FULL PAPER *Pathology*

A Comparative Pathological Study on Canine Necrotizing Meningoencephalitis and Granulomatous Meningoencephalomyelitis

Mari SUZUKI[1)], Kazuyuki UCHIDA[1)]*, Motoji MOROZUMI[2)], Takashi HASEGAWA[3)], Tokuma YANAI[4)], Hiroyuki NAKAYAMA[5)] and Susumu TATEYAMA[1)]

[1)]Departments of Veterinary Pathology and [3)]Veterinary Teaching Hospital, Faculty of Agriculture, Miyazaki University, Miyazaki 889–2155, [2)]Togasaki Animal Hospital, Saitama 341–0044, [4)]Department of Veterinary Pathology, Faculty of Agriculture, Gifu University, Gifu 501–1193 and [5)]Department of Veterinary Pathology, Faculty of Agriculture, The University of Tokyo, Tokyo 113–8657, Japan

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ABSTRACT. Canine necrotizing meningoencephalitis (NME) and granulomatous meningoencephalomyelitis (GME) were compared pathologically. Gross observation exhibited lateral ventricular dilation and discoloration, malacia and/or cavitation of the cerebrum in NME. On the contrary, gross changes were milder in GME, except for occasional visible granulomatous mass formation. Histopathologically, the lesions of NME were distributed predominantly in the cerebral cortex and various degrees of inflammatory and necrotic changes were observed according to clinical stages. Besides, microscopic lesions of GME were mainly distributed in the white matter of the c erebrum, cerebellum and brainstem, which are characterized by perivascular cuffing, multiple granulomas and leptomeningeal infiltrates. Although macrophages and lymphocytes were predominant in the inflammatory lesions of both disorders, macrophages in GME transformed into epithelioid cells and exhibited more massive infiltration. Although lectin RCA-1-reactive cells were numerous in both disorders, lysozyme immunoreactive cells in NME were fewer than that in GME. Parenchymal infiltration of MAC387-positive cells was common in GME and limited in NME. The number of CD3-positive lymphocytes in the GME lesions tended to be greater than in NME, though the difference was not statistically significant. Morphological and immunohistochemical differences of the lesions, in particular, the characteristics of infiltrative macrophages may reflect these different pathogeneses of the two disorders.

KEY WORDS: canine, granulomatous meningoencephalomyelitis, macrophage, necrotizing meningoencephalitis.

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Canine necrotizing meningoencephalitis (NME) is a unique inflammatory disorder in small-sized breed dogs, especially in Pug dogs. The disease is histopathologically characterized by inflammatory changes consisting of lymphocytic, plasmacytic and histiocytic infiltrations and apparent parenchymal necrosis located mainly in the cerebral cortex [9, 15, 23]. The common clinical features are forebrain signs such as partial or generalized seizure, decreased consciousness, abnormal behavior, circling and ataxia [9, 23]. The cause of NME is still unknown. However, our previous report showed that a certain autoantibody against a canine brain tissue was detected in the cerebrospinal fluid (CSF) and serum, which may suggest an autoimmune pathology in NME [25].

The pathological features of NME are often compared with granulomatous meningoencephalomyelitis (GME) that is another inflammatory disease of unknown cause [5, 8, 23]. Although there are some differences such as breed predilection, the distribution of lesions and the presence or absence of necrotic foci, GME and NME show similar histological changes, i.e., meningitis and perivascular cuffing composed of mononuclear cells including lymphocytes and monocyte/histiocyte-lineages [5, 8, 23]. Kipar *et al*. [14] revealed lesions in GME are predominantly composed of CD3 antigen-positive T lymphocytes and a heterogeneous population of activated macrophages with strong MHC class II expression. However, to date, there are a few studies regarding immunohistochemical characterization of inflammatory cells in canine NME. The purpose of the present study is to characterize the inflammatory cells in the lesions of NME and GME and to reveal the pathological differences of these two disorders.

* CORRESPONDENCE TO: UCHIDA, K., Department of Veterinary Pathology, Faculty of Agriculture, Miyazaki University, Miyazaki 889–2155, Miyazaki, Japan.

MATERIALS AND METHODS

Animals and tissue processing: Brain tissues from 15 necropsied dogs, including eleven NME and four GME cases, were obtained from local veterinary practitioners, Veterinary Teaching Hospital of Miyazaki University and Departments of Veterinary Pathology of University of Tokyo and Gifu University. The brains were fixed in 10% buffered formalin or methanol Carnoy's fixatives. Paraffin sections of 6 μm-thick were stained with hematoxylin and eosin (HE).

Immunohistochemistry and lectin histochemistry: Immunohistochemistry was performed using Envision polymer reagent (DAKO-Japan, Kyoto, Japan). Lectin histochemistry was performed by the avidin-biotin peroxidase complex method (ABC, Vector Laboratories, Burlingame, CA, U.S.A.). Sections were heated with autoclave, at 121°C for 5 min, or enzymatic digestion with proteinase K (DAKO-Japan) at room temperature for 10 min, for antigen retrieval. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxidase in methanol at room temperature for 10 min. The sections were incubated for 30 min at

37°C with primary antibodies or biotin-labeled lectin RCA-1 (EY laboratories Inc., San Mateo, CA, U.S.A.). The primary antibodies employed were rabbit antibodies against lysozyme (1:300, DAKO-Japan) and human CD3 (1:20, DAKO-Japan) and canine C3 (1:100, ICN Biomedicals, Inc., Aurora, OH, U.S.A.), and mouse monoclonal antibodies against macrophage, myeloid/histiocyte antigen clone MAC387 (prediluted, DAKO-Japan), canine distemper virus nucleoprotein (CDV-NP, 1:100, VMRD Inc., Pullman, WA, U.S.A.) and canine distemper virus (CDV, 1:200, ViroStat, Portland, MA, U.S.A.). Additionally, biotin-labeled sheep antiserum against canine IgG (1:100, American Qualex, San Clemente, CA, U.S.A.) was used. All sections were incubated with Envision polymer reagent (DAKO-Japan) or ABC reagent (Vector laboratories) at 37°C for 30 min. Reaction products were visualized with 3,3'-diaminobenzidine (Sigma, St Louis, MO, U.S.A.) and the sections were counterstained with hematoxylin.

Quantitative data analysis: The numbers of CD3-positive cells were counted under a magnification of × 400 at selected 10 fields including lesions. Lesions with high cellular density such as meningitis or perivascular cuffing were selected for counting and cases without high cellular density area, were excluded. The mean values and standard deviations were calculated. The mean values were compared by Student *t*-test.

RESULTS

Case histories: Clinical features of 15 dogs examined are summarized in Table 1. The ages of dogs at necropsy ranged from 7 months to 12 years (mean; 3.3 years) in NME and from 2 years and 6 months to 4 years (mean; 3.6 years) in GME. Breeds of NME dogs were 9 Pug dogs, one Papillon and one Maltese. In GME, a Golden Retriever, Shih Tzu, Miniature Dachshund, and Maltease were collected. Generalized seizure was a common clinical sign of NME, and depression, anorexia, circling, tremor, paresis, ananastasia and change of the character were also recorded. In contrast, dogs with GME showed a wide range of clinical neurological signs such as depression, tremor, generalized seizure, hyperesthtesia ataxia and blindness. The titer of neutralizing antibody against CDV in sera or CSF was examined for 5 cases in NME and 2 cases in GME. One NME (Case 5) and two GME (Cases 12 and 15) exhibited high titers, × 2,560, × 5,120 and × 10,240, respectively. Magnetic resonance (MR) imaging or computer tomography (CT) scanning was performed for 7 cases in NME and 3 cases in GME. MR image or CT scan often revealed dilation of the lateral ventricle in various degree, and multifocal or laminar necrosis in the cerebral cortex in NME dogs. The MR imaging depicted higher intensity regions in the brainstem of a GME dog (Case 12). Other GME dogs had a mass at the brain base (Case 14) by CT scan and lateral dilation of the ventricle by MR image (Case 15). Survival time ranged 2 days to 6 years and 5 months in NME and 1 day to 2 months in GME. Ten dogs died spontaneously and five were euthanized.

Gross findings: NME; Eight of 11 cases had the dilation of the lateral ventricles in various degree (Table 1). The septum completely vanished in 2 cases due to severe dilation. At the border between the cerebral cortex and white matter, there were multifocal to scattered discoloration, malacic or cavitation foci. Cortical atrophy was present in 4 cases, and hippocampal and cerebellar atrophy was recorded in Cases 2 and 8, respectively.

GME; Cases 12 and 13 showed mild to severe congestion and hemorrhage in the white matter of the brainstem and cerebellum, respectively. In Case 14, a large granulomatous mass, approximately 1.2 cm in diameter, was found at the optic chiasm, and the optic nerves were swollen severely. Partial discoloration of the white matter in the cerebrum and dilation of the lateral ventricle were observed in Case 15.

Histopathology: NME; The lesions were mainly distributed in the cerebrum. Although both the cortex and white matter were involved, upper and deeper areas of the cerebral cortex suffered severely. Three dogs (Cases 6, 7 and 11) showed severe cerebellar lesions, especially in the white matter. The lesions of NME were classified into three stages according to the severity of necrosis and the intensity of inflammatory reaction. Two dogs (case 1 and 3) had moderate to severe inflammatory changes with a few malacic foci (Fig. 1). The clinical courses between the first clinical onset and death of these dogs ranged from 2 to 68 days. Seven cases (Cases 2, 5, 6, 7, 9–11) exhibited severe malacic foci or cavitation and moderate to severe inflammation concurrently. In these cases, the clinical courses ranged from 59 to 434 days. In the last pattern, necrotic changes including multifocal malacia and large cavitation were dominant, though inflammatory changes were minimal (Cases 4 and 8). The clinical courses of these two dogs ranged from 533 days to 6 years and 5 months. Spongy changes of the neuropil and numerous ischemic neurons were distributed in the cerebral cortex in all cases. Inflammatory lesions mainly existed in the leptomeningeal or perivascular areas and were comprised of a lot of mononuclear cells a small number of neutrophils (Fig. 2). Macrophages severely infiltrated into necrotic and malacic foci. In almost all cases, diffuse astrocytosis was observed especially around the necrotic lesions. There were a large number of gemistocytes, characterized by their hypertrophic eosinophilic cytoplasm. Microglial cells accumulated mildly to moderately.

GME; Although lesions were distributed widely throughout the CNS, the brainstem, cerebellum, and cerebrum were often affected. The spinal cord obtained only from Case 12, had similar lesions. The white matter of the cerebrum, brainstem, and cerebellum suffered most severely (Fig. 3). The gray matter and leptomeninges were affected moderately or mildly. The typical lesions consisted of perivascular cuffing, multifocal granulomas, hemorrhage and leptomeningeal infiltrates. Macrophages, epitheloid cells and lymphocytes were predominant in all lesions (Fig. 4), but the ratio of these cells varied in each lesion. Binucleated or trinucleated epithelioid cells, plasma cells and neutro-

Table 1. Clinical and pathological features of 15 dogs

Case No.	Breed	Age**	Sex	Clinical course	Clinical signs	Gross findings	Pathological diagnosis
1	Pug	8M	F	Died after 2 days	Seizures,salivation	Diffuse hemorrhage on the dura, moderate dilation of the lateral ventricle, discoloration of the deep gray matter of the cerebrum	NME
2	Pug	2Y	F	Died after 129 days	Seizures	Mild dilation of the leteral ventricle, severe hippocampal atrophy	NME
3*	Pug	2Y	M	Died after 68 days	Anorexia, depression, eye discharge, seizures	Severe dilation of the lateral ventricle, discoloration of the deep gray matter of the cerebrum	NME
4*	Pug	9M	M	Died after 533 days	Seizures	Moderate dilation of the lateral ventricle, cortical atrophy,discoloration,malacic area or cavitation in the deep gray matter of the cerebrum	NME
5	Pug	7M	F	Euthanatized after 26 days	Circling, paresis, seizures	Malacic areas in the deep gray matter of cerebrum	NME
6	Pug	1Y	F	Euthanatized after 148 days	Seizures,salivation, tremor, ananastasia,	Severe dilation of the lateral ventricle, severe cortical atrophy, discoloration or cavitaion of the deep gray matter of the cerebrum	NME
7	Pug	8Y	F	Died after 102 days	Seizures	Mild dilation of the lateral venticle, severe cortical atrophy, discoloraiton or malacic areas in the deep gray matter of the cerebrum	NME
8	Maltese	12Y	M	Euthanatized after 6 years 5 months	Seizures	Severe cerebral and mild cerebellar atrophy, severe dilation of the lateral ventricle	NME
9	Pug	2Y 2M	M	Died after 59 days	Seizures	Dilation of lateral ventricle, malacic area at the cerebral cortex	NME
10	Papillon	1Y 5M	M	Euthanatized after 97 days	Circling, tremor, change of character, seizures	Malacic areas at the cerebral cortex	NME
11	Pug	5Y 5M	F	Died after 434 days	Seizures, ananastasia	Mild dilation of the lateral ventricle,discoloration or cavitation of cerebral cortex	NME
12	Golden Retriever	4Y	M	Died after 2 months	Depression, ataxia	Severe disseminated congestion and hemorrhage in the white matter of the cerebellum and brainstem	NME
13	Shih Tzu	4Y	M	Died after 1 day	Circling, seizures	Mild disseminated congestion and hemorrhage in the white matter of the brainstem	GME
14	Miniature Dachshund	4Y	M	Died after 2 months	Blindness, tremor, ataxia,hyperesthesia	Mass at the optic chiasm, swelling of the optic nerves	GME
15	Maltese	2Y 6M	F	Euthanatized after 2 months	Tremor,ataxia	Partial discoloration of cerebral white matter	GME

NME: necrotizing meningoencephalitis, GME: granulomatous meningoencephalomyelitis, Y: years, M: months or male, F: female. * Case 3 and 4 were previously reported in ref 25, ** age: age at necropsy.

phils were occasionally scattered. In Case 14, a large granuloma was observed at the optic chiasm, and the optic nerves suffered from severe granulomatous neuritis. There were no viral inclusion bodies or bacterial organisms in all cases.

Immunohistochemistry and lectin histochemistry: Infiltrative macrophages or microglia in both disorders exhibited intense reactivity to lectin RCA-1 (Figs. 5 and 6). Although one GME case showed limited number of lysozyme-positive cells, lysozyme immunoreactivity was apparent in histiocytes of other GME cases (Fig. 7). On the other hand, granular pattern of immunoreactivity for lysozyme was faint to mild in NME (Fig. 8). Astrocytes and neurons sometimes reacted to this antibody. In both diseases, CD3-positive lymphocytes scattered in the meningitis, perivascular cuffs and parenchymal lesions. The number of CD3-positive cells in GME tended to be greater than that in NME, but the difference was not significant statistically. MAC387-immunoreactivity detected in granulocytes, monocytes and a limited number of macrophages. MAC387-positive cells were

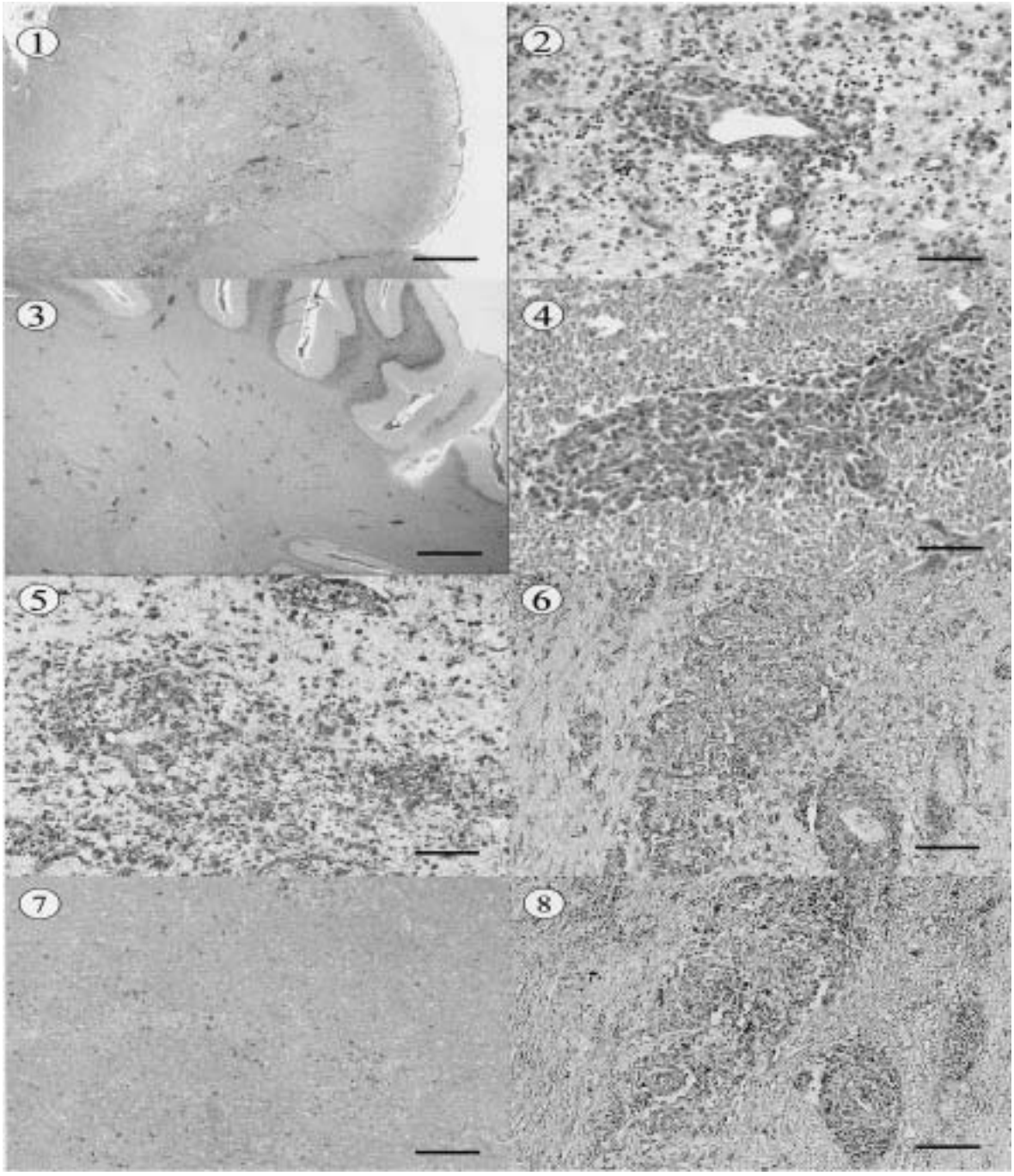

Fig. 1. Cerebrum, Case 1, NME. Inflammatory changes at the border between the cerebral cortex and white matter with multiple malacic foc i. HE Bar=1,000 μm.
Fig. 2. Cerebrum, Case 5, NME. Perivascular and parenchymal infiltration of mononuclear cells including histiocytes and lymphocytes. HE. Bar=0 μm.
Fig. 3. Cerebellum, Case 12. GME. Multifocal perivascular inflammatory changes in the cerebellar white matter. HE. Bar=1,000 μm.
Fig. 4. Pons, Case 13, GME. Perivascular accumulation of epithelioid histiocytic cells. HE. Bar=50 μm.
Fig. 5. Cerebrum, Case 2. NME. A large number of lectin RCA-1-positive cells around the vessels and neuropil. ABC method. Bar=80μm.
Fig. 6. Cerebrum, Case 12. GME. Many lectin RCA-1-positive cells among perivascular infiltrates and granulomas. ABC method. Bar=80 μm.
Fig. 7. Cerebrum, Case 2, NME. A few lysozyme-positive cells in the lesions of Fig. 5. Envision polymer method. Bar=80 μm.
Fig. 8. Cerebrum, Case 12. GME. Relatively large number of lysozyme-positive cells in the lesions of Fig. 6. Envision Polymer method. Bar=80 μm.

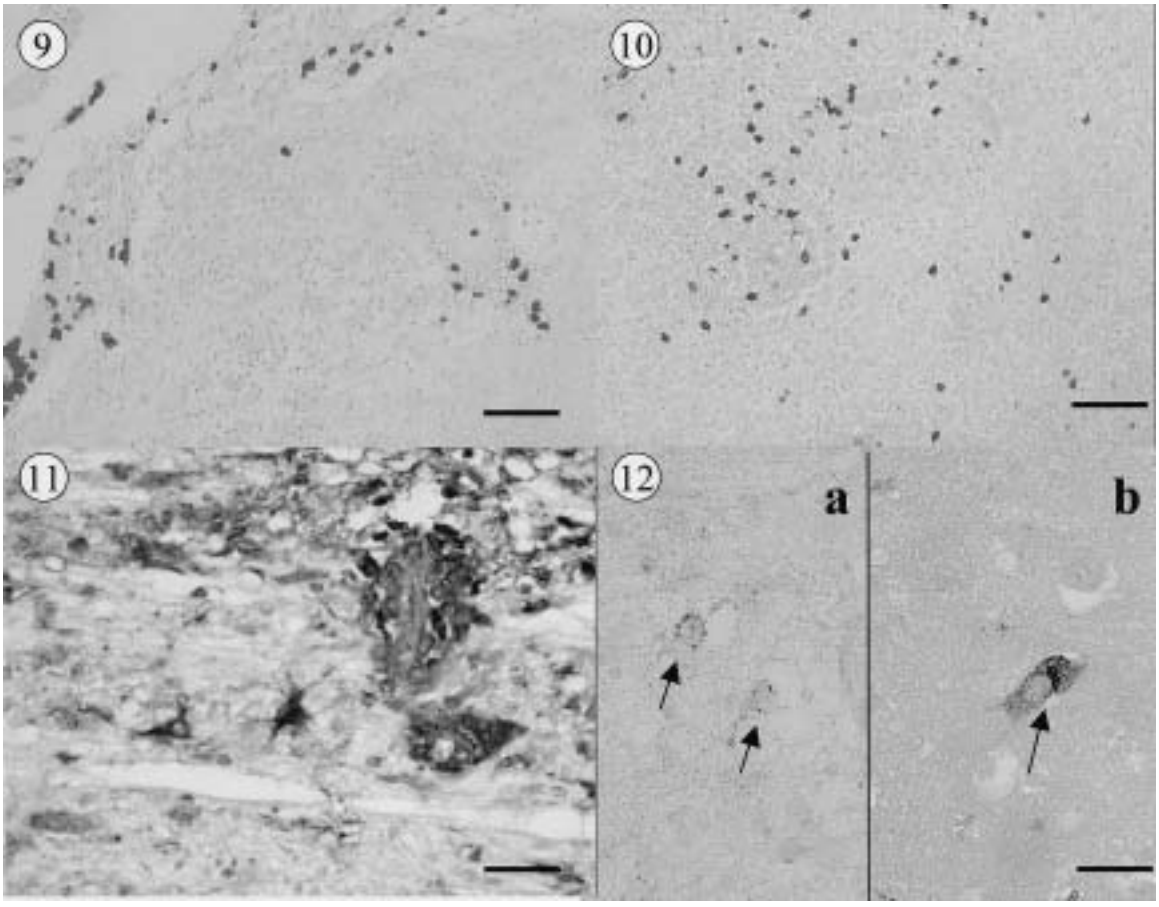

Fig. 9. Cerebrum, Case 6. NME. A small number of MAC387-positive cells in the leptomeninges and in or around the blood vessels. Envision polymer method. Bar=80 μm.

Fig. 10. Cerebrum; Case 13. GME. MAC387-positive cells scatted in the brain parenchyma. Envision polymer method. Bar=80 μm.

Fig. 11. Cerebrum; Case 1. NME. Plasma cells, astrocytes, and macrophages exhibited immunoreactivity for canine IgG. Envision polymer method. Bar=33 μm.

Fig. 12. Cerebrum; Case 2 with NE (a), and Case 12 with GME (b). Granular materials positive for CDV-NP within the cytoplasm of cerebral neurons (arrows). Envision polymer method. Bar=25 μm.

mainly distributed in meninges and in or around blood vessels in NME (Fig. 9). While those in GME were scattered in brain parenchyma as well as perivascular lesions (Fig. 10). IgG and C3 were present in various kinds of cells including plasma cells, astrocytes, histiocytes and neurons (Fig. 11). Necrotic foci as well showed diffuse immunoreactivity to IgG and C3. GFAP-positive astrocytes were increased in number only in the white matter in GME, but distributed widely over the cerebrum in NME. Gemistocytic astrocytes exhibited intense positive reactivity to GFAP in NME. Both antibodies used in this study detected the CDV-positive granules. Neurons possessing CDV-positive cytoplasmic granules were occasionally present in almost cases with both NME and GME (Figs. 12a and 12b), while the number and intensity varied from case to case. These neurons were commonly found in the intact cerebral cortex and the nuclei of the brain stem out side the lesions of both NME and GME.

DISCUSSION

Both NME and GME are canine specific encephalitis of unknown cause and histopathological lesions are sometimes similar to one another [23]. Some clinical features, however, are different between the two disorders. First, Pug dogs are apparently predisposed to NME [9, 15, 23]. In this study, Maltese and Papillon dogs were also affected in NME. Although NME of Maltese dogs was previously reported [22], there has been no report of Papillon dog. It is probable that this disorder may affect more kinds of small pedigrees other than Pug dogs. On the contrary, GME, small-sized breeds are often affected but any breed dogs can develop disease [9, 19]. The age of onset recorded in this study roughly matched with that of the previous reports [9, 15, 22, 23]. Eight of 11 dogs of NME were younger than 2 years old and GME dogs were a little older, suggesting that NME can occur younger dogs than GME. The clinical signs

recorded in this study were similar to those of previous reports [5, 6, 8, 9, 15, 19, 22, 23]. Generalized seizure may reflect severe lesions at the cerebrum in NME. Cordy and Holliday [9] reported that over half of NME cases were acute type, but there were more number of chronic forms in the present study. Case 8 was unusual, because the dog survived for 6 years and 5 months from the onset of disease, while the histopathological features were in conformity with those of chronic form NME [9]. Clinical course was longer in NME than in GME in this study. However, survival time of GME ranged more widely in the previous report [19].

Gross and histopathological findings were quite different between the two disorders. Gross findings of NME were relatively common, including dilation of the lateral ventricle and discoloration, malacia or cavitation in the cerebrum. Gross lesions of GME were mild, expect for occasional granulomatous mass formation. When the clinical course in NME was longer, the necrotic changes were more apparent but inflammatory changes were milder. Cordy and Holliday [9] reported similar phenomenon of NME previously. The association between clinical course of the disease and the severity of the lesions in GME is controversial [10] and was not confirmed in this study. Summers *et al.* [23] described that the microscopic lesions of NME had been confused with GME, especially in acute phase. Acute NME was similar to GME in that severe inflammatory changes with perivascular cuffing existed from the deep cerebral cortex to the white matter and the lesions were mainly composed of mononuclear cells. However, the lesions of GME were marked in the brainstem, were more angiocentric and exhibited massive epithelioid cells infiltration [23]. Although macrophages infiltrated into the lesions of NME, they were more scattered than those of GME. Moreover, granuloma never developed in NME.

The different nature of macrophages between the diseases was revealed by immunohistochemical staining using anti-lysozyme antibody. Lysozyme is a marker for macrophages/histiocytes and myeloid leukocytes in humans. Moore [17] demonstrated that this marker was also useful for the identification of macrophages in canine tissues. In this study, lysozyme-positive cells were predominantly detected in GME but faint to mild in NME. On the other hand, positive cells for lectin RCA-1, another macrophages/histiocytes marker, was intensely detected in both diseases. Lectin RCA-1 is originally employed for detecting microglia [16], and the cells are presumably derived from bone marrow and play histiocyte-like roles in CNS [1]. Microglias can be distinguished from infiltrative macrophages by their characteristic ramified morphology and their localization in normal CNS. However, it is difficult to discriminate microglia from infiltrative macrophages within the inflammatory lesions, because microglia may transform into reactive macrophage-like cells. In human brain, microglia are negative for lysozyme and infiltrative macrophages are positive [26]. Taking the results of immunohistochemical and lectin staining in this study together, microglia may play a more important role than infiltrative histiocytes in NME. The difference of MAC387-positive cell distribution in the two disorders may support this hypothesis because MAC387 is a marker of macrophages, monocytes and granulocytes [7, 30]. In addition, Yamashita *et al.* [29] discussed that cell-mediated immunology plays an important role for the lysozyme synthesis of macrophages in granuloma. Thus, the pathogenesis of GME may be associated with cell-mediated immunology as discussed in previous report [14].

The causes of NME and GME are still unknown. Previously, an autoantibody against astocytes was found in the NME [25], thus an autoimmune pathology may associate with the pathogenesis of this disease. If the autoantibody plays a role for the pathogenesis of NME, severe tissue destruction may be caused by antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). However, immunoreactivity to IgG and C3 could not demonstrate this hypothesis due to their low specificity. Astrocytes in or around the lesions tended to exhibit intense reactivity to IgG in some NME cases, but this phenomenon is found in various types of lesions and is commonly interpreted as non-specific expression [28] or IgG uptake by reactive astrocytes [3, 4, 12, 13]. *In vivo* and *in vitro* experimental models are necessary to examine further correlation between pathogenesis and autoantibody against astrocytes. Complement system is an important factor of CNS dysfunction including autoimmune diseases such as multiple sclerosis in human [18, 20]. Although anti-canine C3 antibody was applied immunohistochemically to a CNS disorder, degenerative myelopathy in German shepherd dogs [2], it may be difficult to interpret the results because C3 is an intermediate component of the complement system and may be detected widely. Moreover, it is known that astrocytes and maybe microglias, neurons or oligodendroglia produce complement in CNS [11, 20]. Kipar *et al.* [14] revealed the bulk of lymphocytes expressed CD3 antigen and discussed T cell-mediated delayed-type hypersensitivity as the pathogenesis of GME. Significant difference of the number of CD3-positive cells between two disorders was not detected between GME and NME. However, it may be not surprising that T cell existed in the lesions of NME because Th cells are necessary for plasma cells to produce antibodies.

On the other hands, infectious agents, especially viruses have been suspected to be the cause or trigger of NME and GME, although they have never been detected [9, 15, 21–23, 27]. In the neurons of almost dogs, CDV-positive granules were present in varied degree. However, the meaning of CDV-positive neurons is still questionable, because these CDV-positive granules in neuron were also found in clinically normal brains examined as controls. Thus, this result may represent non-specific reaction of CDV-antibodies or cross-reactivity to common antigens of CDV within canine neurons. However, in viral infection at young age can prime for and trigger autoimmunity when the virus show molecular mimicry with self-CNS antigens and this phenomenon is influenced by genetic factor [24]. In fact, some NME and GME dogs had very high titer for CDV-antibody. To clarify

the relationship between CDV infection and the pathogeneses of NME and/or GME, further studies will be needed.

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