A HIGH LEVEL OF ANTI-GFAP AUTOANTIBODY IN THE SERUM OF PATIENTS WITH ALZHEIMER'S DISEASE

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ABSTRACT
High titer of autoantibody against glial fibrillary acidic protein (GFAP) was found in the serum from patients with Alzheimer's disease. This was shown by 1) positive immunostaining of GFAP by Western blotting, 2) histochemical immunostaining of astrocytes of human brain, and 3) determination of autoantibody levels by enzyme-linked immunosorbent assay with GFAP as antigen. High anti-GFAP IgG level (quantity of IgG higher than that of 500 ng/ml monoclonal mouse IgG against GFAP) was found in 80% of Alzheimer patients (20/25), but only in 9.1% (3/33) of multi-infarct dementia patients and in 3% (4/126) of the control subjects. Determination of the autoantibody level against GFAP can be useful for diagnosing Alzheimer's disease in the elderly population with dementia.

Alzheimer's disease is primary dementia, causing progressive deterioration of mental function. Although toxic, viral, degenerative, vascular, immunologic and other etiological mechanisms have been proposed in pathogenesis, the cause of Alzheimer's disease is unknown. The pathologic hallmark is a marked increase in senile plaques and neurofibrillary tangles in certain regions of the brain. Even though Alzheimer's disease is the most frequent disease causing dementia in presenile and senile population, the clinical diagnosis of Alzheimer's disease is often obscure without neuropathological findings of the autopsied tissue. It has been a major research goal to find biological or biochemical parameters in patients' biological materials which highly indicate Alzheimer's disease. Recently some evidence suggests that the immune mechanism may play a role in the pathogenesis of Alzheimer's disease (4, 8). In this report we conducted the immunological study to discover a marker parameter for Alzheimer's disease in the serum of the patient.

MATERIALS AND METHODS
Serum
Serum samples were obtained from 25 Alzheimer's disease patients, 20 patients with senile dementia of Alzheimer type (SDAT), 33 patients with multi-infarct dementia, 6 stroke patients without dementia, 12 patients with other neurological diseases, and 126 control subjects without any neuropsychiatric symptoms. The patient characteristics are listed in Table 1. Both Alzheimer's disease and SDAT were the 'probable Alzheimer's disease' according to the diagnostic guideline developed by the NINCDS-ADRDA Workshop (11). The Alzheimer's disease category includes the patients whose onsets are before 65 years old and that of SDAT includes those whose onset is 65 years old or over. The serum samples had been stored at -80°C upto 2 months before the immunological study.
<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean age</th>
<th>Range of age</th>
</tr>
</thead>
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<tr>
<td>AD</td>
<td>25</td>
<td>60.2</td>
<td>48-71</td>
</tr>
<tr>
<td>SDAT</td>
<td>20</td>
<td>79.6</td>
<td>69-91</td>
</tr>
<tr>
<td>M.I.D.</td>
<td>33</td>
<td>78.8</td>
<td>58-94</td>
</tr>
<tr>
<td>Stroke without dementia</td>
<td>6</td>
<td>65.0</td>
<td>38-80</td>
</tr>
<tr>
<td>D.N.D.</td>
<td>9</td>
<td>63.0</td>
<td>48-77</td>
</tr>
<tr>
<td>Others</td>
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<td>53.3</td>
<td>27-72</td>
</tr>
<tr>
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<td>16</td>
<td>74.8</td>
<td>66-93</td>
</tr>
<tr>
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<td>50</td>
<td>52.0</td>
<td>46-64</td>
</tr>
<tr>
<td>Young control</td>
<td>60</td>
<td>35.4</td>
<td>22-44</td>
</tr>
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</table>

AD, Alzheimer's disease; M.I.D., multi-infarct dementia; D.N.D., degenerative neurological diseases, including Huntington's disease (2 cases), Pick's disease (2 cases), olivopontocerebellar atrophy (1 case) and Parkinson's disease (4 cases). Others include 2 cases of normal pressure hydrocephalus and 1 case of systemic lupus erythematosus (SLE).

**Preparation of GFAP**

Bovine spinal cord was homogenised in 0.85 M sucrose/50 mM phosphate buffer (pH 6.5) and myelinated axon was floated by centrifugation at 10,000 g for 30 min at 4°C. The axonal float was treated with 1% Triton X-100 and the axoplasmic protein was precipitated by centrifugation at 10,000 g for 30 min at 4°C. The precipitated protein was solubilized by 8 M urea/10 mM phosphate buffer (pH 6.5) and applied to hydroxyapatite batch treatment. The protein eluted with 8 M urea/140 mM phosphate buffer (pH 7.4) was concentrated by Amicon YM-50 membrane, applied to DE-52 (Whatman) column (1.5 cm x 25 cm) in 50 mM Bis-Tris buffer (pH 6.4) and chromatographed with a gradient of 10 mM to 250 mM NaCl. The protein peak eluted in 25-30 mM salt concentration was collected and used as GFAP (2, 10).

**Immunoblotting**

After sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-FAGE), the protein was transferred to nitrocellulose strip according to the conventional method. The nitrocellulose strip was blocked in 10 mM Tris-buffered saline (TBS)/5% skim milk (DIFCO) for 12 h at 4°C, reacted with human sera (1:30 dilution, unless otherwise indicated) or monoclonal anti-GFAP mouse IgG (Labsystems; 1:300 dilution) and incubated with peroxidase-conjugated goat IgG against human IgG, IgM, or mouse IgG (KPL Laboratories; 1:500 dilution). The bound immunoglobulin was detected by the reaction with 4-chloro-1-naphthol and hydrogen peroxide (7).

**Immunohistochemistry**

The patients' sera were studied by the indirect immunofluorescence method using cryostat sections of human brain. The human brain was obtained at autopsy of a stroke patient (male, 57 years old) and stored in 10% formaldehyde for 7 months. Brain slices were excised from the white matter of the parietal lobe which was apparently free from any neuropathological findings. The section (4 μm thick) was washed with TBS, incubated with 1% skim milk for 30 min, reacted with the patient sera (1:10 dilution) for 2 h at room temperature, and then with fluorescein isothiocyanate conjugated goat anti-human IgG (KPL laboratories; 1:100 dilution) for 2 h at room temperature, and finally viewed in a fluorescence microscope.

**Enzyme-linked Immunosorbent Assay (ELISA)**

An aliquot of 50 μl of purified GFAP (3 μg/ml) in carbonate buffer (pH 9.6) was placed to the microwells of polystyrene microtest plate
(Sumitomo). The protein bound to the well was blocked with 1% skim milk in TBS for 30 min at room temperature. The sample sera were sequentially double-diluted from ×10 to ×320 and an aliquot of 50 μl was applied to the well and incubated for 1.5 h at room temperature. Peroxidase-conjugated goat anti-human IgG (1:500 dilution) was added and incubated for 1.5 h at room temperature. An aliquot of 200 μl/well of o-phenylenediamine (0.04%)/hydrogen peroxide (0.012%)/0.1 M citric acid/0.2 M sodium phosphate was placed to the well and incubated for 30 min. After addition of 50 μl of 10% sulfuric acid, the optical density at 492 nm was measured.

**Absorption Experiment**

Purified GFAP (40 μg/ml) was added to 1:30 diluted patient serum for immunoblotting experiment and 1:10 diluted patient serum for ELISA study, respectively. The serum was incubated at 37°C for 1 h and at 4°C overnight, centrifuged at 10,000 g for 1 h at 4°C and the supernatant was used as the serum preabsorbed by GFAP.

**RESULTS**

**Immunoblotting**

When acetone powder of mouse brain was electrophoresed and stained by Coomassie blue, many protein bands appeared (Fig. 1A, lane a). When this strip was immunostained with monoclonal anti-GFAP antibody, only one protein band with a molecular weight of 45,000 was identified (Fig. 1A, lane b). When the Alzheimer serum was overlaid on the nitrocellulose strip of mouse brain homogenate, many bands were immunostained by the serum (Fig. 1A, lane c). Some of the bands were stained by the control human sera, but the band indicated by arrowheads was strongly stained by Alzheimer sera. The molecular weight of this band was 45,000, corresponding to that of mouse GFAP. The immunostaining of mouse brain homogenate with Alzheimer serum preabsorbed by GFAP lacked this band (Fig. 1A, lane d). The purified bovine GFAP used for the preabsorp-

![Fig. 1](image)

**Fig. 1** A: Acetone powder of mouse whole brain was separated by SDS-PAGE (7.5%) and stained with Coomassie Brilliant Blue (lane a), immunostained with monoclonal anti-GFAP antibody (lane b), with Alzheimer serum (lane c), and with Alzheimer serum preabsorbed by bovine GFAP (lane d). Arrowhead in lane c indicates 45 K M, protein, which is absent in lane d. B: The purified bovine GFAP was electrophoresed and stained by Coomassie Brilliant Blue (lane a). Lane b was immunostained with the monoclonal anti-GFAP antibody. Lanes c and d were reacted with Alzheimer serum and detected by anti-human IgG (lane c), and anti-human IgM (lane d). In lane e Alzheimer serum was preabsorbed, and GFAP was no longer detected by IgG.
tion experiment above showed a single protein band (molecular weight, 50,000) by Coomassie blue staining (Fig. 1B, lane a), and this protein was stained by monoclonal antibody against GFAP (Fig. 1B, lane b), but not by anti-neurofilament antibodies nor anti-tubulin antibody (data not shown). Both IgG and IgM class anti-GFAP antibodies were shown in Alzheimer serum (Fig. 1B, lanes c and d). Since IgM class anti-GFAP antibody was present in essentially all the serum studied (data not shown), the following experiments were focused on IgG class antibody. After Alzheimer serum was preabsorbed by GFAP, the immunostaining of this band with purified GFAP disappeared (Fig. 1B, lane e).

**Immunohistochemistry**

Since an antibody against a protein band with the molecular weight of GFAP was found in Alzheimer and SDAT patients’ sera, the specificity of the sera was studied immunohistochemically. Fig. 2, A and B showed the positive immunostaining of human astrocytes by Huntington’s disease patient serum and SDAT patient serum, respectively. Sera from Alzheimer’s disease (n=8) stained astrocytes, but the sera from control subjects (n=10) did not.

**ELISA**

Sera were assayed for their levels of IgG autoantibody against purified bovine GFAP. When the blocking procedure with skim milk was omitted in this ELISA system, the final optical density was high and the dilution curve of the patient serum did not show any reasonable similarity with that of monoclonal IgG, probably due to the non-specific reaction. ELISA was therefore carried out after blocking the microtest plate with 1% skim milk solution and the human serum was diluted with the same solution. As shown in Fig. 3, the dilution curve of Alzheimer serum (curve B) was almost parallel with that of monoclonal anti-GFAP (curve A) which enabled us to express the autoantibody level by ng/ml equivalent (eq.) to the quantity of monoclonal anti-GFAP IgG (ng/ml). After preabsorption of Alzheimer serum by GFAP, the dilution

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**Fig. 2** Human brain sections are immunostained by the indirect immunofluorescence method with Huntington serum (A) and with SDAT serum (B). Arrows indicate positively stained astrocytes. × 160
curve of Alzheimer serum (curve C) was indistinguishable from the control serum (curve D).

The levels of antibody against GFAP were plotted in Fig. 4. Sera from Alzheimer’s disease, SDAT and other degenerative neurological diseases tended to show high antibody levels against GFAP. In the control subjects only 4 sera showed a high level. Since most of the healthy control serum showed the antibody level below 500 ng/ml eq., this antibody value was arbitrarily taken as the value which demarcated the normal range. The incidence of positive cases (higher than 500 ng/ml eq.) were listed in Table 2. Twenty out of 25 Alzheimer’s disease patients (80%) and 6 of 20 SDAT patients (30%) showed antibody levels higher than 500 ng/ml eq. On the other hand, the anti-GFAP antibody levels in the control sera were almost below 500 ng/ml eq.

DISCUSSION

Autogenous humoral antibodies against neuronal tissues have been demonstrated during aging as well as under various pathological conditions (13). Using rat brain tissue sections, high incidence of the specific antibrain antibody was found among Alzheimer’s patients (57% positive) compared to the normal young and aged healthy controls (none positive) (19), but the nature of the antigen was not characterized. In the investigation of antibrain antibodies in sera of the patients with SDAT, Fillit et al. found antibody against vascular membrane heparan sulfate proteoglycan in 6 of 16 sera from the patients with typical SDAT (4). This phenomenon was observed in our immunohistochemical study showing strong staining of vascular basement membrane by some Alzheimer sera (data not shown). But the frequency of positive staining of vascular basement membrane was lower than that of astrocytes. The presence of circulating autoantibody to the 200 K protein of the neurofilament was demonstrated in the serum of essentially all human individuals (20). Anti-neurofilament antibody level was found high in the control serum as well as in Alzheimer serum (9). Since we also observed the strong immunostaining of neurofilament protein by control sera (data not shown), we concluded that this antibody could not be used as a marker for Alzheimer’s disease.
Fig. 4  The level of anti-GFAP antibody in sera from various neurological patients and control subjects. Circles represent male patients, and triangles female patients. The dilution curves of almost all sera whose antibody levels were below 300 ng/ml eq., did not pararell with curve A in Fig. 3. For AD, MID and D.N.D., see legend to Table 1. S.C., senile control; P.C., presenile control; Y.C., young control

Table 2  The Incidence of 'Positive' Cases

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of positive case/total case</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>20/25</td>
<td>80.0</td>
</tr>
<tr>
<td>SDAT</td>
<td>6/20</td>
<td>30.0</td>
</tr>
<tr>
<td>M.I.D.</td>
<td>3/33</td>
<td>9.1</td>
</tr>
<tr>
<td>Stroke</td>
<td>0/6</td>
<td>0</td>
</tr>
<tr>
<td>D.N.D.</td>
<td>5/9</td>
<td>55.6</td>
</tr>
<tr>
<td>Others</td>
<td>0/3</td>
<td>0</td>
</tr>
<tr>
<td>Senile control</td>
<td>0/16</td>
<td>0</td>
</tr>
<tr>
<td>Presenile control</td>
<td>1/50</td>
<td>2.0</td>
</tr>
<tr>
<td>Young control</td>
<td>3/60</td>
<td>5.0</td>
</tr>
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</table>

For abbreviations, see legend to Table 1.
There have been several reports suggesting the presence of anti-GFAP autoantibody (3, 6, 18). But they have not well documented its specificity. In this study, we demonstrated the specificity of anti-GFAP IgG by immunoblotting, immunohistochemistry and ELISA. Our Western blotting study and absorption experiment revealed that the anti-GFAP IgG was present independently of many other anti-brain antibodies. Though five classes of intermediate filaments have been distinguished biochemically, all of them may share a common antigenic determinant (17), suggesting the possibility that anti-GFAP IgG cross-react with other class intermediate filaments such as neurofilament or vimentin. But from the data of absorption experiment in Western blotting, immunohistochemical staining of human brain and specifically high levels of anti-GFAP IgG in Alzheimer sera in ELISA study, we speculate that the autoantibody reacts with the GFAP specific antigenic determinant which is common among human, mouse and bovine species.

The finding of high incidence of anti-GFAP IgG in Alzheimer’s disease can be useful in the clinical situation. The relatively low incidence of anti-GFAP IgG in SDAT (30%) seems to suggest the possibility that SDAT category contains several clinical entities due to the difficulty of its clinical diagnosis. Since only 9% of patients with multi-infarct dementia and none of stroke patients showed high anti-GFAP level, this parameter can be useful in differential diagnosis between Alzheimer and cerebrovascular type dementia. Among other degenerative neurological diseases, Huntington’s disease (2/2), Pick’s disease (1/2) and olivopontocerebellar atrophy (1/1) showed high anti-GFAP IgG level.

In Alzheimer’s disease, the anti-GFAP autoantibody could have influence on its pathogenesis. It has been reported that immunoglobulins and complements are localized in senile plaques (8), or that IgG may be the precursor of amyloid protein (16). Miyakawa et al. observed by light and electron microscopic study that the amyloid fibrils were spread from the wall of blood vessels into the parenchyma of brain (12). These data are not against the idea that the anti-GFAP autoantibody plays a role in the pathogenesis of senile plaques. Moreover the blood-brain barrier could be injured by the autoantibody, because small vessels of the brain are surrounded by processes of astrocytes. Wisniewski et al. have suggested that the blood-brain barrier may be injured in cases of the Alzheimer type dementia (21).

The reason why specifically high levels of anti-GFAP IgG was found in Alzheimer’s disease is not known. Autoimmune system is known to be activated with aging. In this study the level of anti-GFAP antibody did not increase with aging, nor showed any sex difference in its incidence. Antinuclear and antimicrosomal antibodies were found normal in all Alzheimer sera studied. One patient with systemic lupus erythematosus (SLE) showing dementia did not show a high level of anti-GFAP IgG. It is thus difficult to explain the relationship between the anti-GFAP antibody and Alzheimer’s disease only by the autoimmune mechanism.

It is also possible the endogenous substance with GFAP-like immunoreactivity is recognized by the normally functioning immune system. In fact, a markedly increased amount of a 50,000 molecular weight protein was observed in nuclear fraction of the temporal cortex tissue from Alzheimer patients, which is believed to represent GFAP (15). High level of GFAP in cerebrospinal fluid was reported in dementia patients, and other central nervous system diseases (1, 14). In addition to an increased level of GFAP, any mechanisms which facilitate sensitization of the immune system including rupture of the blood-brain barrier, change in GFAP distribution, change in the solubility of GFAP, would increase the IgG against GFAP. Finally, induction of anti-GFAP antibody will be caused by invasion of exogenous antigen with GFAP-like immunogenicity. Fujinami et al. reported that the phosphoprotein of measles virus and a protein of herpes simplex virus type I cross-react with an intermediate filament, probably vimentin (5).

Though the genesis of high anti-GFAP IgG level in Alzheimer serum is still to be clarified, we stress the significance of this finding as a biological parameter indicating Alzheimer’s disease in clinical situations.

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