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Original

Characterization of T lymphocyte phenotype and phosphorylated axonal neurofilament subunit H level associated with presumptive and diagnosed progressive myelomalacia in dogs

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Abstract

The aim of this study was to characterize the T lymphocyte phenotype and phosphorylated axonal neurofilament subunit H (pNF-H) in dogs diagnosed with progressive myelomalacia (PM) and dogs with presumptive PM. A retrospective case series of six dogs with confirmed PM and 8 dogs with presumptive PM was in vesrigared, conducted and clinical signs, magnetic resonance imaging (MRI), the somatosensory evoked potential, and T lymphocyte phenotype in clinical records and pNF-H levels in the peripheral blood were evaluated. pNF-H levels were determined in both study dogs and healthy controls (beagles). PM was clinically diagnosed based on: (Berger et al. 2007) MRI of disc-associated spinal cord compression, (Boylan et al. 2009) clinical progression from initial paraparesis or paraplegia, to thoracic limb lower motor neuron paresis, to tetraplegia associated with cranial migration to the extent of cutaneous trunci reflex loss and analgesia, leading to death via respiratory paralysis, and (Ceron et al. 2005) histological examination. All PM dogs were paraplegic and had signs of lower motor neuron lesions. The $CD4^+/CD8^+$ ratio in 13 out of the 14 dogs (92.9%) was significantly higher than that in healthy controls ($p<0.001$). pNF-H was only detected in the peripheral blood of PM dogs. In some PM dogs, we did not observe signal hyperintensity on T2-weighted MRI. Our study results indicate that the detection of pNF-H and a high $CD4^+/CD8^+$ ratio in the peripheral blood may Facilitate earlier diagnosis of PM than is possible with MRI.

Key words : CD4+/CD8+ratio, dog, myelomalacia, pNF-H, spinal cord

Introduction

Currently, the pathogenic mechanism of canine progressive myelomalacia (PM) remains unknown, and no effective treatment is available. Affected individuals typically die of respiratory failure within several days of symptom onset (Okada et al. 2010). PM, which does not occur in humans, has become one of the most important spinal disorders in dogs and has been reported in approximately 10% of dogs suffering from thoracolumbar Intervertebral disc herniation (IVDH), featuring a loss of pain perception (Olby et al. 2003; Scott et al. 1999). The symptoms associated with canine thoracolumbar IVDH are typically classified into 5 stages: pain with no other symptoms (Grade 1), ambulatory paresis (Grade 2), non-ambulatory paresis (Grade 3), paraplegia (Grade 4),

and paraplegia with loss of nociception (Grade 5) (Schulz et al.1998). Symptom severity is dependent on the extent and degree of compression and damage to the spinal cord. Because PM does not manifest in dogs suffering from thoracolumbar IVDH without paraplegia, canine PM is thought to be a feature only in severe cases of IVDH (i. e., not observed in Grades 1 to 3).

The degree of spinal cord injury can vary in Hansen type I disc herniation, which appears in dogs as an acute protrusion of the nucleus into the spinal canal. Acute spinal cord injury is divided into primary and secondary injuries. Primary injury is caused by direct physical or mechanical pressure exerted onto the neurons and blood vessels by prolapse of the nucleus pulposus. The severity of the primary lesion is dependent on the speed and volume of the nucleus pulposus protruding into the spinal canal as well as the extent and duration of spinal cord compression (Scott et al. 1999). Spinal cord ischemia caused by primary injury can induce a reaction cascade that primarily involves tissue-damaging biochemical changes, which, in turn, can lead to secondary injury. Within 10 to 20 minutes after spinal cord injury, minor bleeding spots develop due to increased vascular permeability. Within hours, the appearance of edema, ischemia, hemorrhagic foci in the gray and white matter, failure of local microcirculation, and a lack of nutrients and oxygen result in the progressive necrosis of neurons in the vicinity. Additionally, as soon as the microcirculation fails (minutes after injury), the continuous overproduction of free radicals, the excitatory neurotransmitter glutamine, and inflammatory cytokines for several weeks thereafter leads to further tissue damage, including apoptosis (Saiwai et al. 2010). The cytokines are produced by the neurons and astrocytes themselves and by neutrophils and macrophages that migrate from the vascular system upon the breakdown of the blood-spinal cord barrier (Gonzalez et al. 2003). Although it is likely that changes in the injured spinal cord microenvironment result in PM development and lesion expansion, the mechanism underlying this process has not yet been elucidated, and no effective treatment is available for PM.

In PM dogs presenting with severe IVDH, surgical extraction of the protruded disc material is not an effective means for preventing disease progression, and it inevitably results in death. Nevertheless, early surgery remains the primary intervention for severe cases of IVDH in dogs, and this is where practicing veterinarians face difficulty in treatment.

The number of reports available on clinical cases and studies of canine PM is extremely limited. In this study, we explored the possible correlations between PM and the results obtained from electrophysiological testing and magnetic resonance imaging (MRI) in addition to a new approach that involves Phosphorylated axonal neurofilament subunit H (pNF-H) analysis along with an examination of the T lymphocyte phenotype. These evaluations were conducted in 14 dogs that were presumed or confirmed to have incurable PM.

Materials and Methods

Case selection

The study was conducted involving 14 dogs diagnosed with acute paraplegia at Aikouishida Animal Hospital from January 2009 to March 2012 that were later presumed or confirmed to have PM. A presumptive diagnosis of PM was made based on clinical symptoms from initial paraplegia, to thoracic limb lower motor neuron (LMN) paresis to tetraplegia associated with cranial migration to the extent of cutaneous trunci muscle reflex loss, leading to death due to respiratory paralysis, in accordance with a report by *Okada et al.* (2010). A definitive diagnosis of PM was made based on histopathological findings in cases in which necropsy was consented to. Where PM was strongly suspected, euthanasia was recommended because of the poor prognosis associated with this disease.

The study was performed in accordance with the Japanese Regulations for Animal Welfare issued by The Ministry of Education, Culture, Sports, Sciences, and Technology of Japan.

Medical records review

The following parameters were obtained from the medical records of each dog: breed, age, sex, body weight, clinical findings from a neurologic examination, existence of pelvic limb nociception prior to MRI examination, existence of vertebral column hyperesthesia, interval between onset of clinical signs and signs of LMN lesions in the pelvic limbs or cranial migration of the neurologic deficits, interval between onset of clinical signs and MRI examination, existence of Horner syndrome, and interval from the onset of clinical signs to death.

C-reactive protein measurement

The C-reactive protein (CRP) level was mea-

sured with immunonephelometry (Laser CRP-2, Arrows, Osaka, Japan) using 30 μL of serum. The range of measurement was set as 0.05-20 mg/dL.

Flow cytometric analysis of peripheral blood

The analysis was performed in rolring on 14 dogs with PM and 6 healthy beagles. The relative ratios (Accuri C6 flow cytometer, Accuri Cytometers Inc., MI, USA) of $CD3^+$ (Tcells), $CD4^+$ (helper Tcells), $CD8^+$ (cytotoxic Tcells), and $CD21^+$ (Bcells) were obtained against the canine-specific antibodies listed in Table 3.

Blood samples (200 μL) were mixed with 2 μL of lysis solution (FACS lysis solution, Becton, Dickinson and Company, NJ, USA) and incubated for 15 minutes at room temperature. Peripheral WBCs (white blood cells) were isolated by centrifugation (200 *g*) for 5 minutes at room temperature. The WBC (white blood cell) fraction was rinsed before a sufficient amount of antibody (Antibodies, Serotec, Oxford, UK) was added. The samples were incubated in the dark for 15 minutes at room temperature. A suitable antibody concentration was calculated beforehand.

Once the reaction was complete, 2 mL of phosphate-buffered saline solution was added to remove the excess primary antibody. Finally, the cells were suspended in 500 μL of sheath fluid (FACSFlow, Becton, Dickinson and Company, NJ, USA) and maintained at 4℃ until analysis (CFlow Plus software, Accuri Cytometers Inc., MI, USA). The cell phenotypes of peripheral lymphocytes were obtained by gating on the screen with a forward scatter versus side scatter dot plot (FACSCalibur, Becton, Dickinson and Company, NJ, USA). For each sample, data from 5,000 events in the lymphocyte gates were recorded, from which the percentages of $CD3^+$, $CD4^+$, $CD8^+$, and $CD21^+$ were categorized according to the different lymphocyte surface markers over the 5,000 events. The absolute values for the lymphocyte subsets were calculated using counts obtained from WBC analysis in combination with the flow cytometer. These procedures were conducted in accordance with *Tamura et al.* (2012).

Quantification of serum pNF-H levels

Serum samples were obtained from 6 healthy beagles (controls) that were purchased for other purposes and 14 PM dogs and were stored at −30℃. The assay was performed using a commercial ELISA kit (ELISA-pNFH; BioVendor Laboratory Medicine Inc., Brno, Czech Republic). Frozen serum samples were thawed, and the samples obtained from each specimen were loaded onto an ELISA plate. The assay was performed according to the manufacturer's protocol.

Neurologic examination

Examinations consisted of tests to assess posturad responses (proprioception, placing reaction, hopping reaction, and extensor postural thrust), spinal reflexes (patellar reflex, cranial tibial reflex, gastrocnemius reflex, flexion reflex, crossed-extension reflex, and panniculus reflex), nociception, and the animals' ability to self-urinate.

Diagnostic imaging

T1 (T1-weighted magnetic resonance imaging) — and T2 (T2-weighted magnetic resonance imaging) — weighted MRI (0.2 T Vet-MR, Esaote S. p. A, Genova, Italy) wase performed under general anesthesia. The findings were reviewed by 3 veterinarians until an agreement was reached regarding the MRI interpretations (including the site and side of IVDH and the locations of abnormal signal intensities within the spinal cord). Abnormal signal intensities within the spinal cord were characterized as hyperintense, isointense, or hypointense compared with healthy spinal cord parenchyma on sagittal and transverse T1WI and T2WI. The lengths of the abnormal signal intensities were measured in comparison with the body length of L2.

Somatosensory evoked potential

The somatosensory evoked potential test was performed under general anesthesia with isoflurane. Somatosensory evoked potential (SEP) was measured with a system (Neuropack MEB-9102, Nihon Koden, Tokyo, Japan) that adopts surface-disc electrodes as recording and reference electrodes. The recording electrode was placed at the juncture of the coronal and sagittal sutures, which was considered to be adjacent to the somatosensory area, and the reference electrode was placed on the spinous process of the axis following the method of *Uzuka et al.* (1995). Electrical stimulation was applied at a frequency of 3 Hz for 0.2 milliseconds to obtain an average total of 500 responses according to the method described by *Okuno et al.* (2005).

Statistical analyses

The significance of the values was determined using the Mann-Whitney *U*-test and other statistical analyses (StatMate III for Windows, Atms, Tokyo, Japan). Values with $P<0.001$ were considered significant.

Results

Dogs

This study was conducted using 14 dogs (8 males and 6 females): including 12 miniature dachshunds, one French bulldog, and one American cocker spaniel (Table 1). The mean age of the dogs was 5.42±2.9 years, and that of the body weight was 6.22±3.0 kg (mean±SD).

Clinical signs

The chief complaint in all dogs was the sudden onset of paraplegia. Eleven of the 14 dogs had developed paraplegia within 12 hours of the onset of symptoms including discomfort, anorexia, or paraparesis at the owner properties, and the remaining dogs had developed paraplegia 24 hours after the onset of paraparesis. The body temperature was≥39.5℃ in 3 of the 14 dogs. High CRP levels (over 1.0 mg/dL) were observed in 2 of the 14 dogs. In 3 of the 14 dogs, the WBS counts were higher than 20,000 cells/μL. According to their clinical histories, each dog lacked nociception in the pelvic limbs at the time of referral for evaluation. All dogs eventually developed LMN signs in the pelvic limbs, cranial migration of neurologic deficits, or both. Eight of the 14 dogs developed bilateral Horner syndrome on the day prior to their death (Table 2). The mean duration from the onset of PM until death was 7.43±2.9 days (mean±SD). Ten dogs died due to disease progression, and the remaining dogs were euthanized.

Diagnosis

A definitive diagnosis of PM was made in 6 dogs. This sample included 4 dogs that were euthanized and 2 dogs that died naturally from disease progression. A pathological diagnosis was made during post-mortem examination with consent from each owner. The remaining 8 dogs were presumed to have PM based on findings that were consistent with the case definition.

Magnetic resonance imaging

The median interval from the onset of clinical signs to MRI was 2.0 days (range: 1 to 4 days). IVDH was detected in all of the dogs (Table 2), and diffuse abnormal signal intensities were evident in the spinal cord at the site of IVDH. The T1WI of the affected areas revealed isointense signals that were absent in healthy spinal cords, whereas hyperintense signals were detected on T2WI (Figure 1). The length of abnormal hyperintensity

Table 1 Clinical characteristics of dogs with confirmed or presumptive PM

Dog No.	Breed	Age (y)	Sex	BW (kg)	Nociception	CRP (mg/dl)	WBC (μl)	BT (℃)	pNF-H (ng/ml)
1	Miniature Dachshund	12	M	5.9	−	0	10500	38.9	8.11
2	Miniature Dachshund	4	M	3.7	−	4.2	18700	38.8	6.76
3	Miniature Dachshund	3	M	5.7	−	0.45	13000	38.6	9.88
4	Miniature Dachshund	3	F	8.1	−	0	21700	39.7	7.15
5	Miniature Dachshund	5	M	4.9	−	0	19000	38.6	6.45
6	Miniature Dachshund	4	M	5.6	−	0.65	14600	39.2	8.76
7	French Bulldog	2	M	14	−	0.25	17900	39.8	9.01
8	American Cocker Spaniel	2	F	13	−	0	18700	39.4	6.43
9	Miniature Dachshund	6	F	3.4	−	1.1	14900	38.5	5.65
10	Miniature Dachshund	5	F	5.6	−	0.8	9400	38.6	9.54
11	Miniature Dachshund	4	M	7.4	−	0	20800	39.2	9.12
12	Miniature Dachshund	11	F	5.5	−	0	16700	38.6	8.99
13	Miniature Dachshund	6	F	5.8	−	0	10100	38.9	9.71
14	Miniature Dachshund	6	M	5.2	−	0.4	25500	39.5	9.59
15	Miniature Dachshund	5	M	4.7	+	0	11500	38.7	0
16	Miniature Dachshund	3	M	6.1	+	0	8700	38.8	0
17	Miniature Dachshund	3	M	4.5	+	0	12500	38.4	0
18	Miniature Dachshund	3	F	4.9	+	0	11800	38.7	0
19	Miniature Dachshund	6	F	5.1	+	0	9100	38.6	0
20	Miniature Dachshund	7	F	4.4	+	0	10500	38.8	0

C=Confirmed (based on necropsy or gross surgical inspection). CRP=C-reactive protein. F=Female. M=Male. BW=Body weight. BT=Body temperature. WBC=White blood cell. pNF-H=phosphorylated axonal neurofilament subunit H.
−=Absent. +=Present.
PM: Dog Nos. 1-14.
Healthy: Dog Nos. 15-20.

Table 2 Findings of MRI and neurologic signs in dogs with confirmed or presumptive PM

Dog No.	Site of IVDH	Length of signal hyperintensity[a]	Interval between onset of clinical signs and MRI (d)	SEP[b]	Horner syndrome	Survival duration (d)	Diagnosis
1	T13-L1	2	2	−	+	6	P
2	T12-13	3	2	−	+	8	C
3	T12-13	NR	NR	−	+	6	C
4	T13-L1	1.5	2	−	+	6	C
5	T13-L1	NR	NR	−	NR	8	P
6	T11-12	NR	NR	−	+	5	P
7	L2-3	NR	NR	−	+	3	P
8	T13-L1	5	4	−	NR	14	P
9	T13-L1	NR	NR	−	+	13	P
10	T13-L1	4	2	−	NR	6	C
11	T13-L1	9	3	−	+	6	C
12	L1-2	NR	NR	−	NR	6	C
13	L1-2	0	1	−	NR	9	P
14	T12-13	0	1	−	NR	8	P

a) Expressed as multiples of the length of the body of L2 at the site of IVDH. b) Somatosensory evoked potential.
C=Confirmed (based on necropsy or gross surgical inspection). IVDH=Intervertebral disk herniation. NR=Not recorded. P=Presumptive (based on characteristic clinical signs of PM).
−=Absent. +=Present.
Euthanasia: Dog Nos. 2, 4, 10, 12.

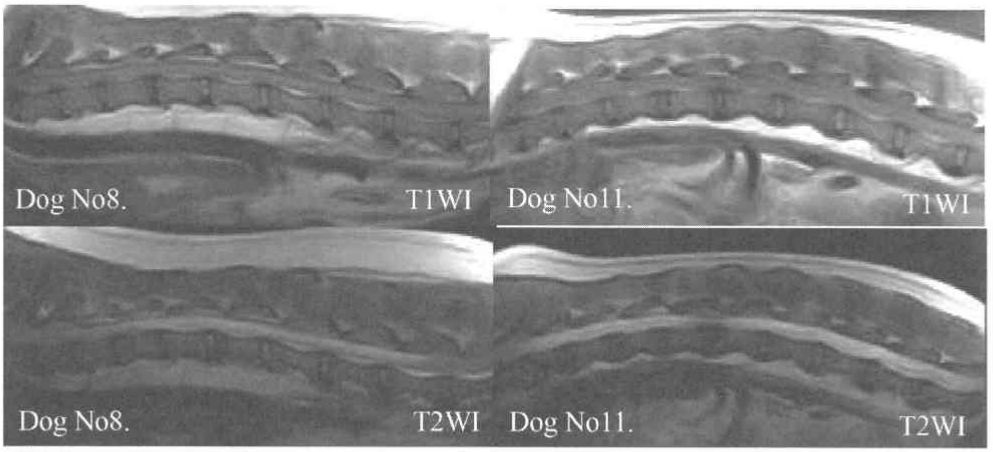

Fig. 1 Magnetic resonance imaging. T1-weighted and T2-weighted images of Dog No. 8 and Dog No. 11. In the T2-weighted image of Dog No. 8, the length of signal hyperintensity was 5 times that of L2. In the T2-weighted image of Dog No. 11, the length of signal hyperintensity was 9 times that of L2.

Table 3 Monoclonal antibodies used in the present study

Phenotype	Specificity	Host	Isotype	Conjugate	Clone
CD21	B-cells	Mouse	IgG1	RPE[a]	CA2.1D6
CD3	T-cells	Mouse	IgG1	FITC[b]	CA17.2A12
CD4	Helper T-cells	Rat	IgG2a	FITC	YKIX302.9
CD8	Cytotoxic T-cells	Rat	IgG1	RPE	YCATE55.9
NC[c]	Mouse IgG1	Mouse	IgG1	RPE	DAK-GO1
NC	Mouse IgG1	Mouse	IgG1	FITC	DAK-GO1

a) RPE, Rhodophyceae phycoerythrin; b) FITC, Fluorescein isothiocyanate isomer I; c) Negative control.
Antibody concentrations: As in manual.

of the spinal cord on sagittal T2WI ranged from approximately 0 to 9 times that of thelumbar (L2) vertebral body.

Somatosensory evoked potential

Somatosensory evoked potential amplitudes were undetectable in the pelvic limbs of all dogs.

Flow cytometric analysis of peripheral blood

Peripheral blood was analyzed by flow cytometry to determine the ratios of surface antigen markers. In PM dogs, the median relative rates (all % Values=tares) of surface markers were as follows : 76.0% $CD3^+$ (ranging from 69.9 to 83.0%), 6.7% $CD21^+$ (3.1 to 11.5%), 55.9% $CD4^+$ (46.0 to 65.0%), and 17.8% $CD8^+$ (13.4 to 27.7%). The median $CD4^+/CD8^+$ ratio was 3.2 (ranging from 1.8 to 4.8) (Table 4). Comparatively, the median relative ratios of surface markers in healthy dogs were as follows: 73.6% $CD3^+$ (ranging from 70.3 to 80.7%), 7.2% $CD21^+$ (5.9 to 15.7%), 46.8% $CD4^+$ (39.2 to 54.1%), and 24.3% $CD8^+$ (21.4 to 28.7%). The median $CD4^+/CD8^+$ ratio was 1.84 (ranging from 1.66 to 2.39). PM dogs exhibited significantly higher peripheral $CD4^+/CD8^+$ ratios compared with healthy dogs ($p<0.001$).

Quantification of serum pNF-H levels

pNF-H was detected in the peripheral blood of all PM dogs, and the mean concentration was 8.23 ±1.39 (SD) ng/mL. However, pNF-H was not detected in the blood of 6 healthy control beagles. The concentration of peripheral pNF-H levels in PM dogs was significantly different from that of the controls ($p<0.001$).

Discussion

This is the first study to examine T lymphocyte phenotypes and pNF-H levels in the peripheral blood of dogs with PM.

The manifestation of clinical signs of PM indicates imminent death. Horner syndrome, which was identified in 8 (57.1%) of the 14 dogs in this study, is one of the clinical symptoms unique to PM. The appearance of this syndrome indicates that the ascending progressive neuropathy has moved along the spinal cord and reached the head and thoracic region. All dogs that developed Horner syndrome died within a few days. Veterinarians should thus inform owners of the impending death of their animals when PM is suspected based on evidence of Horner syndrome.

In dogs with PM, T2WI MRI of the spinal cord

Table 4 Phenotypes on T lymphocytes in dogs with confirmed or presumptive PM

Dog No.	$CD3^+$ lymphocytes*	$CD21^+(CD3^-)$ lymphocytes*	$CD4^+(CD8^-)$ lymphocytes*	$CD8^+(CD4^-)$ lymphocytes*	$CD4^+/CD8^+$ ratio
1	69.9	10.0	49.7	19.8	2.51
2	75.8	5.3	50.6	14.2	3.56
3	79.6	3.1	65.0	20.6	3.16
4	76.7	10.9	62.0	16.2	3.83
5	70.1	8.0	64.1	13.4	4.78
6	80.4	3.1	63.7	14.6	4.36
7	80.7	5.9	51.1	27.7	1.84
8	75.8	5.3	50.6	20.2	2.50
9	70.5	11.5	55.1	20.6	2.67
10	76.2	7.4	60.0	15.7	3.82
11	78.7	3.7	58.9	18.2	3.24
12	83.0	8.9	56.7	17.1	3.32
13	74.1	5.9	49.5	17.6	2.81
14	70.8	8.2	46.0	18.0	2.55
15	72.1	6.8	46.9	27.2	1.72
16	76.8	6.6	48.7	25.1	1.94
17	76.1	9.4	44.3	22.9	1.93
18	70.9	12.1	47.1	23.1	2.04
19	71.5	11.9	44.5	22.9	1.94
20	72.2	7.8	48.1	23.8	2.02

* Values are given in percentages.
PM: Dog Nos. 1-14.
Healthy: Dog Nos. 15-20.

typically shows hyperintensity. *Okada et al.* reported that T2 signal hyperintensity with a length 6 to 20 times that of the L2 vertebral body was observed on MRI of all 12 PM animals examined (Okada et al.2010). In our study, T2 signal hyperintensity was observed in 6 of 8 animals that underwent MRI. The length of the T2 signal hyperintensity ranged from 1.5 to 9 times that of the L2 vertebral body (Table 2), and only 1 animal showed a T2 signal hyperintensity with a length of more than 6 times that of the L2 vertebral body. The fact that our results differ from those reported by *Okada et al.* is likely to be attributable to the difference in the elapsed time from the onset of clinical signs to MRI (Okada et al. 2010). The median interval between the onset of clinical signs and MRI was 2.0 days (ranging from 1 to 4 days) in this study, whereas the interval was 3.7 days (ranging from 2 to 7 days) in the study by *Okada et al* (2010). The MRI of 2 animals that did not exhibit T2 signal hyperintensity in our study was performed within 24 hours of the onset of paraplegia. Therefore, extensive T2 signal hyperintensity, which is considered to be a feature unique to PM, may not be a characteristic finding if MRI is performed soon after onset.

An acute elevation of serum CRP levels reflects of inflammation and progressive tissue damage (Ceron et al. 2005). CRP is currently used as an inflammatory marker to determine the prognosis associated with a number of neoplastic (Shimada et al. 2003) diseases, myocardial infarction, and stroke in humans (Clearfield et al. 2005; Winbeck et al. 2002). Several recent reports on CRP levels in dogs demonstrated elevated CRP levels associated with certain disease states, including inflammatory bowel disease (Jergens et al. 2003), immune-mediated arthritis (Moore et al. 1992), and autoimmune hemolytic anemia (Tecles et al. 2005). However, because the rise in CRP was evident in only a few of the PM dogs in our study, it was deemed unsuitable as a specific diagnostic marker.

In dogs, SEP can be recorded on the scalp by applying electrical stimulation to peripheral nerves on the scalp. The recorded SEP waveform reflects the activation of the sensory cortex. However, in humans, the short-latency SEP recorded on the scalp by applying electrical stimulation yields several waveforms that are considered to be induced in the peripheral nerve, spinal cord, brain stem, and cerebral sensory cortex (Poncelet et al. 1993; Uzuka et al. 1995; Vanderzant et al. 1989). The waveform amplitude can be reduced or lost in dogs by experimentally compressing the spinal cord and blocking the blood supply to it (Okuno et al. 2005). Previously, we reported a loss of SEP amplitude in clinical cases of neurological grade 5 IVDH in dogs (Okuno et al. 2012). In this study, loss of the SEP amplitude was confirmed in all PM dogs, indicating that a complete lack of amplitude is also observed during SEP monitoring in PM dogs.

Flow cytometers are used to analyze cell surface markers and can be functionally classified as either cell sorters, which have a monitor and can selectively isolate the cells of interest, or cell analyzers, which are only capable of monitoring cell surface markers. In this study, we utilized a cell analyzer to analyze T lymphocyte phenotypes. T lymphocytes are classified into 2 major subsets: $CD4^+$ ($CD8^-$) helper T lymphocytes and $CD8^+$ ($CD4^-$) cytotoxic T lymphocytes. The ratio of the 2 subsets in the peripheral blood of adult dogs is very similar to that of humans (Faldyna et al. 2001; Moore et al. 1992). According to Harris et al., the $CD4^+/CD8^+$ ratio is 1.7 in healthy humans (Harris et al. 1992), and, in our study, the median $CD4^+/CD8^+$ ratio was 1.84 (ranging from 1.66 to 2.39) in healthy dogs. Furthermore, the lymphocyte subset ratio in the peripheral blood in our study revealed similarities between dogs and humans. Therefore, we conclude that the peripheral blood lymphocyte subset ratios are very similar in healthy dogs and humans. *Gulizia et al.* (1993) reported that the $CD4^+/CD8^+$ ratio was significantly higher in human patients with immune-mediated diseases compared with that of healthy subjects. In our study, the elevated $CD4^+/CD8^+$ ratio observed in 13 out of the 14 (92.9%) dogs with PM (Table 4) was significantly higher compared with that of healthy dogs ($p<0.001$). These results suggest that the $CD4^+/CD8^+$ ratio could be a new diagnostic criterion for canine PM and should be explored further in future research. We note that CD3 and CD21 are additional T lymphocyte cell surface markers that can also be used for characterization.

Monitoring certain biomarkers in the blood or cerebrospinal fluid may be effective for determining the severity and stage of PM. A number of proteins produced in neurons and glial cells, including S100B, neuron-specific enolase, myelin basic protein, and glial fibrillary acidic protein, have been proposed as biomarkers of central nerve damage from traumatic brain injury in humans (Berger et al. 2007; Honda et al. 2010). However, reports of functional biomarkers for canine PM have yet to be published.

A correlation between serum pNF-H levels and

disease outcomes has been described in recent studies that employed an animal spinal cord injury model (Shaw et al. 1999) and patients with aneurysmal subarachnoid hemorrhage (Loy et al. 2005). pNF-H is a major structural protein complex of axons and detected in the plasma when axons are injured. Because the phosphorylated form is resistant to protease degradation, pNF-H functions as an effective marker to monitor the extent of the injury. The serum pNF-H concentration reaches its peak 3 days after spinal cord injury, in contrast to other neuron-derived biomarkers such as S100B and neuron-specific enolase, the concentrations of which peak within 24 hours after the injury (Loy et al. 2005; Shaw et al. 1999). Such changes in serum pNF-H levels over time appear to reflect the progressive loss of axons as a result of secondary damage. In experiments using a rat spinal cord injury model, serum pNF-H levels correlated with the strength of the initial impact (primary insult) applied via a spinal cord impactor (Shaw et al. 1999). Based on these findings, we analyzed the serum pNF-H levels were in canine PM to examine whether pNF-H could serve as a biomarker. In our study, pNF-H was not detected in the peripheral blood of healthy animals but was clearly detected in the peripheral blood of all PM dogs. Correlations between serum pNF-H levels and the prognosis have already been reported in studies conducted in aneurysmal subarachnoid hemorrhage patients and amyotrophic lateral sclerosis patients (Boylan et al. 2009; Lewis et al. 2008). Further studies are required to verify whether the pNF-H concentration can be used as an effective clinical biomarker of PM. The establishment of pNF-H as a biomarker of PM may facilitate the development of new therapies and improve the prognosis associated with currently incurable PM.

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