

## Detection of an Autoantibody from Pug Dogs with Necrotizing Encephalitis (Pug Dog Encephalitis)

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**Abstract.** An autoantibody against canine brain tissue was detected in the cerebrospinal fluid (CSF) and serum of two Pug dogs (Nos. 1 and 2) by indirect immunofluorescence assay (IFA). Dog No. 1, a 2-year-old male, exhibited severe depression, ataxia, and generalized seizures and died 2 months after the onset of symptoms. Dog No. 2, a 9-month-old male, exhibited severe generalized seizures and died 17 months after the onset of symptoms. Histopathologic examination revealed a moderate to severe multifocal accumulation of lymphocytes, plasma cells, and a few neutrophils in both the gray and white matter of the cerebrum in dog No. 1. In dog No. 2, the cellular infiltrates were mild, but there was a severe, diffuse, and multifocal necrosis in the cerebral cortex with prominent astrocytosis. With the aid of IFA using fluorescein isothiocyanate-labeled anti-dog IgG goat serum and a confocal imaging system, specific reactions for glial cells were detected in the CSF of these Pug dogs but not in six canine control CSF samples. Double-labeling IFA using CSF from these Pug dogs and a rabbit antiserum against glial fibrillary acidic protein (GFAP) revealed that the autoantibody recognized GFAP-positive astrocytes and their cytoplasmic projections. By immunoblot analysis, the autoantibody from CSF of these Pug dogs recognized two common positive bands at 58 and 54 kd, which corresponded to the molecular mass of human GFAP. The role of this autoantibody for astrocytes is not yet clear. However, if the presence of the autoantibody is a specific feature of Pug dog encephalitis, it will be a useful clinical diagnostic marker and a key to the pathogenesis of this unique canine neurologic disease.

*Key words:* Astrocytes; autoantibody; dogs; necrotizing encephalitis; Pug dog encephalitis.

Necrotizing nonsuppurative meningoencephalitis, which is characterized by inflammatory changes with lymphocytic, plasmacytic, and histiocytic infiltration and apparent parenchymal necrosis in the cerebrum, has been reported to occur predominantly in Pug dogs<sup>2,3,10,11,17</sup> and rarely in other breeds, such as Maltese dogs,<sup>18,19</sup> and is therefore also known as Pug dog encephalitis. The clinical neurologic lesions result in generalized seizure, ataxia, and depression in young Pug dogs. The cause of Pug dog encephalitis is unknown. Previous reports have suggested that some viral infections such as canine herpes type 1 or canine distemper may play an initial role in the pathogenesis of the necrotizing encephalitis.<sup>3</sup> However, no direct evidence has been produced to support this hypothesis.

In humans, there are several immune system-mediated neurologic diseases, including multiple sclerosis and Guillain-Barré syndrome.<sup>4,5,7</sup> These disorders are characterized by prominent central and peripheral nerve demyelination, respectively, and some immune system responses to the constituents of myelin are proposed as important pathologic events.<sup>4,5,7,21</sup> In dogs, such immune system-mediated neurologic disorders are very rare, although canine idiopathic polyradicu-

loneritis may have a pathogenesis similar to that of human Guillain-Barré syndrome.<sup>10,20</sup> In some patients with these immune system-mediated diseases, several types of autoantibodies have been isolated, but their pathogenic significance has not always been clarified.

In the present study, an autoantibody against astrocytes was identified in the cerebrospinal fluid (CSF) and serum of two Pug dogs with necrotizing meningoencephalitis, and the target cells and proteins of the antibody were examined.

### Materials and Methods

#### Dogs

Dog No. 1, a 2-year-old male Pug, exhibited severe depression, ataxia, and generalized seizures and died 2 months after the onset of the disease. Dog No. 2, a 9-month-old male Pug, exhibited severe generalized seizures. This dog was treated with phenobarbital (2 mg/kg) and prednisolone (0.7 mg/kg) but died 17 months after the onset of the disease. Examinations of the CSF and serum revealed no increase of neutralizing antibody against canine distemper in comparison with the reference values (<1/64). In addition, both animals had been vaccinated for canine distemper virus. At necropsy, samples of CSF and brain tissues were collected for autoantibody assay and histopathologic examination, re-

spectively. Tissue samples for routine histology were fixed with 10% formalin, and selected brain tissues were fixed with methanol Carnoy's solution for immunohistochemistry. In addition, CSF samples from six dogs without neurologic signs were used as controls. Serum samples from two normal dogs were also examined. As antigen for the fluorescence assay, 10- $\mu$ m-thick cryostat sections from a clinically normal 3-year-old mixed-breed dog were used. The cryostat sections were taken from the frontal cerebral cortex and were fixed with acetone at  $-20$  C for 5 minutes. Homogenate samples from the cerebral cortex were also employed in an immunoblot assay.

### Histopathology

Paraffin-embedded sections 6  $\mu$ m thick were stained with hematoxylin and eosin (HE). Selected sections were stained with Luxol fast blue. Immunohistochemistry for astrocytes, T lymphocytes, and plasma cells and/or B lymphocytes was performed using a fluorescence assay or the Envision polymer method (Dako Japan, Kyoto, Japan). The following antibodies were used: rabbit antisera against human glial fibrillary acidic protein (GFAP, prediluted, Dako), human CD3 (1 : 50, Dako), and fluorescein isothiocyanate (FITC)-labeled sheep antiserum against canine IgG (1 : 100, American Qualex, San Clemente, CA). To visualize microglia, sections were stained with biotinylated lectin *Ricinus communis agglutinin-1* (RCA-1, 1 : 400, EY Laboratories, San Mateo, CA).

### Indirect fluorescence assay

To detect the autoantibody against canine brain tissues, cryostat sections of the cerebrum of a clinically normal dog were prepared. Before the indirect fluorescence assay (IFA), the collected CSF and serum samples of both the Pug dogs and the negative controls were incubated at 36 C for 30 minutes to inactivate complement. Cryostat sections were incubated at 37 C for 30 minutes with serially diluted CSF or serum samples (1 $\times$ , 10 $\times$ , and 100 $\times$ ). Sections then were incubated with an FITC-labeled sheep antiserum against canine IgG (1 : 100, American Qualex) and subsequently observed with the aid of a fluorescence microscope. As a positive control antibody, a rabbit antiserum against human GFAP (prediluted, Dako) was used with a FITC-labeled goat antiserum against rabbit IgG (1 : 200, Dako).

### Double labeling IFA

For the determination of the autoantibody binding sites, double labeling IFA was performed with FITC- and rhodamine-labeled antibodies. Cryostat sections were incubated with CSF samples from Pug dogs and rabbit antiserum against human GFAP at 37 C for 30 minutes. Sections then were reacted with a FITC-labeled sheep antiserum against canine IgG (American Qualex) and a rhodamine-labeled goat antiserum against rabbit IgG (1 : 100, Kirkegaard and Perry Laboratories, Gaithersburg, MD) at 37 C for 30 minutes. The sections were observed and evaluated with a confocal imaging system (MRC-600, Nippon BioRad Laboratories, Tokyo, Japan).

### Immunoblot analysis

Homogenized brain samples from a clinically normal dog were employed for sodium dodecyl sulfate polyacrylamide gel electrophoresis. Blotting was performed using clear blot membrane-P (Atto, Tokyo, Japan). Immunoblot analysis was carried out using CSF samples from both the Pug dogs and the normal controls, a biotin-labeled sheep antiserum against canine IgG (1 : 100, American Qualex), and an avidin-biotin peroxidase complex reagent (PK-4000, Vector Laboratories, Burlingame, CA). The reaction products were visualized with 3,3'-diaminobenzidine (Sigma, St. Louis, MO). As positive control sera, a mouse monoclonal antibody against human GFAP (1 : 50, Dako) and a rabbit antiserum against human GFAP (prediluted, Dako) were employed together with biotinylated secondary antisera against the mouse IgG (1 : 200, Dako) or rabbit IgG (1 : 200, Dako).

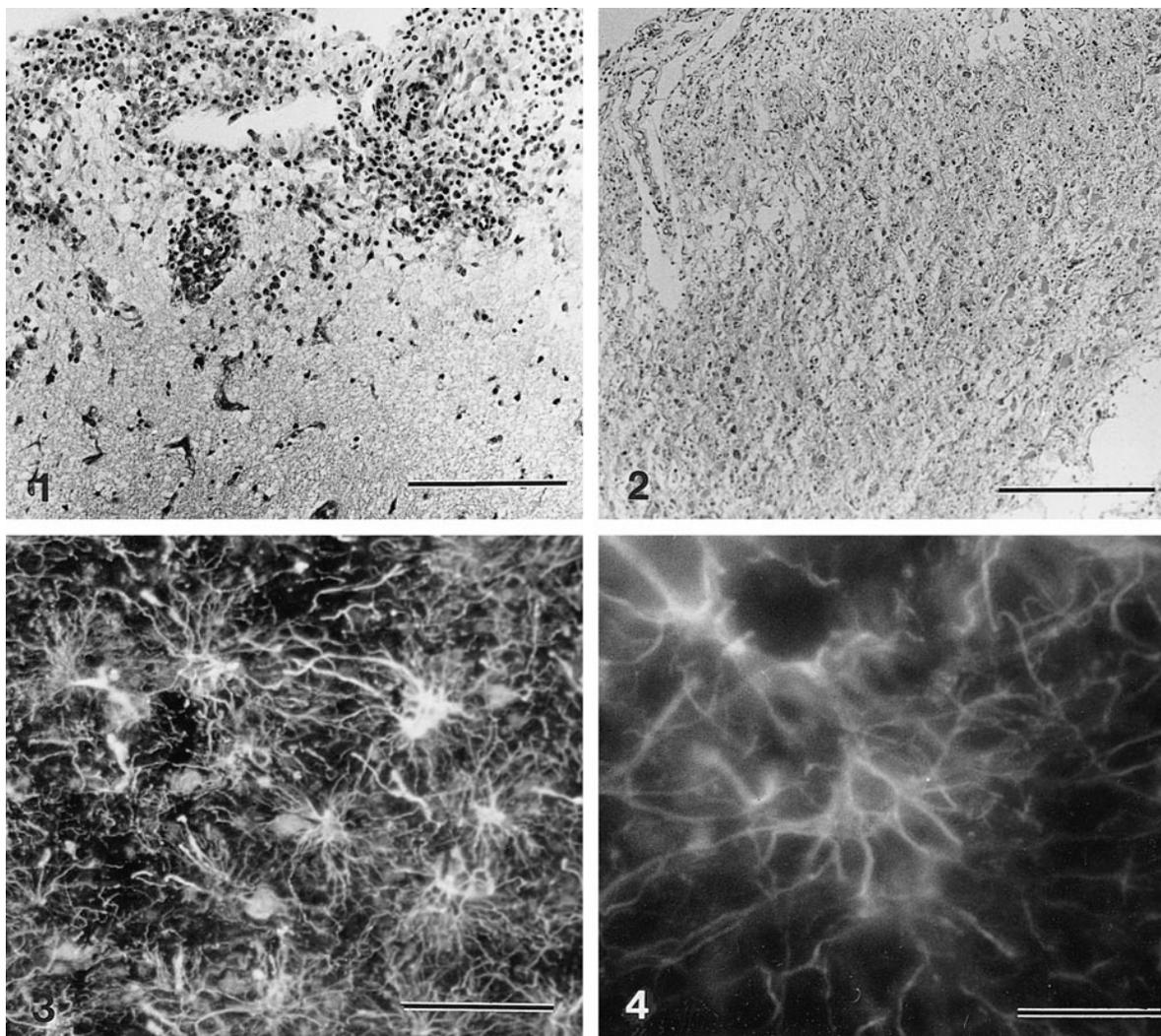
## Results

### Gross findings

At necropsy, dog No. 1 exhibited mild to moderate dilation of the lateral ventricles. Several scattered pale foci were distributed in the deep cerebral cortex. In the visceral organs, the lungs showed severe diffuse congestion, but there were no significant gross lesions in the other organs. In dog No. 2, the lateral ventricles were dilated with mild diffuse cortical atrophy. In the cerebral cortex, there were multifocal yellowish, pale malacic areas, sometimes with cavitation. There were no obvious gross lesions in the visceral organs except for severe, diffuse pulmonary congestion and edema.

### Histopathologic findings

The cerebrum of the dog No. 1 exhibited moderate to severe multifocal accumulation of mononuclear cells and a few neutrophils (Fig. 1). The lesions were located bilaterally in both the gray and white matter of the cerebrum but not in the other brain regions, such as thalamus, midbrain, cerebellum, spinal cord, and spinal roots. These inflammatory changes were most prominent in the subleptomeningeal area and the border between the gray and white matter. Among these infiltrative cells, a large number of cells were positive for canine IgG, suggesting that they were B lymphocytes or plasma cells, and a few cells showed a positive reaction for CD3. In addition, IgG-positive cells predominantly accumulated around blood vessels, and a few CD3-positive cells diffusely infiltrated the brain parenchyma. Moderate spongy changes of the neuropil and numerous ischemic neurons were also distributed in the cerebral cortex. In these foci, there was a mild proliferation of reactive astrocytes that were positive for GFAP and a mild to moderate multifocal accumulation of microglial cells that were labeled by lectin RCA-1. In dog No. 2, the cellular infiltrates were mild but severe diffuse cortical necrosis, multifocal cortical malacia with large cavitation, and diffuse astrocytosis



**Fig. 1.** Cerebrum; Pug dog No. 1. Severe accumulations of mononuclear cells together with a few neutrophils in the subleptomeningeal area of the cerebral cortex. HE. Bar = 125  $\mu$ m.

**Fig. 2.** Cerebrum; Pug dog No. 2. Severe diffuse cortical necrosis, multifocal cortical malacia with large cavitation, and diffuse astrocytosis in the cerebral cortex. HE. Bar = 125  $\mu$ m.

**Fig. 3.** Cerebrum; Pug dog No. 1. Immunofluorescence staining of acetone-fixed cerebrum using CSF and FITC-labeled secondary antiserum against canine IgG reveals cytoplasm and process of glial cells. IFA. Bar = 60  $\mu$ m.

**Fig. 4.** Cerebrum; Pug dog No. 1. Higher magnification of Fig. 3. IFA. Bar = 30  $\mu$ m.

(Fig. 2). There was a large number of gemistocytes, characterized as hypertrophic large, plump astrocytes with abundant eosinophilic cytoplasm that were positive for GFAP and had multiple nuclei. A small number of gitter cells also accumulated in the malacic area. Lectin RCA-1 staining revealed moderate, diffuse, and multifocal proliferation of microglial cells. In the subleptomeningeal area of the cortex, there was a mild perivascular to diffuse infiltration of plasma cells. There were also small perivascular cuffs consisting of macrophages and plasma cells in both the gray and white matter. In neither dog were there inclusion bodies or bacterial organisms. From these findings, both

of the Pug dogs were diagnosed with necrotizing non-suppurative meningoencephalitis (Pug dog encephalitis).

#### Autoantibody assay

IFA of the CSF samples from both of the Pug dogs and of the serum from dog No. 2 revealed the specific yellowish-green fluorescence of FITC in the cytoplasm and processes of the glial cells (Figs. 3, 4). The strongly IFA-positive cells were distributed mainly in the subleptomeningeal and perivascular areas of the gray matter and localized diffusely in the white matter. The distribution pattern of these cells was almost the same

as that of the GFAP-positive cells examined as a positive control. These specific fluorescence signals were detectable at a 10× dilution of CSF and serum from dog No. 2 and a 100× dilution of CSF from dog No. 1. In the other six control canine CSF and serum samples, no specific reactions were detected, but a small number of neurons had nonspecific granular yellowish fluorescence in the cytoplasm.

Double-labeling IFA methods confirmed that the autoantibody from the two Pug dogs was bound to the GFAP-positive cells, suggesting that they were astrocytes. The secondary antibodies used in this study failed to react with brain tissue without CSF from Pug dogs and rabbit antiserum against human GFAP. By confocal imaging microscopy, the specific sites for autoantibody in the CSF were observed as a green signal (Fig. 5A), and the positive sites for GFAP were represented by a red signal (Fig. 5B). Common sites labeled with both the autoantibody and the antiserum against GFAP were represented as yellow signals and were located in the cytoplasm and process of astrocytes (Fig. 5C). The specific sites for the autoantibody were considerably broader than those for antiserum against GFAP and were distributed widely in the processes of astrocytes.

Immunoblot analysis revealed that the autoantibody in the CSF of both cases occurred in two positive bands at 58 and 54 kd (Fig. 6, lanes 3 and 5). These positive bands were also detectable in the 100× dilution of CSF from dog No. 1 (Fig. 6, lane 4). In contrast, in the control CSF no specific positive bands were detected (Fig. 6, lane 6). In the positive control lanes, the mouse monoclonal antibody against GFAP was detected in a single band at 54 kd (Fig. 6, lane 1). In addition, incubation with the rabbit antiserum against GFAP produced four intensely positive bands at around 50 kd (Fig. 6, lane 2). Several bands at about 80 kd observed in most samples, including negative controls, were considered nonspecific.

### Discussion

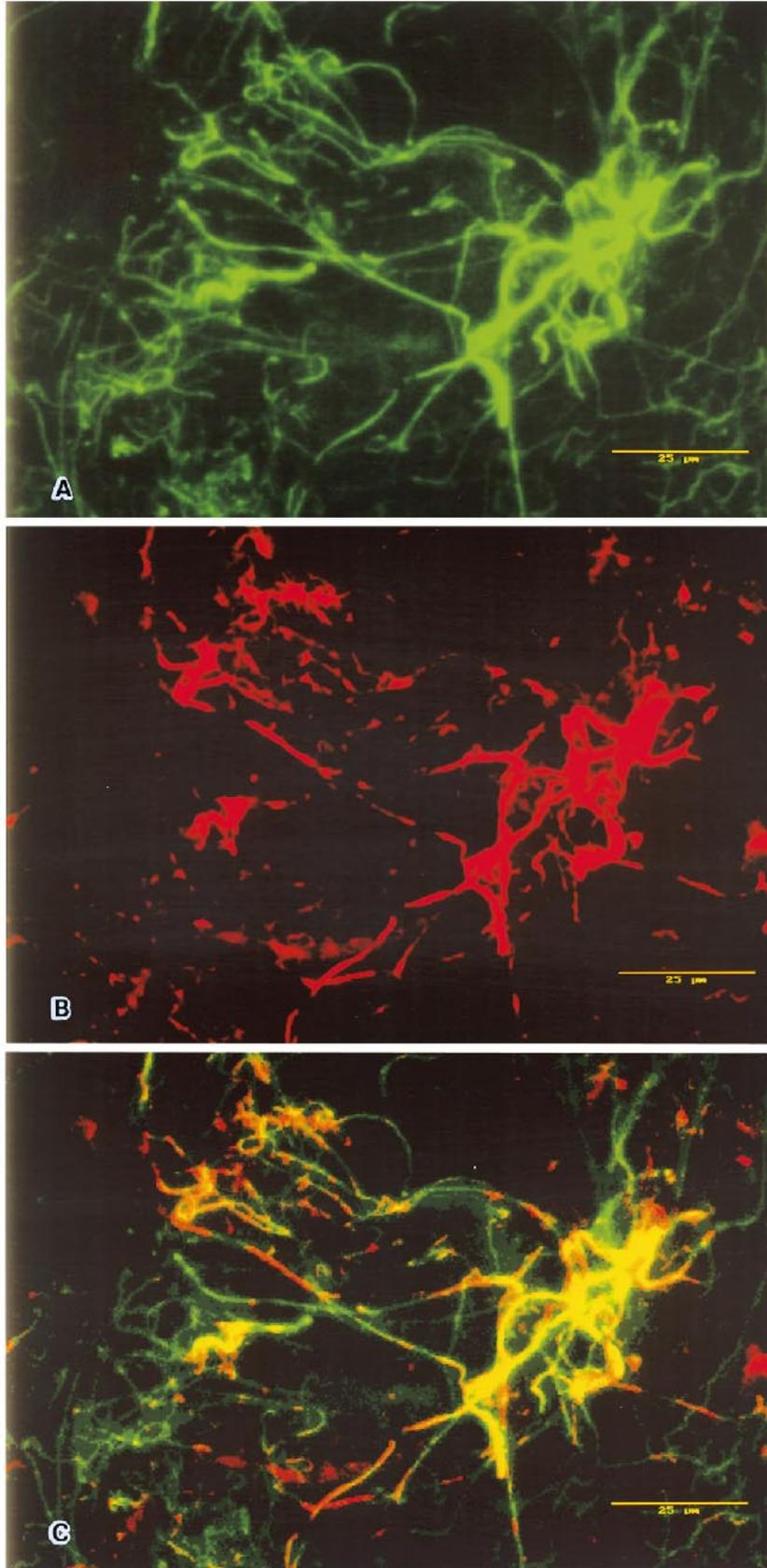
The histologic brain lesions of the two Pug dogs were somewhat different, but the distribution pattern of the lesions was similar. In dog No. 1, there were moderate to severe inflammatory reactions consisting of abundant mononuclear cells and a few neutrophils with moderate necrotic lesions containing ischemic neurons. In contrast, dog No. 2 exhibited severe, dif-

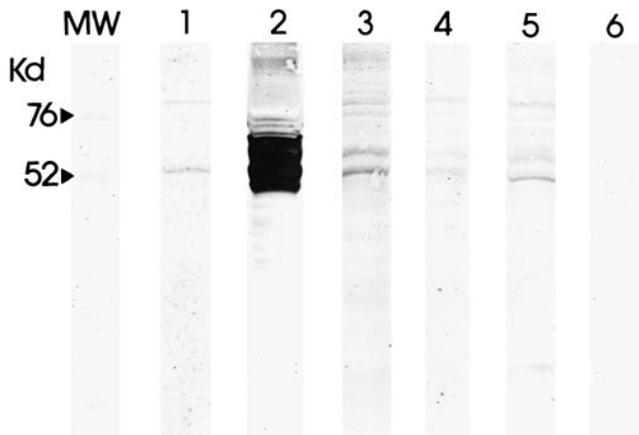
fuse, and multifocal cortical necrosis with abundant astrogliosis and moderate microgliosis, but the cellular infiltrates were very mild. The differing histologic appearance of these brain lesions might be due to the different clinical histories; dog No. 1 survived for only 2 months and dog No. 2 survived for 17 months after clinical onset. Thus, dog No. 1 represents a subacute stage and dog No. 2 represents a chronic course of Pug dog encephalitis.<sup>3</sup> Moreover, immunosuppressive treatment using prednisolone might play a role in producing chronic clinicopathologic changes in dog No. 2 in addition to lower IFA titer of autoantibody. Some lesions, such as laminar cortical necrosis with ischemic neuronal changes, might be secondary and due to prolonged seizures. However, the clinical and essential pathologic features in both dogs are almost the same as those previously described for Pug dog encephalitis.<sup>2,3,11</sup>

The cause of necrotizing meningoencephalitis in Pug dogs is still unknown.<sup>10,17</sup> Although several authors have suggested that some viruses such as canine distemper virus or the canine herpes virus may be responsible for the initial pathogenesis of Pug dog encephalitis,<sup>3</sup> there has been no evidence to support an infectious etiology in this unique disease. Even in these two Pug dogs, there were no findings to suggest infectious agents. Several differential etiologies other than viral infections have been studied, including canine granulomatous meningoencephalitis, toxoplasmosis, seizure-related cortical necrosis, toxic or metabolic causes, and circulatory disturbances due to cardiac arrest,<sup>3</sup> but these would be unlikely primary events in Pug dog encephalitis. However, no previous reports have included the possibility that Pug dog encephalitis is an immune system-mediated disorder. Thus, in the dogs discussed here, the presence of an autoantibody in CSF or serum was examined because autoantibody initiates and/or induces lesions in several autoimmune diseases.<sup>9</sup> In the present study, an autoantibody against canine astrocytes was found in CSF and serum in the Pug dogs, which had suffered from necrotizing, nonsuppurative encephalitis. To our knowledge, there have been no other reports concerned with the presence of autoantibodies in cases of Pug dog encephalitis. A monoclonal antibody against human GFAP labeled a canine 54-kd protein, which corresponded to the molecular mass of human GFAP. In addition, rabbit antiserum against human GFAP la-

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**Fig. 5.** CSF; dog. Double-labeling immunofluorescence method with a confocal imaging system reveals that the specific sites for autoantibody in the CSF are observed as a green signal by FITC (A), the positive sites for GFAP are represented by a red signal by rhodamine (B), and common sites labeled with both antibodies are represented as yellow signals (C) which are located in the cytoplasm and fibers of astrocytes. Bar = 25 μm.





**Fig. 6.** Immunoblot analysis using homogenized brain sample with the incubation of a mouse monoclonal antibody against human GFAP (lane 1), a rabbit antiserum against human GFAP (lane 2), 10 $\times$  diluted CSF from dog No. 1 (lane 3) and dog No. 2 (lane 5), 100 $\times$  diluted CSF from dog No. 1 (lane 4), and nondiluted control canine CSF (lane 6).

beled four intensely positive bands at around 50 kd on immunoblots from canine brain extracts. These facts indicate that the autoantibody recognizes some astrocytic proteins and suggest the possibility that the target proteins of the autoantibody may be a subset of GFAPs. Because the autoantibody also labeled a canine 58-kd protein on immunoblot and a few GFAP-negative sites in astrocytes by confocal imaging microscopy, the autoantibody might have polyclonality and recognize some astrocytic protein other than GFAP.

The significance of the autoantibody against astrocytes in the pathogenesis of Pug dog encephalitis is unclear. The presence of the autoantibody may be interpreted in one of two ways. An autoimmune response responsible for producing the autoantibody may play an important role in the pathogenesis of Pug dog encephalitis. Serum of patients with several immune system-mediated disorders, such as lupus erythematosus, pemphigus disease, hemolytic anemia, polymyositis, and several autoimmune endocrine diseases, contains various types of autoantibodies that are supposed to be related to the pathogenesis of the disease.<sup>9</sup> Among the neurologic disorders, circulating antibodies for myelin components are thought to play a role in the demyelination that occurs in Guillain-Barré syndrome<sup>4,12</sup> and multiple sclerosis<sup>21</sup> with the cellular immunity mediated by T cells. In these immune system-mediated diseases, autoimmunity for peripheral or central myelin can explain the demyelinating white matter lesions in the spinal nerve roots or brain stem. In contrast, autoimmunity for astrocytes might explain the diffuse gray and white matter lesions in Pug dog encephalitis, but dominant cerebral involvement re-

mains to be clarified. A significant increase of anti-neurofilament and anti-GFAP antibodies has been found in autistic individuals, suggesting that these autoantibodies might be related to autoimmune pathology in autism.<sup>17</sup> To confirm whether the autoimmune response is an important pathologic event in Pug dog encephalitis, further immunologic studies, including evaluation of cellular immunity, are required. In Pug dog encephalitis, some primary event such as a viral infection, may act as a trigger and the subsequently acquired autoimmune response may then enhance the degenerative lesions. Some inherited abnormality of the immune system also might be present in this particular canine breed. However, an autoantibody against astrocytes may arise secondarily as the result of prolonged destructive brain changes induced by some initial cause and may actually have only a limited role to play in the pathogenesis of Pug dog encephalitis. For example, various types of autoantibodies against neurons and glial cells including GFAP have been reported to occur in individuals with Alzheimer's disease and in nondemented aged people and animals,<sup>6,8,14</sup> although the significance of these antibodies has not yet been determined. The production of these autoantibodies against nerve tissues may be an age-related event rather than being specific for neurologic diseases.<sup>16</sup> The presence of anti-GFAP antibodies in aged people with or without dementia might be a secondary phenomenon to blood-brain barrier disruption. Even in patients with multiple sclerosis, various autoantibodies detected in the serum and CSF are considered to arise as the result of the proliferation of B cells in the nervous system and do not contribute to the central demyelination.<sup>5,7</sup> In addition, a natural monoclonal autoantibody that promotes central remyelination has been observed to occur in a murine model of multiple sclerosis and Theiler's virus infection.<sup>1,15</sup> These data suggest that the autoantibody for astrocytes observed in relation to Pug dog encephalitis may arise as the result of the severe degenerative changes accompanied by abundant inflammation. In this scenario, the autoantibody would have only a limited role to play in the pathogenesis of this particular disease. However, if the production of the autoantibody is a specific feature of Pug dog encephalitis and does not occur in other neurologic diseases such as viral infections, granulomatous meningoencephalitis, seizure-induced necrosis, or metabolic disorders, the detection of autoantibodies will be a useful clinical diagnostic marker together with other CSF findings described previously.<sup>3</sup> To elucidate the pathologic roles of the autoantibody for astrocytes in Pug dog encephalitis, some adequate murine models with a similar strategy of experimental allergic encephalitis,<sup>13</sup> established as a model of human multiple sclerosis, will be needed.

An autoantibody found in cases of Pug dog encephalitis recognizes the 54- and 58-kd proteins of canine brain extracts. These findings are preliminary and require further biochemical and immunologic studies using murine models to fully elucidate the significance of this autoantibody in the pathology of Pug dog encephalitis. In addition, comparative studies using a larger number of cases of Pug dog encephalitis and other neurologic disorders will clarify the usefulness of this autoantibody as a clinical diagnostic marker.

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