5.95
2	5y3m	M	0	11.4	0	29.5	3 months	0.68	3.33
3	8y3m	M	3	22.1	17	49.5	4 months	0.88	1.77
4	11y3m	sF	0	18.8	0	40.5	15months	0.38	5.94
5	10y 7m	cM	1	16.5	N/A	28.4	6 months	0.81	3.36
6	6y6m	sF	0	21.8	2	56.6	4 month	0.6	3.36
7	12y	cM	1	17.6	0	41.6	12 months	0.73	1.81
8	9y6m	sF	0	11.4	0	36.3	7 months	0.81	2.84
9	5y 6m	cM	3	24.7	3	52.3	2 months	0.67	2.59

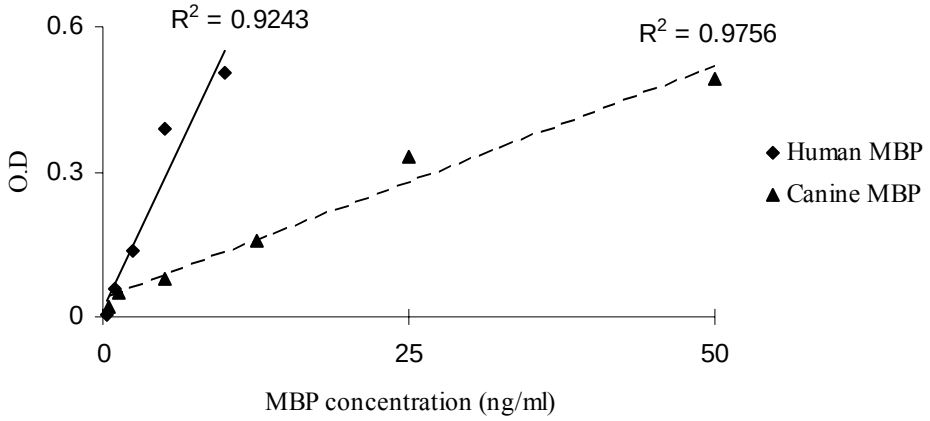
CM = cisterna magna; LC = lumber cistern; NC = nuclear cell count; MBP = myelin basic protein; reference range, * < 32.4 mg/dl, #< 42.5mg/dl



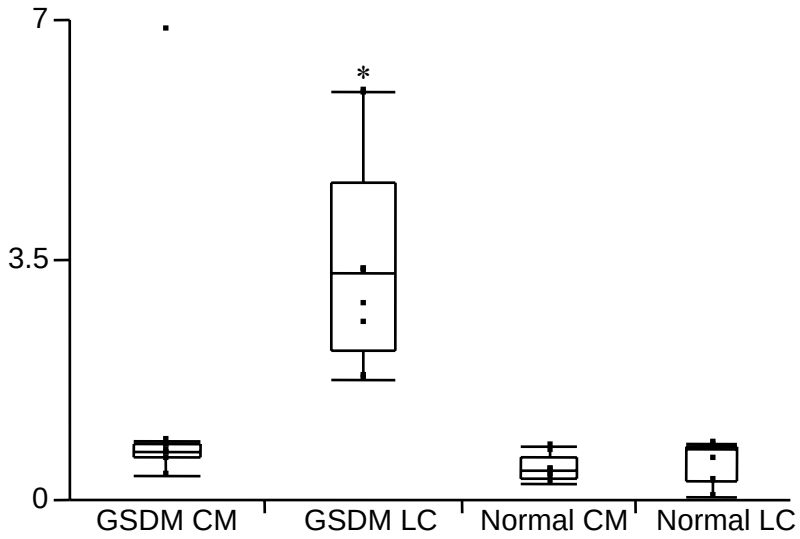
GSDM = German Shepherd degenerative myelopathy
CM = cisterna magna, LC = lumber cistern
* Significantly different. p<.01.
♦ Significantly different. p<.05.
Figure 2-1. Medians and ranges of total protein concentration (mg/ml) in the CSF.



Lane 1, Broad range molecular weight markers
Lane 2, Bovine MBP (18.5KDa)
Lane 3, Isolated canine MBP
Figure 2-2. The cross-reactivity of the anti-human MBP (myelin basic protein) to the canine MBP was demonstrated by immunoblotting.



MBP = myelin basic protein, O.D. = optical density
Figure 2-3. Sensitivity (O.D.) of the isolated canine MBP in the human MBP ELISA.



CM = cisterna magna, LC = lumber cistern.
* Significantly different. $p<.01$.

Figure 2-4. Medians and ranges of the MBP concentration (ng/ml) in the cerebrospinal fluid (CSF) of 9German shepherd degenerative myelopathy (GSDM) and normal dogs

CHAPTER 3
ANTI-MBP ANTIBODY DETECTION IN THE CSF WITH GSDM

Introduction

As an immunodominant T cell antigen, the injection of MBP into experimental animals induces similar demyelinative lesion to multiple sclerosis (Kies et al., 1965). In human, therefore, the presence of the anti-MBP antibody has been suggested as a cause of the immune-mediated reaction in demyelinative neurological disorders. However, the results of reports regarding the autoantibody against MBP in the CSF of MS patients has varied widely (Garcia-Merino et al., 1986; Reindl et al., 1999; Chamczuk et al., 2002; Berger et al., 2003; O'Connor et al., 2003).

In veterinary literature, Vandevelde et al. reported the presence of the antibody against MBP in the serum and CSF of dogs with canine distemper encephalitis (Vandevelde et al., 1982). They proposed that the humoral immunological reaction caused the demyelinative lesions in canine distemper virus infection.

In GSDM, an immune-mediated reaction to the CNS has been suspected as an etiology of the demyelinative lesions and the deposition of the IgG and complement in the lesions were reported (Waxman et al., 1980; Waxman et al., 1980; Barclay and Haines 1994). However, no direct evidence supporting the presence of autoantibody has been reported.

The purpose of this study was to demonstrate the presence of anti-MBP autoantibody in canine CSF for the evaluation of autoimmune mediated pathoetiology in GSDM.

Materials and Methods

Sample population and preparation applied to this study were described in chapter 2. The CSF obtained from the lumbar cistern was employed.

In order to assess the presence of the anti-MBP antibody in canine CSF, we first developed an ELISA based assay. In brief, Microtiter Plates (MaxiSorp™, Nunc, Rochester, NY) were coated with 100ul of 5ug/ml solution of bovine MBP in 50mM carbonate/bicarbonate buffer pH 9.6. The plate was incubated overnight at 4 C. After coating, the wells were washed three times with 50mM Tris-Buffered Saline (TBS), and then blocked with 50mM TBS containing 1% bovine serum albumin (BSA) for one hour at room temperature. After washing, 100ul of the CSF samples were applied to the wells. For the standard curve creation, the anti-human MBP antibody (monoclonal mouse anti-Myelin basic protein, Hytest ltd, Turku, Finrand) diluted to 16.4, 32.81, 65.63, 131.25, and 262.5ng/ml in distilled water were applied. The plate was incubated for one hour at room temperature and washed five times, and reacted with ALP-conjugated anti-Dog IgG diluted 1:30,000 or ALP conjugated anti-mouse IgG diluted 1:30,000 for one hour at room temperature. After washes five times, the plate was developed with p-NPP substrate and quantitated by plate reader at 405nm.

Result

Monoclonal anti-human MBP antibodies used for the standard curve were sufficiently reacted in the ELISA (Figure 3-1). However, no significant reaction was observed in canine CSF from normal dogs and dogs diagnosed GSDM (Figure 3-2).

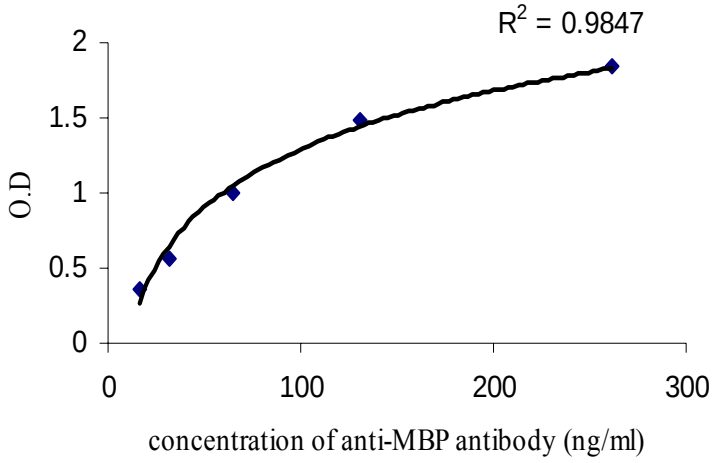
Discussion

In the current study, although we hypothesized the presence of the autoantibody against the myelin sheath in the CSF of GSDM, no anti-MBP antibody was detected.

However, it was unfeasible to suggest the inexistence of autoantibody in the CSF due to the lack of the precise positive control. For the immunological detection of the autoantibody, the bovine MBP was used as a coating antigen in our study. In contrast to the anti-human MBP antibody used for the standard curve, the autoantibody in canine CSF may not react with bovine MBP. Moreover, in direct ELISA based assay, the antigen is immobilized on the plastic plate. It may result in the loss of epitopes detected by the antibodies. Therefore, the autoantibody against MBP in canine CSF may be undetectable in this method if it presented in dogs with GSDM. Also, it may be possible to detect the anti-MBP antibody in an ELISA and immunoblotting with precise canine MBP as a test antigen.

In order to elucidate the immune-mediated etiology of GSDM, a further study would be recommended. In multiple sclerosis, other proteins including myelin oligodedrocyte glycoprotein (MOG), myelin associated glycoprotein (MAG), and proteolipid protein (PLP) have been predicted as a target antigen of an autoantibody (Reindl et al., 1999; O'Connor et al., 2003).

Finally, the etiology of GSDM was not assessed in the present study because of a negative result in the anti-MBP antibody assay. In addition to the improvement of the methodology for immune-detection, the other etiologic approach such as genetic DNA analysis is recommended in the future study.



O.D. = optical density, MBP = myelin basic protein
Figure 3-1. Standard curve of monoclonal anti-human MBP antibody in the ELISA

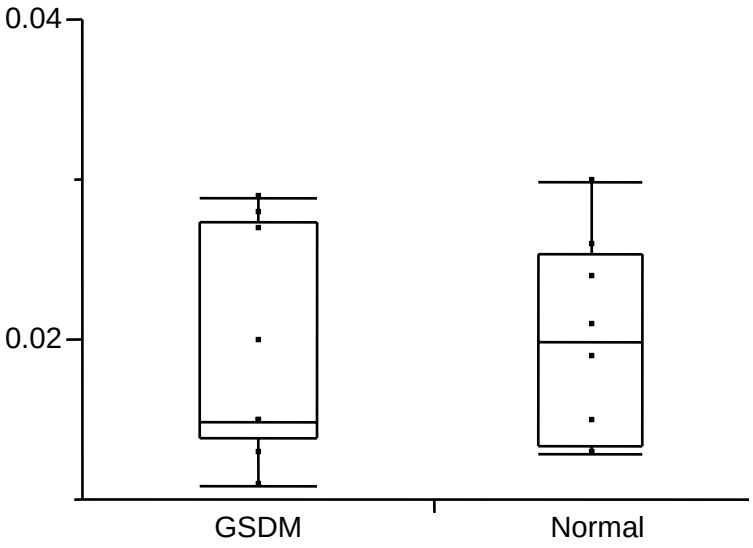


Figure 3-2. The concentrations of the anti-MBP (O.D.) antibody in canine CSF

CHAPTER 4
OLIGOCLONAL BAND DETECTION IN THE CEREBROSPINAL FLUID OF DOGS WITH DEGENERATIVE MYELOPATHY

Introduction

German shepherd degenerative myelopathy (GSDM) is a progressive neurological disorder characterized by widespread demyelination of the spinal cord with the thoracolumbar segment being the most frequently and severely affected area (Averill 1973; Braund and Vandevelde 1978). The clinical signs of this disease are primarily represented by pelvic limbs ataxia including a conscious proprioceptive deficit and signs of hypermetria (Clemmons 1992). Clinical examinations are normal except for an elevated protein level in the cerebrospinal fluid (CSF) collected from the lumbar cistern (Clemmons 1992). Therefore, the diagnosis of GSDM is usually made by ruling out other disorders affecting the spinal cord. As a cause of demyelinated lesions in the central nervous system (CNS), immune-mediated reactions have been suspected from previous immunological and genetic studies of GSDM (Waxman et al., 1980; Waxman et al., 1980; Barclay and Haines 1994; Clemmons 2006).

In order to evaluate the immune-mediated etiology of the CNS, determination of intrathecal IgG synthesis plays a central role (Correale et al., 2002). Detection of oligoclonal bands in CSF, as a qualitative analysis of IgG, was recently established as a reliable means to demonstrate intrathecal IgG synthesis. Detection of the presence of oligoclonal IgG by use of isoelectric focusing (IEF) and a sensitive immunodetection

method has been proposed as the “golden standard” diagnostic procedure in multiple sclerosis in humans (Andersson et al., 1994; Freedman et al., 2005).

The presence of oligoclonal IgG bands analyzed by IEF-immunofixation in canine CSF was previously reported in two individual studies (Callegari 2002; Ruaux 2003). Oligoclonal IgG banding in CSF with viral meningioencephalitis and suspicious DM was demonstrated by Callegari et al. Ruaux et al. reported that oligoclonal bands were detected in definitive, suspicious GSDM.

The Protean IEF cell™ was designed for the IEF dimension of two-dimensional electrophoresis. ReadyStrip™ IPG strips, as application gels for the Protean IEF cell™, are high-purity IPG monomers and thoroughly tested for quality and reproducibility. These strips are supported by a plastic film to facilitate simple manipulation and are characterized by a stable various pH range, stringent gel length tolerances of ±2 mm for consistent pI separations.

Theoretically, high reproducible and quality banding patterns are expected for oligoclonal banding detection in IEF with the Protean IEF cell™ and ReadyStrip™ IPG strips. To our knowledge, however, no study employing this instrument for oligoclonal detection has been reported.

The purpose of this study was to develop a new application of the Protean IEF cell™ and ReadyStrip IPG strip™ instrument for oligoclonal IgG banding detection in the canine CSF.

Materials and Methods

Optimization of the IEF Protocol

In order to adapt the Protean IEF cell™ and ReadyStrip™ for the analysis of canine serum and CSF, we first tested different running conditions and sample volume sizes to

develop the optimal protocol that yielded adequate separation and detection of IgG. Tested parameters were 1) amounts of IgG in CSF, 2) conditions for the final focusing step, 3) pH ranges of immobilized pH gradient strips, and 4) conditions of paired CSF/serum samples for the detection of oligoclonal bands.

Serum and lumbar CSF samples were collected from an 11year old, female Boxer with a clinical diagnosis of degenerative myelopathy and used for IEF optimization. The diagnostic criteria have been described elsewhere (Clemmons 1992). CSF was centrifuged at 14,000 rpm for 10 min and the supernatant was collected. Serum and CSF were frozen until IEF was performed. Collection of serum and CSF samples from animals included in this study was approved by the Institutional Animal Care and Use Committee of the University of Florida (IACUC protocol number E335).

General procedures for IEF: General procedures for IEF were based on manufacture's instruction for two-dimensional electrophoresis. Serum and CSF samples were diluted in ultra-pure deionized water (NANOpure Diamond™, Barnstead, Dubuque, IA). Immobilized pH gradient strips (ReadyStripTM IPG strip, Bio-Rad Laboratories, Inc. Hercules, CA) were rehydrated with diluted samples in focusing buffer containing 8M urea, 2% CHAPS, 0.2% carrier ampholytes (pH 3-10 or pH 5-8), 15mM DTT (Bio-Rad Laboratories, Inc. Hercules, CA), and bromophenol blue at 50V for 12 hrs. After the rehydration step, strips were subjected to sequential focusing steps. These steps were composed of the conditioning step (250V 20 min), voltage ramping step (4,000V 2hr), and final focusing step (4,000V, 1,000Vhrs or 10,000Vhrs current limit 50uA) (Choe and Lee 2000).

After IEF, the strips were placed on dry filter papers with the gel side facing up to blot an excess mineral oil used during the IEF protocol. The strips were further blotted with distilled water-wetted filter papers to completely remove the mineral oil. The strips were press-blotted onto a nitrocellulose membrane (0.45µm, Bio-Rad Laboratories, Inc. Hercules, CA) through 5 sheets of filter papers soaked with Tris-buffered saline (TBS) under a 2 kg weight for 1 hr at room temperature (RT). The membrane was blocked in 3% bovine serum albumin (BSA) in TBS containing 0.01% Tween 20 (T-TBS) for 1 hr (RT). Incubation of the membrane with alkaline phosphatase-conjugated rabbit anti-dog IgG (1:10,000, Alkaline phosphatase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L), Jackson immuoresearch Laboratory, Inc. West grove, PA) was performed overnight at RT. The membranes were then washed in T-TBS twice. Finally, the membranes were developed in a solution containing nitro blue tetrazolium and bromo-coloroindoleyl phosphate in 100mM Tris-HCl, 50mM MgCl2, 100mM NaCl, pH9.5 (Sadaba et al., 2004). Digital images of the membranes were taken by a molecular imager (Fluor-S Multiimager™, Bio-Rad Laboratories). Visualization of IgG band patterns was performed by using imaging analysis computer software (Quantity one 1-D analysis software™, Bio-Rad Laboratories, Inc. Hercules, CA,).

Determination of the optimal IgG amount in CSF: To determine the optimal amount of IgG in the CSF sample that yields clear detection of IgG banding patterns by IEF, we tested CSF samples containing three different amounts of IgG (50ng, 100ng, 200ng). The amount of IgG in the CSF sample was determined by a commercially available IgG quantification kit (Dog IgG ELISA Quantization Kit, Bethyl, Inc. Montgomery, TX) according to manufacture's instruction. The optical density was

recorded at 450nm by use of a microplate reader (EL340 Biokinetics Reader, Bio-Tek Instruments, Winooski, VT). IEF was performed as described above with a pH 3-10 ReadyStripTM IPG strip. The condition for the final focusing step was 4,000V with 10,000Vhrs.

Determination of the optimal condition for the final focusing step: Two different final focusing conditions were tested; 4,000V with 1,000Vhrs and 4,000V with 10,000Vhrs. IEF was performed as described above with a pH 3-10 ReadyStripTM IPG strip. The amount of IgG in the sample was 100ng.

Determination of the optimal pH gradient of ReadyStripTM IPG strip: Two immobilized pH gradient strips were tested; one with a broad pH range (pH 3-10) and the other with a narrow pH range (pH 5-8). IEF was performed as described above. The condition for the final focusing step was 4,000V with 10,000Vhrs, and the amount of IgG in the sample was 100ng.

Determination of the optimal sample conditions for the detection of oligoclonal bands: Paired serum/CSF samples were normalized based either on their protein or IgG content. Three different sample conditions were tested; paired samples were normalized to contain either 2µg protein or 100ng IgG or 200ng IgG. The amounts of protein in samples were determined by Bradford protein assay kit (Bio-Rad Protein assay, Bio-Rad Laboratories, Inc. Hercules, CA,). The condition for the final focusing step was 4,000V with 10,000Vhrs, and a pH 3-10 ReadyStripTM IPG strip was used.

Detection of Oligoclonal Bands in GSDM Patients

With the use of the optimized protocol, we then tested whether oligoclonal bands can be detected in paired serum/CSF samples from GSDM patients. Six German shepherd dogs, ranging from 5 years 6 months to 12 years in age (median 9 years 6

months and 10 years 7 months) were included (Table 4-1). These dogs were diagnosed as GSDM based on the previously described criteria (Clemmons 1992). Under general anesthesia, CSF was collected from the lumbar cistern by use of an18G spinal needle. Peripheral blood was collected and serum separated. CSF and serum samples were also collected from 6 mongrel canine cadavers obtained from a local animal shelter which served as normal control samples. These samples were collected immediately after euthanasia. CSF samples were collected from the cisterna magna in 2 dogs and from the lumbar cistern in 4 dogs. CSF and serum samples were subjected to IEF with the optimal running and sample conditions determined as described. IgG banding patterns in the paired CSF/serum samples were visually evaluated after immunoblotting of the strips and with the aid of densitometric analyses. The criterion for oligoclonal bands is the demonstration at least two more bands in CSF but not present in serum (Correale et al., 2002).

Results

Optimization of the IEF Protocol

To obtain reproducible results with IEF for the analysis of canine CSF, we first determined the optimal IgG content in the CSF samples. The results revealed that three CSF samples with different IgG contents (50ng, 100ng, 200ng) produced similar banding patterns (Fig 4-1 A). As expected, the intensities of the detected bands on membranes were proportional to the loaded IgG contents. Densitometric analysis further confirmed successively increased intensities of the detected bands in the three samples while maintaining their banding patterns (Fig 4-1 B). The optimal IgG content in the CSF sample was determined to be 100ng in which individual bands were readily appreciated. In contrast, the banding pattern was difficult to detect in the CSF sample containing a

lower IgG content (50ng) because of insufficient signals obtained after immunoblotting. In the CSF sample with a higher IgG content (200ng), a high background tended to obscure individual bands, particularly at the basic region of the strip.

The comparison of two different conditions for the final focusing step was performed with a broad pH strip (pH 3-10) on a CSF sample containing 100ng of IgG. When a lower voltage (1,000Vhr) was applied to the sample, only a polyclonal pattern was observed at the basic region of the strip (Fig 4-2 A right side and B pink line). In contrast, with a higher voltage (10,000Vhr), a clear banding pattern was observed (Fig 4-2 A life side and B blue line); therefore, a high voltage was required for the final focusing step.

The results of the comparison of two immobilized strips with different pH ranges showed the broad pH range strip (pH 3-10) to be more suitable for CSF analysis. Although the number of detected bands was higher with the narrow pH range strip (pH 5-8), bands focused on the basic regions of the strip were not adequately resolved, resulting in a thick intense band at the edge of the membrane (Fig 4-3 A right and B pink line). With the broad pH strip (pH 3-10), individual bands were more evenly separated, and it was easier to interpret the overall banding pattern (Fig 4-3 A left and B blue line).

Detection of oligoclonal bands is based on comparison of IgG banding patterns present in paired serum and CSF samples. This comparison requires the paired samples to be normalized based either on their protein or IgG content. The results showed that when paired samples contained 100ng of IgG, a clear separation of IgG was achieved after immunoblotting (Fig 4-4 A). Paired samples containing 200ng IgG also resulted in separation of IgG clones, but individual bands were not clearly focused due to

overloading of IgG (Fig 4-4 C). When paired samples contained 2μg of total, although the CSF sample showed a clear IgG banding pattern, a high background observed in the serum precluded comparison of banding patterns between the paired samples (Fig 4-4 E). The results of densitometric analyses also supported that normalizing paired CSF/serum samples to 100ng IgG allows reliable comparison of IgG banding patterns between CSF and serum (Fig 4-4 B, D, F).

Detection of Oligoclonal Bands in GSDM Patients

Analyses of paired CSF/serum samples from GSDM patients and normal controls were performed with the optimized IEF protocol. Specifically, we normalized paired samples to 100ng IgG, and the samples were focused on broad pH range ReadyStripTM IPG strips with the final focusing condition of 4,000V, 10,000Vhr. We found that normal control samples showed similar IgG patterns in paired CSF/serum samples (Fig 4-5). In 2 normal control samples, an additional IgG band was observed in CSF which was focused on the acidic region of the strip. In contrast, 4 of 6 paired samples from GSDM patients showed additional IgG bands in CSF (Fig 4-6 and Fig 4-7). Three of these patients showed more than 2 additional bands in CSF (2 additional bands in 2 patients and 3 additional bands in 1 patient), thus considered to have oligoclonal bands. These additional bands tended to be located randomly on the pH gradients of the strips.

Discussion

Optimization of the IEF Protocol

In the current study, we accommodated the Protean IEF cell™ with the oligoclonal band detection. For setting up the construction of reagents and the basic IEF programming, manufacture's instruction and previous studies for serum application were referred (Choe and Lee 2000).

In order to evaluate the reproducibility and sensitivity of the IEF method, the same samples obtained from a dog with DM were consistently used through the entire study. As we expected, several banding intensity were demonstrated in paired CSF/serum samples.

To evaluate the sensitivity of the methods, the optimal IgG content in CSF samples was first tested. Similar banding patterns of IgG were observed in all three samples with a volume dependent manner. This suggested that our methods were sufficiently sensitive to detect oligoclonal bands in CSF samples containing IgG as little as 100ng. The high sensitivity of the methods allowed testing of a small volume of CSF samples with low levels of IgG. Furthermore, since no difference of the banding pattern was observed among the tested samples, this technique was thought to be highly reproducible.

The adequate focusing time for IgG separation was evaluated. For minimizing the solubility problem of the target protein, the strips were rehydrated for twelve hours prior to the focusing phase. Two different focusing conditions (1000Vh, 10000Vh) were examined in our study. The time and current dependent of IgG migration was observed in IEF with the Protean IEF cell™. The theory of the IgG migration in the IEF gels was previously described (Keir et al., 1990; Walker 1994). Under no equilibrium procedure in the previous study (Keir et al., 1990), the formations of the banding pattern were demonstrated before the IgG migration. With rehydration step, however, proteins are equilibrated over entire gels and migrate following the current flow in the focusing phase. Under the 1000Vh focusing step, IgG were roughly migrated to the basic region based on their pI; however, complete focusing was not achieved. In contrast, under the 10000Vh focusing step, the isotypes of IgG were completely separated and focused on the IPG gel.

In our preliminary study, no differences were observed between the focusing times of 5000Vh and 10000Vh (data not shown), but the 10000Vh focusing condition seemed to provide more reproducible results.

We examined two IPG strips with different pH ranges to compare the efficacy of pH gradients for IgG separation. In IEF, the target proteins migrate through the pH gradient present in the gels. Therefore, in order to maximally resolve target proteins, it is important to use strips with an appropriate pH gradient based on the predictd pI of the target proteins (Pirttila et al., 1991). The broad pH range (pH 3-10) gradient has been generally recommended for resolving proteins when their pI ranges are unknown. The pI ranges of the IgG isotypes were expected between 5.5 and 9.5 from previous studies (Walker 1994; Keren 2003). In the present study, we examined the broad ranged (pH3-10) and basic narrow ranged (pH5-8) strips. As we expected, the IgG isotypes were separated by the charge dependent heterogeneity. The number of detected IgG banding in the narrow ranged strips is higher with attenuated background than that of broad ranged strips The efficacies of the narrow basic pH gradient for the specific IgG isotypes detection were previously presented, because of its sensitivity (Pirttila et al., 1991; Lamers et al., 1995; Kleine and Damm 2003; Kleine and Damm 2003) . However, the pH gradient of the basic range strips limits up to pH 8 and the residues of IgG isotypes were observed in our study. We recommended employing the broad range pH strips for comparison the whole IgG profile in the paired CSF/serum samples.

In all methods used for the detection of intrathecal Ig synthesis, CSF must be compared directly with matched serum (Andersson et al., 1994; Freedman 2004). For a simple comparison of the banding patterns between CSF and matched serum, the fixed

dilution of serum against set volume of the CSF, similar amounts of the total protein, and IgG in paired samples were applied in previous studies (Keir et al., 1990; Correale et al., 2002). In the present study, the equal volume of total protein in paired samples was first examined because of the simple preparation. Several simple methods including bincinchoninic acid (BCA) assay, Lowry protein assay, and Bradford protein assay for measurement of total protein in the CSF and serum have been reported (Keller and Neville 1986; Rostrom et al., 2004). These methods are sensitive enough to measure a low concentration of protein in CSF and no special equipment was required. However, the interpretation of paired samples was complicated because of the strong background in the serum samples. Thus, equal volumes of IgG in matched samples are generally required for a simple interpretation of oligoclonal bands. The volume of 20ng to 1200ng IgG have been recommended for oligoclonal band detection (Keir et al., 1990). In the current study, clear and sharp band patterns were observed in serum and CSF when both samples were normalized to 100ng IgG. However, as the intensity of the detected bands is also influenced by immunoblotting methods, a wider range of IgG concentrations may be acceptable with more sensitive immunoblotting.

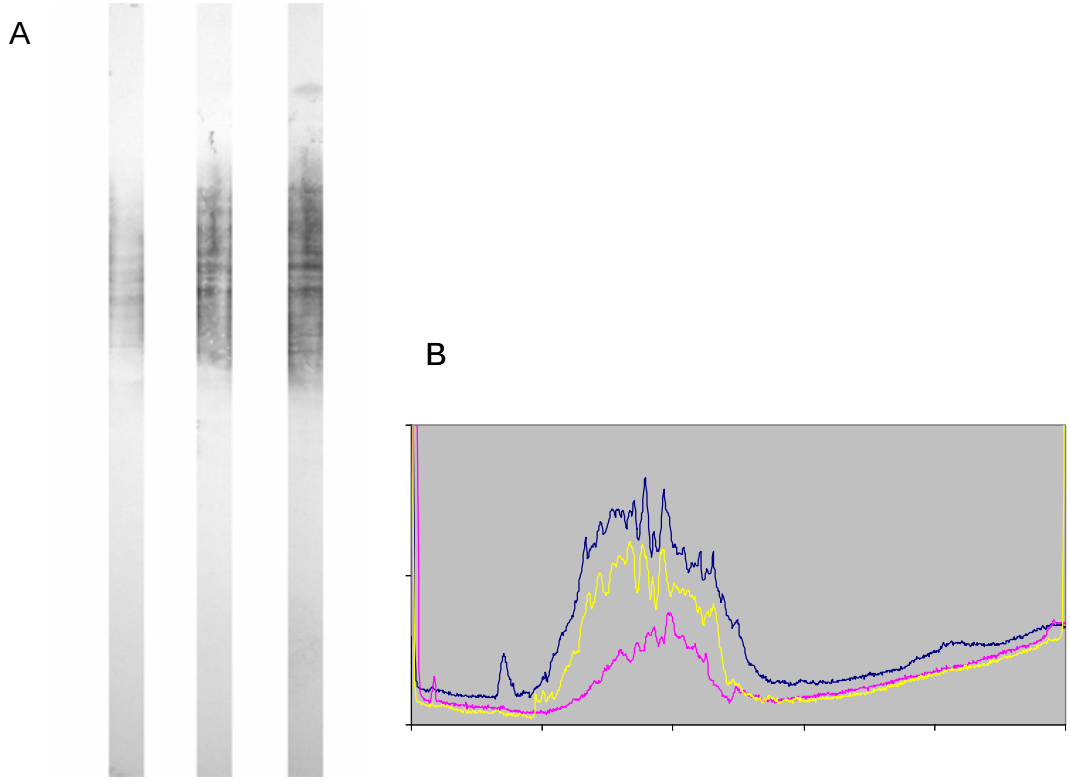
Following the IEF procedure, the separated proteins were transferred to nitrocellulose membranes by press-blotting with little modification (Keir et al., 1990). In non-covalent binding, up to 50% of the proteins initially bound to the membrane are known to be washed off by the detergent through the subsequent incubation procedure (Keir et al., 1990). However, sufficient reactivity with the antibody was observed in our study without a fixation step.

Finally, we reported the application of the Protean IEF cell™ and ReadyStrip™ IPG strip for oligoclonal band detection. Although a few disadvantages were considered in our study including consuming time through the entire method, wearing gloves for prevention of acrylamide toxicity, and cost for the strips, oligoclonal banding detection by this method may provide more sensitive and reliable information regarding the presence of intrathecal IgG synthesis.

Detection of Oligoclonal Bands in GSDM Patients

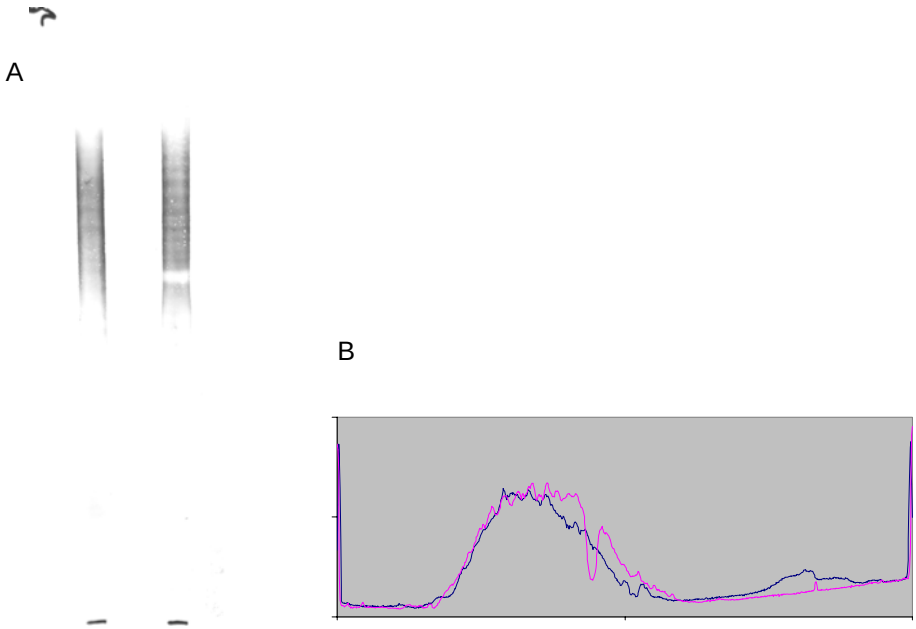
Qualitative analysis of the intrathecal IgG synthesis in GSDM was performed by the IEF-immunofixation. In the current study, we demonstrated more than two distinct additional bands in the CSF from three dogs with GSDM. Moreover, several identical banding patterns were observed in all paired CSF/serum samples. This finding was comparable with the previous study reported by Ruaux et al. In this previous study, additional banding patterns in the CSF were reported in five of ten dogs with GSDM and in three of six control dogs. They also suggested that dogs with GSDM tended to present a strong immunoglobulin band compared with the faint band formation demonstrated in other dogs including control group. In human, the presence of identical oligoclonal banding in paired samples indicates a systemic disorder with leakage of the BBB. In our current study, however, no evidence of systemic disorders was observed in all cases. In addition, the identical banding patterns observed in normal dogs were more consistent than those in GSDM. Therefore, these banding patterns may represent typical IgG isotypes normally present in canine paired samples. The reasons for these banding patterns observed in normal dogs are unknown. A unique living circumstance of the dog, such as frequent vaccinations, may lead to alternation of the BBB permeability thereby allowing the coexistence of identical IgG isotypes in CSF and serum.

In this study, we described simple and reliable methods for oligoclonal band detection by use of Protean IEF cell™ and ReadyStrip™ IPG strips, and suggested the presence of oligoclonal bands in the CSF of GSDM patients. The specificity and sensitivity of the methods need to be determined in a future study with a large sample size and histological confirmation of GSDM. With the methods described in our study, further investigations may provide important insights into the pathoetiology of GSDM.



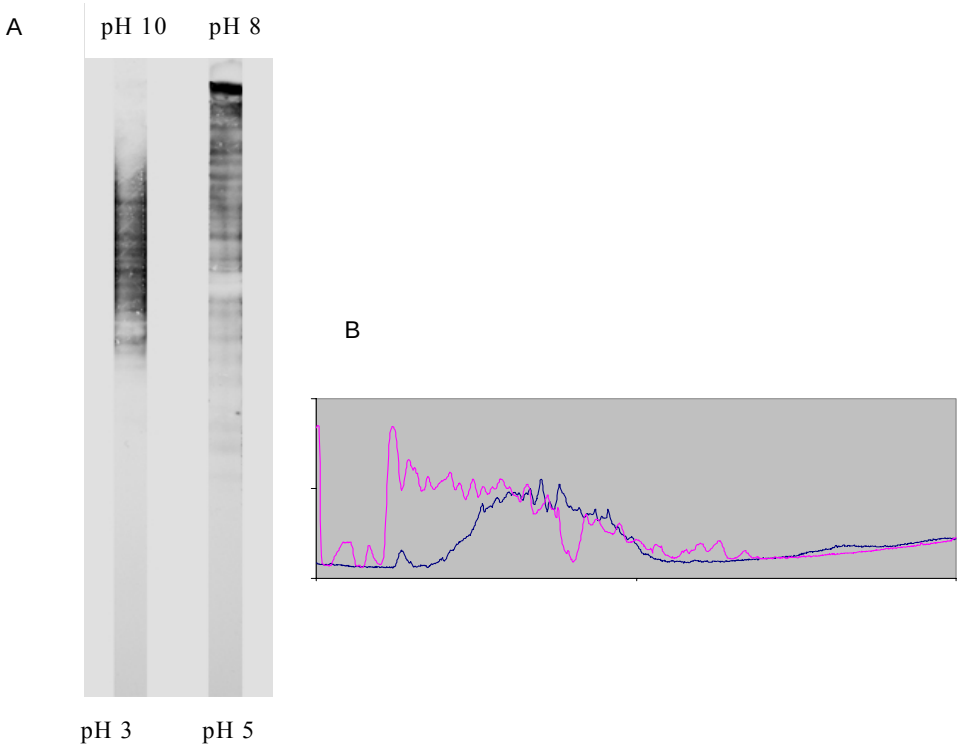
A) 50ng 100ng 200ng B) pink, 50ng; yellow, 100ng; blue, 200ng

Figure 4-1. The CSF containing 50ng, 100ng, and 200ng of IgG were applied in IEF-immunoblotting. A dose dependent intensity was observed. The banding patterns presented by immunoblotting (A) were analyzed by the densitometry (B).



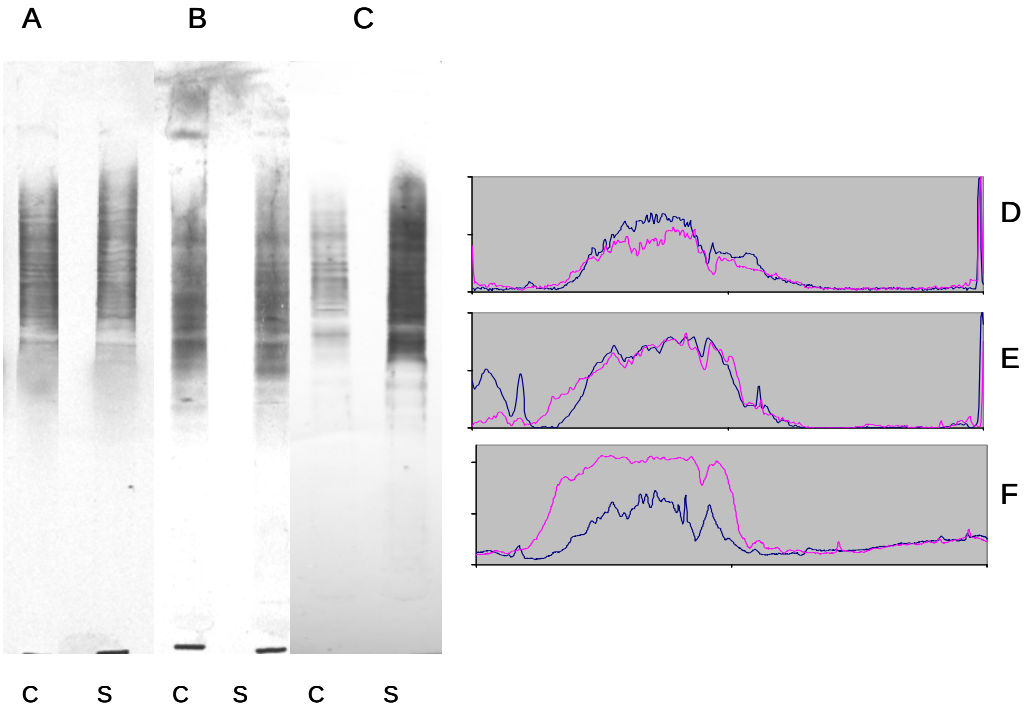
A) IEF-immunofixation, B) Densitometory analysis; blue 1000Vh, pink 10,000Vh

Figure 4-2. The CSF containing 100ng of IgG was focused at 1000Vh and 10,000Vh. No banding pattern was observed in the condition of 1000Vh.



A) IEF-immunofixation, B) Densitometory analysis; blue pH 3-10, pink pH 5-8

Figure 4-3. The paired CSF/serum samples containing 100ng of IgG were applied to the broad range (pH 3-10) and narrow range (pH 5-8) strips. The resolution of banding patterns was clear in the narrow range strip.



C, CSF; S, serum; blue, CSF; pink, serum

Figure 4-4. Three focusing conditions of the paired samples were examined. 100ng of IgG A), 200ng of IgG B), and 2µg of total protein C) were contained in the paired samples. The banding patterns were analyzed by densitometry D), E), and F).

Table 4-1. Clinical observations and CSF analysis of 6 German shepherd dogs with degenerative myelopathy

Dog	age	sex	NCC (cells/µl)	Protein (mg/ml)	AQ	IgG index	OB	Duration of clinical sign
1	11y3m	sF	0	40.5	0.65	0.34	1	15 months
2	10y7m	cM	N/A	28.4	0.55	0.41	0	6 months
3	6y6m	sF	2	56.6	0.51	0.55	2	4 months
4	12y	cM	0	41.6	0.77	0.40	2	12 months
5	9y6m	sF	0	36.3	0.9	0.17	0	7 months
6	5y 6m	cM	3	52.3	0.65	0.69	3	2 months

sF, spayed female; cM castrated male; NCC, nuclear cell count; AQ, albumin quanta; OB, the number of additional oligoclonal band; reference range, * <42.5mg/dl

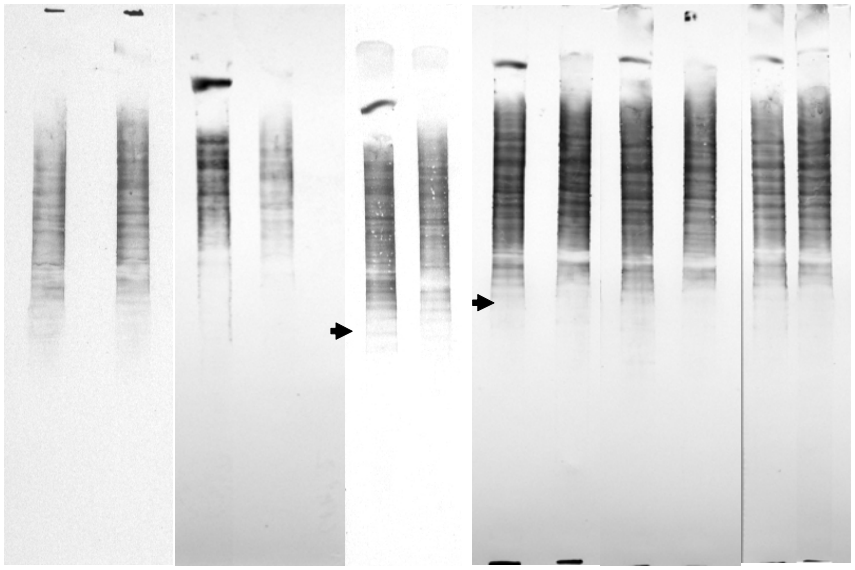


Figure 4-5. The CSF and matched serum samples of six normal dogs were examined by IEF-immunoblotting.

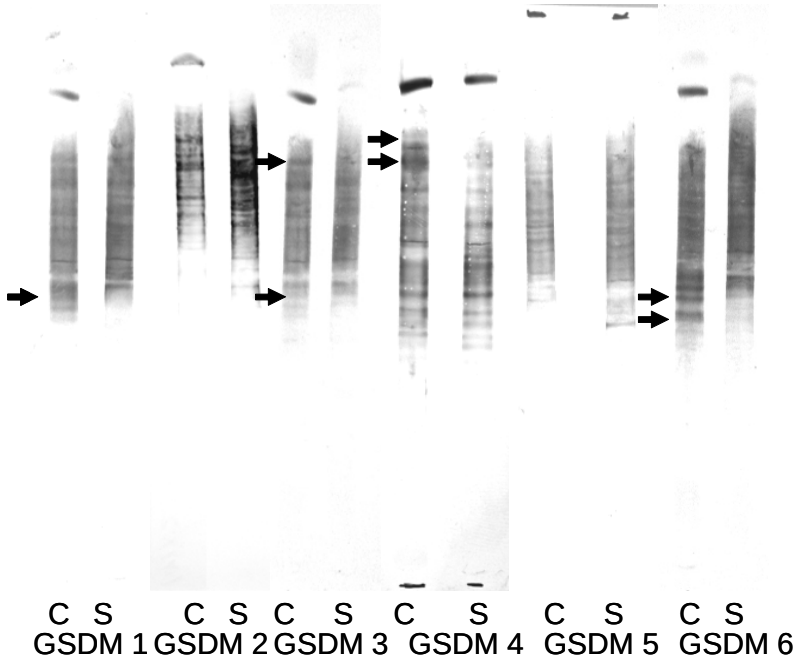


Figure 4-6. The CSF and matched serum samples of six dogs with GSDM were examined by IEF–immunoblotting. Oligoclonal additional bands (arrow) were observed in four cases.

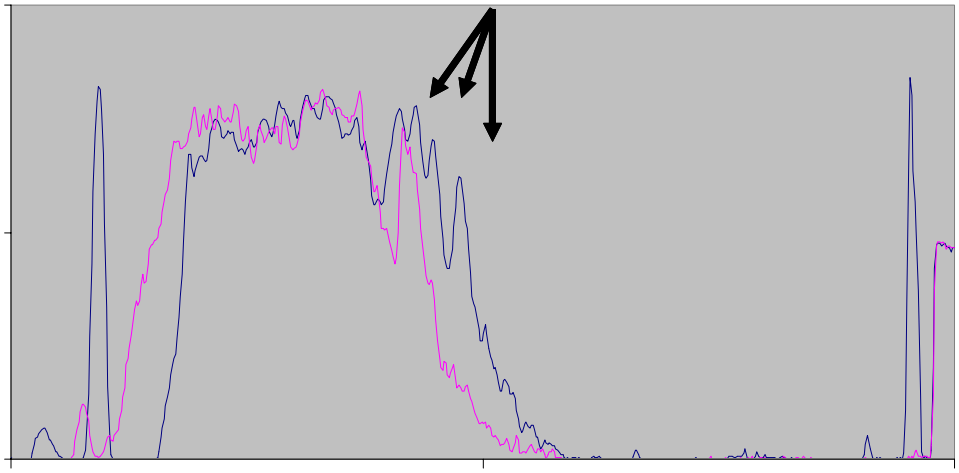


Figure 4-7. The band intensity of GSDM 6 was represented by optical density. Three additional peaks (arrow) were observed

CHAPTER 5
INTRATHECAL IGG SYNTHESIS IN GSDM

Introduction

Previous studies suggested that demyelination and axonal loss are characteristic pathological features of GSDM (Averill 1973). Although precise mechanisms leading to the development of spinal cord lesions have not been elucidated, several lines of evidence exist which support the hypothesis that GSDM is caused by autoimmune responses to the CNS. This hypothesis has been primarily based on early studies reporting altered immune systems in GSDM (Waxman et al., 1980; Waxman et al., 1980), and an immunohistological study demonstrating IgG and complement deposition in demyelinative lesions in GSDM (Barclay and Haines 1994).

In the current study, we hypothesized that the immune-mediated etiology of GSDM is analogous to MS in human beings. In order to assess the intrathecal IgG synthesis, IgG index and albumin quanta (AQ) were calculated based on quantitative ELISA; the qualitative IgG analysis was examined by IEF-immunofixation method.

Materials and Methods

Albumin and IgG concentrations of serum and CSF were determined by quantitative ELISA. The CSF and serum samples assayed in the current study were obtained from six German shepherd dog in chapter 4.

The amount of IgG in the sample was determined by a commercially available IgG quantification kit (Dog IgG ELISA Quantization Kit, Bethyl, Inc. Montgomery, TX) according to manufacture's instruction. The optical density was recorded at 450nm by

use of a microplate reader (EL340 Biokinetics Reader, Bio-Tek Instruments, Winooski, VT).

In the albumin quantitative ELISA, the micro-titer plate (MaxiSorp™, Nunc, Rochester, NY) was coated by Goat anti-Dog albumin antibody (1:100) (Bethyl, Inc. Montgomery, TX). The sample was adequately diluted in distilled water (serum, 1:500,000; CSF, 1:5,000). As a detection antibody, HRP-conjugated goat anti-dog albumin antibody (1:150,000) (Bethyl, Inc. Montgomery, TX) was applied. The dog serum albumin (Sigma-Aldrich, St. Louis, MO) was used for the standard curve.

Albumin quanta (AQ) and IgG index were calculated according the formula as follows;

AQ = ALB CSF/ALB serum × 100, IgG index = IgG CSF/IgG serum /AQ

The reference range of AQ and IgG index are less than 0.3 (Behr et al., 2006) and 0.7 (Tipold et al., 1993) respectively. The results are given as mean and ± SEM.

Results and Discussion

Albumin Quanta

The mean values of AQ and total protein concentration in GSDM ranged from 0.51 to 0.90 (mean 0.67) and from 28.4 to 56.6mg/ml (mean 42.6), respectively (Table 4-1). The total protein concentration obtained in this study was within the reference range, except for two dogs (dog 3 and dog 6). The albumin quantification of various neurological disorders in the canine CSF/serum paired samples have been recently reported by Behr et al (Behr et al., 2006). This study described a linear correlation between the total protein concentration and the AQ value using high resolution protein electrophoresis (Behr et al., 2006). The AQ value calculated in our current study was higher than that of the reference range in the previous study. In addition to the increased

concentration of the total protein in CSF, the high AQ value also suggested a destruction of blood-CSF barrier in demyelinative lesions of GSDM. However, since the AQ values are reported to be dependent on age and methods that laboratory employs, breed and age matched normal controls are required in a future study.

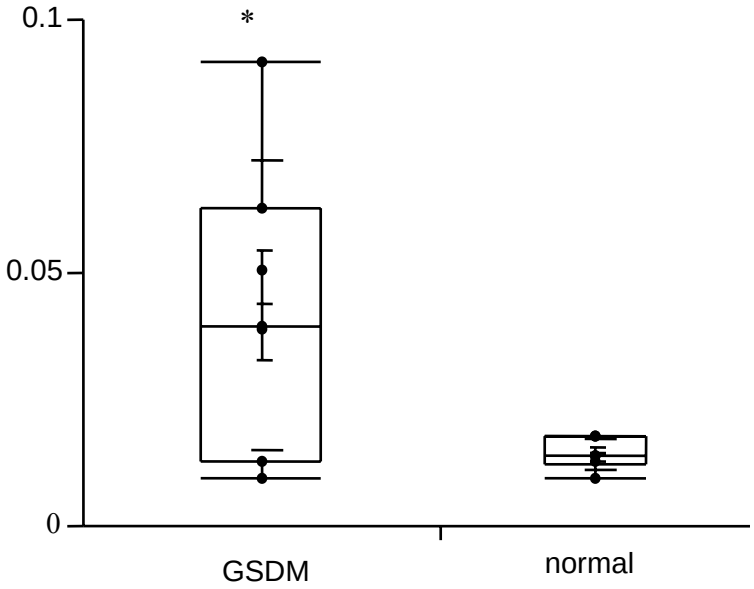
IgG Index

The mean value of IgG concentration was significantly higher in GSDM (0.042 mg/ml±0.026) than in normal group (0.014mg/ml±0.014) (Figure 5-1). The IgG index was calculated by comparing the amount of IgG in CSF with that in serum using albumin as a reference protein. Measurements of IgG in paired samples were performed by quantitative IgG ELISA. The mean value (0.42±0.17) of the IgG index in GSDM was within a reference range (<0.7) described in a previous study with quantitative ELISA (Table 4-1) (Bichsel et al., 1984; Tipold et al.,1993). No intrathecal IgG synthesis was suspected in our study. However, the sensitivity of the IgG index for the detection of intrathecal IgG synthesis has been controversial in human medicine because of its frequent false values with the high AQ (Behr et al., 2006). Hence, for the detection of the intrathecal IgG synthesis, quantitative assays calculated by IgG index are considered less sensitive than qualitative IgG assays such as demonstration of the presence of oligoclonal bands. In our study, the high AQ values observed in GSDM were described above. Therefore, the presense of intrathecal IgG synthesis were not refuted with the normal range of the IgG index.

In chapter 4, we presented that there were more than two additional bandings in the CSF from three dogs with GSDM. Interestingly, the additional bands were observed in dogs with a relatively high IgG index ranged between 0.40 and 0.65 (dog 3, 4, and 6). Two of these dogs also presented a relatively short clinical duration and higher range of

the total protein concentration. These results may indicate the presence of active lesions in the spinal cord in these three cases. Furthermore, the additional IgG bands in the CSF were clearly observed in these cases. Therefore, we demonstrated the presence of intrathecal IgG synthesis at least in these three cases. Because of the small sample size in the current study, statistical analyses were not performed. In addition, histological confirmation of GSDM was not performed in the current study.

In order to confirm the sensitivity and specificity for the diagnosis of GSDM, a further study with a larger sample size and histological confirmation of GSDM is warranted. Based on the results of this study, we suggested the presence of intrathecal IgG synthesis in GSDM. This finding will contribute to the development of a novel diagnostic schema of GSDM. Further investigations on intrathecal IgG synthesis in GSDM will also provide important insights into the immune-mediated etiology of GSDM.



GSDM, Gereman shepherd degenerative myelopathy
Figure 5-1. The concentration of IgG in lumbar CSF (mg/ml)

CHAPTER 6
LIMITATIONS IN THE STUDY AND CONCLUSION

Limitations

In this study, two methods for CSF analysis of GSDM were described. These were sufficiently reproducible and sensitive for canine CSF analysis. In chapter 2, the clinical application of human MBP ELISA was described. We demonstrated a simple laboratory test to assess active demyelinating lesions in the CNS of GSDM patients. In chapter 3, with the use of IEF and immunoblotting, the presence of intrathecal IgG synthesis in GSDM patients was demonstrated by detection of oligoclonal bands.

Limitation in Sampling Groups

There were several limitations in this study. First, all samples of GSDM were obtained from clinically diagnosed patients. Pathological confirmation of GSDM was not performed in this study. In order to evaluate the sensitivity and specificity of the assays described in this study for GSDM diagnosis, pathological confirmation of GSDM would be required in future studies.

Second, normal control samples were obtained from canine cadavers in this research. Clinical history and age were not known in all cadavers. The CSF samples with abnormal protein concentration were excluded from the normal group. All samples were collected immediately after euthanasia to avoid factors influencing the permeability of blood brain barrier (BBB). Age-dependent changes in permeability of the BBB may occur which could alter our results. Congenital immune deficiency or some other inheritance disorders have been reported in the GSD (Batt et al., 1991; Rosser 1997),

which may result in the alternated values of the AQ and the IgG index in this breed. For the evaluation of the reference range in aged German shepherd dogs, therefore, age and breed matched control samples may be needed.

The number of samples obtained for this study was relatively small and other canine disorders affecting the spinal cord were not examined. A larger sample size of the dogs would have provided more reliable statistical measures. Comparing the results for GSDM patients with other neurologic diseases, both inflammatory and non-inflammatory in nature, would have provided useful information

Cerebral spinal fluid and serum samples were collected from GSDM patients in various stages of the disease. Is it likely that the changes seen would change over time and might even help categorizing disease stages.

Limitation in Immune Cross-Reactivity of Human MBP ELISA

Cross-reactivity of the human MBP ELISA to canine MBP was confirmed by immunoblotting. Canine MBP was isolated by organic extraction (Maatta et al., 1997). Following electrophoresis, isolated MBP was detected by Coomasie staining at 18.5KDa, known as a monomeric molecular weight of MBP in mammals. In addition, we demonstrated a sufficient reaction of the polyclonal anti-human MBP antibody to isolated MBP in western blotting and ELISA. Comparing with the human and bovine MBP, however, the reaction of the anti-human MBP antibody was less sensitive to the isolated canine MBP. We suspect the loss of epitopes of isolated MBP during extraction procedure. As a result of the alternate splicing of the MBP transcript, four isoforms of MBP are recognized in human and the size and conformation of MBP vary in pH of the aqueous solutions. On the other hand, only one isoform (18.5kDa) of canine MBP was

extracted by the acidic aqueous solution in our study. It may be cause of less immune-reactivity demonstrated in the isolated canine MBP.

Limitation in IEF-Immunofixation

IEF-immunofixation technique has been known as the most sensitive method for oligoclonal band detection in laboratory test. We developed the novel IEF-immunofixation techniques with the plastic back IPG strips.

Recently, the international formed committee published a consensus statement of IgG qualitative criteria in human. In this statement, twelve recommendations regarding the analysis were stated below (Freedman et al., 2005).

1. The single most informative methods is a qualitative assessment of CSF for IgG, best performed using IEF together with some form of immunodetection approved by food and drug administration.
2. This qualitative analysis should be performed using un-concentrated CSF and must be compared a serum sample run simultaneously in the same assay in an adjacent truck.
3. Optimal runs use similar amounts of IgG from paired serum sample and CSF.
4. Recognized positive and negative controls should be run with each set of the samples and entire gel rejected if oligoclonal bands in the positive controls are poorly developed or the negative controls are over loaded.
5. Cerebrospinal fluid reports of qualitative analysis should be made in terms of 1 of the 5 recognized staining patterns of oligoclonal banding.
6. Interpretation should be made by an individual experienced in the technique used.
7. Clinicians need to consider the results of all other test performed as part of the CSF panel (cell count, protein, glucose, and lactate and others).
8. In certain cases, an evaluation using light chains for immunodetection can help to resolve equivocal oligoclonal IgG pattern.
9. Consideration should be given to repeating the lumber puncture and CSF analysis if clinical suspicion is high but results of CSF are equivocal, negative, or show only a single band.

10. Quantitative IgG analysis is an informative complementary test but is not considered a substitute for qualitative IgG assessment, which has the highest sensitivity and specificity.

11. When performed, nonlinear formulas should be used to measure intratheal IgG level that considered the integrity of the BBB by also measuring the ratio of albumin in CSF to serum.

12. Laboratories performing routine CSF analysis should be those that ensure their own internal quality control and participate in external quality assessment controls to assure maintenance of a high standard of reliability and performance, as has been recommended in some international consensus report.

(Freedman et al., 2005)

In order to assess the accuracy of the qualitative IgG analysis, a negative and positive quality control was required. However, no adequate samples for a positive and negative control were established in our study. A further study containing the other neurological disorders may provide empirical information for the quality control of canine oligoclonal band detection.

Summary

MBP in Human Neurological Disorders and GSDM

German shepherd degenerative myelopathy is a chronic, demyelinating neurodegenerative disease of the CNS. In order to investigate underlying pathogenesis of this disorder, we have hypothesized an immune-mediated etiology similar to MS in human beings. In this study, we demonstrated the elevation of MBP in CSF of GSDM. In human, the concentration of MBP in CSF of MS patients was described by Ohta et al. with sensitive ELISA. In that study, an increased MBP was presented in 81% of active MS patients in contrast to 19% of patients with inactive MS and other neurological disorders. They also demonstrated the maximum MBP concentration during the recurrent exacerbation phase of the disease by the serial sampling. In addition, the elevation of MBP has been demonstrated in patients with acute neurological diseases including active

phase of RRMS, leukodystrophies, myelopathies, encephalopathies, cerebrovascular disease, brain surgery, and head injury (Massaro and Tonali 1998; Whitaker 1998; Michalowska-Wender et al., 2001; Ohta et al., 2002), and rarely found in chronic demyelination disorders such as amyotrophic lateral sclerosis, Parkinson deisease (Ohta et al., 2000). In human, therefore, the determination of MBP in CSF is not only used for the ancillary diagnosis of the active demyelination but also for monitoring the disease phase and therapeutic efficacy. In the current study, the elevation of MBP was demonstrated in all cases of GSDM "chronic demyelinative canine disorder" without the correlation of the clinical stage. Although no histological confirmation was performed, our results suggested the presence of active demyelinative lesions in the spinal cord in GSDM patients. In contrast to chronic demyelinaton diseases in human, the clinical course of GSDM is relatively short and a more rapid decline in function. It may cause an active and progressive break-down of myelin sheath during the entire disease phase in GSDM.

Primary progressive MS is not a highly active disease and, therefore, elevated MBP is less likely to occur. On the other hand, GSDM, although also a chronic neurodegenerative disease, has a compressed time course. Primary progressive MS has a 7-10 year course of disease from initial signs until severe disability; whereas GSDM progressed over 9-18 months. Based upon life-span of dogs compared to human beings, these times represent a similar "life years". It might be expected that the levels of all reactive components would, therefore, be higher in GSDM patients compared to PPMS patients.

Oligoclonal Band in Human Neurological Disorders and GSDM

In the current study, oligoclonal bands were detected in three CSF samples of GSDM patients that presented relatively high values of the IgG index. For the laboratory test, the frequency of the oligoclonal bands was reported by Mayringer et al. in human neurological disorders (Mayringer et al., 2005). In that study, oligoclonal bands were detected in 98.4% of demyelinating diseases, 68.4% of inflammatory neurological disesases, and 70.7% of the other neurological disorders including vascular and metabolic diseases; a strong correlation between the IgG index and this frequency was also demonstrated. In contrast to the IgG index dependent frequency of the oligoclonal band detection in inflammatory and other neurological disorders, there is no dependency on the IgG index in demyelinating diseases. In that study, therefore, they concluded that it was not necessary to analyze the IgG index, but perform the oligoclonal band detection in patient with suspicious demyelinative diseases. Moreover, McLean et al reported that the diagnostic probability of MS, other inflammatory disorder, and other diagnosis disorders were 66%, 20%, and 14%, respectively, when oligoclonal bands were demonstrated in CSF (McLean et al., 1990). In other studies describing the sensitivity and specificity of qualitative IgG analysis, Lunding et al. reported that oligoclonal bands were detected with 100% sensitivity of MS patients and 9.5% of other neurological disorders, giving the test specificity of 91% for MS patients (Lunding et al., 2000). Importantly, the presence of elevated IgG and unique CSF oligoclonal bands of IgG are similar in PPMS as we have described in GSDM patients.

In the current study, we demonstrated the presence of unique intrathecal oligoclonal bands in the CSF in some dogs. This is in contrast to a study in dogs (Ruaux 2003) where oligoclonal bands were found in all dog CSF samples, but which were not unique. The

fact that the IgG index is elevated in dogs with GSDM indicates that IgG is accumulated in the CSF of affected dogs. While some clearly have de novo synthesis of IgG, demonstrated by the unique oligoclonal bands. Those who have elevated IgG without unique bands may indicate that most GSDM patients selectively accumulate IgG into the CSF. Perhaps the BBB has mechanisms to accumulate IgG which have not previously been appreciated, analogous to the 2-pore theory of protein regulation in the kidney.

Conclusion

German Shepherd DM patients do have changes in CSF IgG concentrations and some patients have the presence of unique CSF oligoclonal bands of IgG. In addition, MBP concentration in CSF of these patients is elevated. These facts support the immune basis of GSDM and indicate that GSDM is one of the immune-related neurodegenerative diseases. While the changes are more pronounced than in PPMS, they are less severe than RRMS which is a more active form of MS. The age of onset, the time course, the lack of sex predisposition, the location and extent of neurologic damage, the type of neurologic pathology, and the changes demonstrated in this study all support that fact that GSDM appears to be analogous to PPMS in human. Further studies will be needed to understand the significance of these findings and how they relate to the pathophysiology of GSDM, but this study has demonstrated the relevance of GSDM as an important animal model for neurodegeneration in human beings.

LIST OF REFERENCES

Alling C, Karlsson B and Vallfors B (1980). Increase in myelin basic protein in CSF after brain surgery. J Neurol 223(4): 225-30.

Andersson M, Alvarez-Cermeno J, Bernardi G, Cogato I, Fredman P, Frederiksen J, Fredrikson S, Gallo P, Grimaldi LM and Gronning M (1994). Cerebrospinal fluid in the diagnosis of multiple sclerosis: a consensus report. J Neurol Neurosurg Psychiatry 57(8): 897-902.

Antel JP, Arnason BG and Medof ME (1979). Suppressor cell function in multiple sclerosis: correlation with clinical disease activity. Ann Neurol 5(4): 338-42.

Averill DR, Jr. (1973). Degenerative myelopathy in the aging German Shepherd dog: clinical and pathologic findings. J Am Vet Med Assoc 162(12): 1045-51.

Barclay KB and Haines DM (1994). Immunohistochemical evidence for immunoglobulin and complement deposition in spinal cord lesions in degenerative myelopathy in German shepherd dogs. Can J Vet Res 58(1): 20-4.

Bashir K and Whitaker JN (1999). Clinical and laboratory features of primary progressive and secondary progressive MS. Neurology 53(4): 765-71.

Batt RM, Barnes A, Rutgers HC and Carter SD (1991). Relative IgA deficiency and small intestinal bacterial overgrowth in German shepherd dogs. Res Vet Sci 50(1): 106-11.

Behr S, Trumel C, Cauzinille L, Palenche F and Braun JP (2006). High resolution protein electrophoresis of 100 paired canine cerebrospinal fluid and serum. J Vet Intern Med 20(3): 657-62.

Berger T, Rubner P, Schautzer F, Egg R, Ulmer H, Mayringer I, Dilitz E, Deisenhammer F and Reindl M (2003). Antimyelin antibodies as a predictor of clinically definite multiple sclerosis after a first demyelinating event. N Engl J Med 349(2): 139-45.

Bichsel P, Vandevelde M, Vandevelde E, Affolter U and Pfister H (1984). Immunoelectrophoretic determination of albumin and IgG in serum and cerebrospinal fluid in dogs with neurological diseases. Res Vet Sci 37(1): 101-7.

Bitsch A, Dressel A, Meier K, Bogumil T, Deisenhammer F, Tumani H, Kitze B, Poser S and Weber F (2004). Autoantibody synthesis in primary progressive multiple sclerosis patients treated with interferon beta-1b. J Neurol 251(12): 1498-501.

Blennow K (2004). Cerebrospinal fluid protein biomarkers for Alzheimer's disease. NeuroRx 1(2): 213-25.

Braund KG and Vandevelde M (1978). German Shepherd dog myelopathy--a morphologic and morphometric study. Am J Vet Res 39(8): 1309-15.

Brokstad KA, Page M, Nyland H and Haaheim LR (1994). Autoantibodies to myelin basic protein are not present in the serum and CSF of MS patients. Acta Neurol Scand 89(6): 407-11.

Callegari Daniela CI, Luisa De Risio, Bianchi Ezio and Martelli Paolo (2002). Dogs Cerebrospinal Flu Total Protein Concentrations and IgG Isoelectrofocusing (IEF) Patterns. 27th WSAVA congress, Granada.

Chamczuk AJ, Ursell M, O'Connor P, Jackowski G and Moscarello MA (2002). A rapid ELISA-based serum assay for myelin basic protein in multiple sclerosis. J Immunol Methods 262(1-2): 21-7.

Choe LH and Lee KH (2000). A comparison of three commercially available isoelectric focusing units for proteome analysis: the multiphor, the IPGphor and the protean IEF cell. Electrophoresis 21(5): 993-1000.

Chrisman CL (1992). Cerebrospinal fluid analysis. Vet Clin North Am Small Anim Pract 22(4): 781-810.

Clemmons RM, Cheeseman J, Kamishina H and Oji T (2006). Genetic analysis of a spontaneous canine model of primary progressive multiple sclerosis. Experimental biology 2006, San francisco, CA.

Clemmons RM (1992). Degenerative myelopathy. Vet Clin North Am Small Anim Pract 22(4): 965-71.

Confavreux C and Vukusic S (2006). Natural history of multiple sclerosis: a unifying concept. Brain 129(Pt 3): 606-16.

Correale J and de los Milagros Bassani Molinas M (2002). Oligoclonal bands and antibody responses in multiple sclerosis. J Neurol 249(4): 375-89.

Coyle P (2001). Meeting the Challenge of Progressive Multiple Sclerosis. New York, Demos.

Dasgupta MK, Catz I, Warren KG, McPherson TA, Dossetor JB and Carnegie PR (1983). Myelin basic protein: a component of circulating immune complexes in multiple sclerosis. Can J Neurol Sci 10(4): 239-43.

Dasgupta MK, McPherson TA, Catz I, Warren KG, Dossetor JB and Carnegie PR (1984). Identification of myelin basic protein (MBP) in circulating immune complexes (CIC) from some multiple sclerosis (MS) patients. Prog Clin Biol Res 146: 353-8.

Dujmovic I, Mesaros S, Pekmezovic T, Levic Z and Drulovic J (2004). Primary progressive multiple sclerosis: clinical and paraclinical characteristics with application of the new diagnostic criteria. Eur J Neurol 11(7): 439-44.

Ebers GC (2004). Natural history of primary progressive multiple sclerosis. Mult Scler 10 Suppl 1: S8-13; discussion S13-5.

Fechner H, Johnston PE, Sharp NJ, Montague P, Griffiths IR, Wang X, Olby N, Looman AC, Poller W and Flegel T (2003). Molecular genetic and expression analysis of alpha-tocopherol transfer protein mRNA in German shepherd dogs with degenerative myelopathy. Berl Munch Tierarztl Wochenschr 116(1-2): 31-6.

Fischman HR (1982). Multiple sclerosis: a new perspective on epidemiologic patterns. Neurology 32(8): 864-70.

Franciotta D, Zardini E, Bergamaschi R, Grimaldi LM, Andreoni L and Cosi V (2005). Analysis of Chlamydia pneumoniae-specific oligoclonal bands in multiple sclerosis and other neurologic diseases. Acta Neurol Scand 112(4): 238-41.

Freedman MS (2004). Primary progressive multiple sclerosis: cerebrospinal fluid considerations. Mult Scler 10 Suppl 1: S31-4; discussion S34-5.

Freedman MS, Thompson EJ, Deisenhammer F, Giovannoni G, Grimsley G, Keir G, Ohman S, Racke MK, Sharief M, Sindic CJ, Sellebjerg F and Tourtellotte WW (2005). Recommended standard of cerebrospinal fluid analysis in the diagnosis of multiple sclerosis: a consensus statement. Arch Neurol 62(6): 865-70.

Garcia-Merino A, Persson MA, Ernerudh J, Diaz-Gil JJ and Olsson T (1986). Serum and cerebrospinal fluid antibodies against myelin basic protein and their IgG subclass distribution in multiple sclerosis. J Neurol Neurosurg Psychiatry 49(9): 1066-70.

Griffiths IR and Duncan ID (1975). Chronic degenerative radiculomyelopathy in the dog. J Small Anim Pract 16(8): 461-71.

Grigoriadis N and Hadjigeorgiou GM (2006). Virus-mediated autoimmunity in Multiple Sclerosis. J Autoimmune Dis 3: 1.

Haegert DG and Marrosu MG (1994). Genetic susceptibility to multiple sclerosis. Ann Neurol 36 Suppl 2: S204-10.

Harding AE, Macevilly CJ and Muller DP (1989). Serum vitamin E concentrations in degenerative ataxias. J Neurol Neurosurg Psychiatry 52(1): 132.

Johnston PE, Barrie JA, McCulloch MC, Anderson TJ and Griffiths IR (2000). Central nervous system pathology in 25 dogs with chronic degenerative radiculomyelopathy. Vet Rec 146(22): 629-33.

Johnston PE, Knox K, Gettinby G and Griffiths IR (2001). Serum alpha-tocopherol concentrations in German shepherd dogs with chronic degenerative radiculomyelopathy. Vet Rec 148(13): 403-7.

Jones JC, Inzana KD, Rossmeisl JH, Bergman RL, Wells T and Butler K (2005). CT myelography of the thoraco-lumbar spine in 8 dogs with degenerative myelopathy. J Vet Sci 6(4): 341-8.

Keir G, Luxton RW and Thompson EJ (1990). Isoelectric focusing of cerebrospinal fluid immunoglobulin G: an annotated update. Ann Clin Biochem 27 (Pt 5): 436-43.

Keller RP and Neville MC (1986). Determination of total protein in human milk: comparison of methods. Clin Chem 32(1 Pt 1): 120-3.

Keren DF (2003). Protein electrophoresis in clinical diagnosis. New York, Oxford University Press.

Kies MW, Thompson EB and Alvord EC, Jr. (1965). The Relationship of Myelin Proteins to Experimental Allergic Encephalomyelitis. Ann N Y Acad Sci 122: 148-60.

Kleine TO and Damm T (2003). Distinct heterogeneity of IgG immune response in cerebrospinal fluid (CSF) detected by isoelectric focusing (IEF) with extended immunofixation. Brain Res Bull 61(3): 309-20.

Kleine TO and Damm T (2003). Molecular heterogeneity, detected by two electrophoretic micro procedures, of IgG in human cerebrospinal fluid (CSF). Anal Bioanal Chem 375(8): 1000-5.

Lamers KJ, de Jong JG, Jongen PJ, Kock-Jansen MJ, Teunesen MA and Prudon-Rosmulder EM (1995). Cerebrospinal fluid free kappa light chains versus IgG findings in neurological disorders: qualitative and quantitative measurements. J Neuroimmunol 62(1): 19-25.

Lim ET, Sellebjerg F, Jensen CV, Altmann DR, Grant D, Keir G, Thompson EJ and Giovannoni G (2005). Acute axonal damage predicts clinical outcome in patients with multiple sclerosis. Mult Scler 11(5): 532-6.

Lunding J, Midgard R and Vedeler CA (2000). Oligoclonal bands in cerebrospinal fluid: a comparative study of isoelectric focusing, agarose gel electrophoresis and IgG index. Acta Neurol Scand 102(5): 322-5.

Maatta JA, Coffey ET, Hermonen JA, Salmi AA and Hinkkanen AE (1997). Detection of myelin basic protein isoforms by organic concentration. Biochem Biophys Res Commun 238(2): 498-502.

Mantegazza R, Cristaldini P, Bernasconi P, Baggi F, Pedotti R, Piccini I, Mascoli N, La Mantia L, Antozzi C, Simoncini O, Cornelio F and Milanese C (2004). Anti-MOG autoantibodies in Italian multiple sclerosis patients: specificity, sensitivity and clinical association. Int Immunol 16(4): 559-65.

Massaro AR and Tonali P (1998). Cerebrospinal fluid markers in multiple sclerosis: an overview. Mult Scler 4(1): 1-4.

Mayringer I, Timeltaler B and Deisenhammer F (2005). Correlation between the IgG index, oligoclonal bands in CSF, and the diagnosis of demyelinating diseases. Eur J Neurol 12(7): 527-30.

McDonald WI, Compston A, Edan G, Goodkin D, Hartung HP, Lublin FD, McFarland HF, Paty DW, Polman CH, Reingold SC, Sandberg-Wollheim M, Sibley W, Thompson A, van den Noort S, Weinshenker BY and Wolinsky JS (2001). Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. Ann Neurol 50(1): 121-7.

McDonnell GV, Kirk CW, Hawkins SA and Graham CA (2000). An evaluation of interleukin genes fails to identify clear susceptibility loci for multiple sclerosis. J Neurol Sci 176(1): 4-12.

McDonnell GV, Mawhinney H, Graham CA, Hawkins SA and Middleton D (1999). A study of the HLA-DR region in clinical subgroups of multiple sclerosis and its influence on prognosis. J Neurol Sci 165(1): 77-83.

McLean BN, Luxton RW and Thompson EJ (1990). A study of immunoglobulin G in the cerebrospinal fluid of 1007 patients with suspected neurological disease using isoelectric focusing and the Log IgG-Index. A comparison and diagnostic applications. Brain 113 (Pt 5): 1269-89.

Michalowska-Wender G, Losy J and Wender M (2001). Biological markers to confirm diagnosis and monitor the therapy in multiple sclerosis patients. Folia Neuropathol 39(1): 1-5.

Montalban X (2005). Primary progressive multiple sclerosis. Curr Opin Neurol 18(3): 261-6.

O'Connor KC, Chitnis T, Griffin DE, Piyasirisilp S, Bar-Or A, Khoury S, Wucherpfennig KW and Hafler DA (2003). Myelin basic protein-reactive autoantibodies in the serum and cerebrospinal fluid of multiple sclerosis patients are characterized by low-affinity interactions. J Neuroimmunol 136(1-2): 140-8.

Ohta M, Ohta K, Ma J, Takeuchi J, Saida T, Nishimura M and Itoh N (2000). Clinical and analytical evaluation of an enzyme immunoassay for myelin basic protein in cerebrospinal fluid. Clin Chem 46(9): 1326-30.

Ohta M, Ohta K, Nishimura M and Saida T (2002). Detection of myelin basic protein in cerebrospinal fluid and serum from patients with HTLV-1-associated myelopathy/tropical spastic paraparesis. Ann Clin Biochem 39(Pt 6): 603-5.

Pirttila T, Mattila K and Frey H (1991). CSF proteins in neurological disorders analyzed by immobilized PH gradient isoelectric focusing using narrow PH gradients. Acta Neurol Scand 83(1): 34-40.

Pozzilli C, Palmisano L, Mainero C, Tomassini V, Marinelli F, Ristori G, Gasperini C, Fabiani M and Battaglia MA (2004). Relationship between emotional distress in caregivers and health status in persons with multiple sclerosis. Mult Scler 10(4): 442-6.

Ramsaransing GS, Heersema DJ and De Keyser J (2005). Serum uric acid, dehydroepiandrosterone sulphate, and apolipoprotein E genotype in benign vs. progressive multiple sclerosis. Eur J Neurol 12(7): 514-8.

Reindl M, Linington C, Brehm U, Egg R, Dilitz E, Deisenhammer F, Poewe W and Berger T (1999). Antibodies against the myelin oligodendrocyte glycoprotein and the myelin basic protein in multiple sclerosis and other neurological diseases: a comparative study. Brain 122 (Pt 11): 2047-56.

Revesz T, Kidd D, Thompson AJ, Barnard RO and McDonald WI (1994). A comparison of the pathology of primary and secondary progressive multiple sclerosis. Brain 117 (Pt 4): 759-65.

Rima BK and Duprex WP (2006). Morbilliviruses and human disease. J Pathol 208(2): 199-214.

Ristic S, Lovrecic L, Brajenovic-Milic B, Starcevic-Cizmarevic N, Jazbec SS, Sepcic J, Kapovic M and Peterlin B (2005). Mutations in the hemochromatosis gene (HFE) and multiple sclerosis. Neurosci Lett 383(3): 301-4.

Rolak LA (1996). The diagnosis of multiple sclerosis. Neurol Clin 14(1): 27-43.

Romatowski J (1984). Degenerative myelopathy in a German shepherd. Mod Vet Pract 65(7): 535-7.

Rosser EJ, Jr. (1997). German shepherd dog pyoderma: a prospective study of 12 dogs. J Am Anim Hosp Assoc 33(4): 355-63.

Rostrom B, Grubb A and Holmdahl R (2004). Oligoclonal IgG bands synthesized in the central nervous system are present in rats with experimental autoimmune encephalomyelitis. Acta Neurol Scand 109(2): 106-12.

Rot U and Mesec A (2006). Clinical, MRI, CSF and electrophysiological findings in different stages of multiple sclerosis. Clin Neurol Neurosurg 108(3): 271-4.

Ruaux CG, Coats JR, March PA and WilliamsDA (2003). Analisis of oligoclonal banding in CSF and serum from dogs with degenerative myelopathy. ACVIM forum, North. Carolina.

Sadaba MC, Gonzalez Porque P, Masjuan J, Alvarez-Cermeno JC, Bootello A and Villar LM (2004). An ultrasensitive method for the detection of oligoclonal IgG bands. J Immunol Methods 284(1-2): 141-5.

Salvadori C, Cantile C, De Ambrogi G and Arispici M (2003). Degenerative myelopathy associated with cobalamin deficiency in a cat. J Vet Med A Physiol Pathol Clin Med 50(6): 292-6.

Summers BA, Whitaker JN and Appel MJ (1987). Demyelinating canine distemper encephalomyelitis: measurement of myelin basic protein in cerebrospinal fluid. J Neuroimmunol 14(2): 227-33.

Thompson AJ, Montalban X, Barkhof F, Brochet B, Filippi M, Miller DH, Polman CH, Stevenson VL and McDonald WI (2000). Diagnostic criteria for primary progressive multiple sclerosis: a position paper. Ann Neurol 47(6): 831-5.

Tienari PJ, Kuokkanen S, Pastinen T, Wikstrom J, Sajantila A, Sandberg-Wollheim M, Palo J and Peltonen L (1998). Golli-MBP gene in multiple sclerosis susceptibility. J Neuroimmunol 81(1-2): 158-67.

Tienari PJ, Terwilliger JD, Ott J, Palo J and Peltonen L (1994). Two-locus linkage analysis in multiple sclerosis (MS). Genomics 19(2): 320-5.

Tipold A, Pfister H and Vandevelde M (1993). Determination of the IgG index for the detection of intrathecal immunoglobulin synthesis in dogs using an ELISA. Res Vet Sci 54(1): 40-4.

Traber MG, Pillai SR, Kayden HJ and Steiss JE (1993). Vitamin E deficiency in dogs does not alter preferential incorporation of RRR-alpha-tocopherol compared with all rac-alpha-tocopherol into plasma. Lipids 28(12): 1107-12.

Vandevelde M, Kristensen F, Kristensen B, Steck AJ and Kihm U (1982). Immunological and pathological findings in demyelinating encephalitis associated with canine distemper virus infection. Acta Neuropathol (Berl) 56(1): 1-8.

Vandevelde M and Spano JS (1977). Cerebrospinal fluid cytology in canine neurologic disease. Am J Vet Res 38(11): 1827-32.

Walker JM (1994). Basic protein and peptide protocols, Humana Press.

Warren S, Greenhill S and Warren KG (1982). Emotional stress and the development of multiple sclerosis: case-control evidence of a relationship. J Chronic Dis 35(11): 821-31.

Waxman FJ, Clemmons RM and Hinrichs DJ (1980). Progressive myelopathy in older German shepherd dogs. II. Presence of circulating suppressor cells. J Immunol 124(3): 1216-22.

Waxman FJ, Clemmons RM, Johnson G, Evermann JF, Johnson MI, Roberts C and Hinrichs DJ (1980). Progressive myelopathy in older German shepherd dogs. I. Depressed response to thymus-dependent mitogens. J Immunol 124(3): 1209-15.

Whitaker JN (1978). Immunochemical comparisons among myelin basic proteins. Comp Biochem Physiol B 59(4): 299-306.

Whitaker JN (1998). Myelin basic protein in cerebrospinal fluid and other body fluids. Mult Scler 4(1): 16-21.

Whitaker JN, Lisak RP, Bashir RM, Fitch OH, Seyer JM, Krance R, Lawrence JA, Ch'ien LT and O'Sullivan P (1980). Immunoreactive myelin basic protein in the cerebrospinal fluid in neurological disorders. Ann Neurol 7(1): 58-64.

Zabad RK, Patten SB and Metz LM (2005). The association of depression with disease course in multiple sclerosis. Neurology 64(2): 359-60.

BIOGRAPHICAL SKETCH

Takashi Oji was born on March 2, 1974, in Fukuoka, Japan. He received his Bachelor of Veterinary Medical Science degree from Yamaguchi University, Japan, in March 1999. He then worked in a small animal practice for five years. After that, he came to University of Florida and did research on canine CSF. From January 2005 to present, he has been a master's student in veterinary medical science at University of Florida. He also works as a research assistant under the guidance of Dr. Roger M. Clemmons.