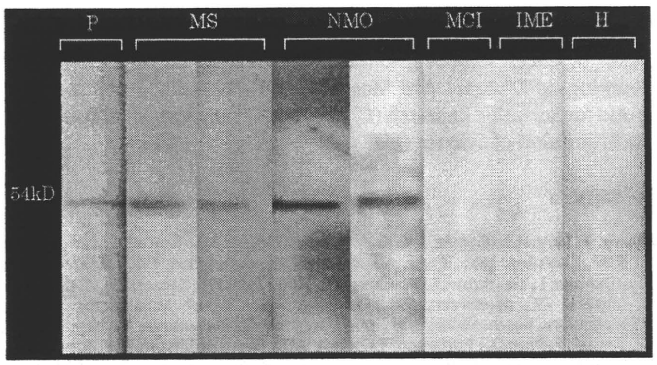

Fig. 2. Immunoblotting of human phosphoglycerate mutase 1 (PGAM1) full-length recombinant protein with GST. The recombinant protein (0.28 μg) was applied to each well. P, 1:300-diluted anti-PGAM1 monoclonal antibody (Abnova); MS, 1:1500-diluted sera from patients with multiple sclerosis; NMO, 1:1500-diluted sera from patients with neuromyelitis optica; MCI, 1:1500-diluted sera from patients with multiple cerebral infarctions; IME, 1:1500-diluted sera from patients with infectious meningoencephalitis; H, 1:1500-diluted sera from healthy controls.

analyzed on a MALDI TOF/TOF instrument, AXIMA Performance (Shimadzu). By utilizing information on the *x–y* positions of spotted samples on AccuSpot, autoexperiments using AXIMA Performance were performed to analyze the samples on the plates. Every autoexperiment and protein identification were performed using an integrated software, Kompact Ver.2.8. Protein identification was carried out using the MS/MS ion search database, Mascot (http://www.matrixscience.com/; Matrix Science Ltd.).

2.6. Immunoreactivity of sera from patients with various neurological diseases against human PGAM1 full-length recombinant protein

We examined the anti-PGAM1 antibodies in sera from 21 MS, 13 NMO, 21 PD, 20 MCI, to 19 IME patients, and 17 healthy controls by 1DE immunoblotting using the commercially available human PGAM1 full-length recombinant protein with GST (Abnova). Immunoblotting was carried out as described in Section 2.3. The screening dilution of sera from all patients and healthy controls was 1:1500.

2.7. Statistical analysis

We used the chi-square test with Yates' continuity correction to assess the difference in the prevalence of the anti-PGAM1 antibody between groups. Differences were considered significant at $P<0.05$.

3. Results

3.1. Screening and identification of target antigen of MS patients' autoantibodies (Fig. 1)

We examined the target antigen that reacted selectively with MS patients' sera. We detected by 1DE immunoblotting an approximately 30 kDa band corresponding to a protein that reacted with antibodies in sera from two out of five MS patients, but not with sera from five healthy controls. The same sample was subjected to 2DE, and one spot (observed MW/pI: 26,000/6.9) with a similar molecular weight reacted with the sera from these two MS patients but not with the sera from the healthy controls. We analyzed this spot by MALDI TOF-MS. This protein spot was identified as PGAM1 (accession number, P25113; score/coverage identification (%), 660/40; number of matched peptides, 11; theoretical MW/pI, 28,948/6.67).

3.2. Immunoreactivity of sera from patients with various neurological diseases against human PGAM1 full-length recombinant protein (Figs. 2 and 3)

To investigate whether the anti-PGAM 1 antibody is specific for MS, we examined this autoantibody in sera from patients with various neurological diseases (21 MS patients, 13 NMO patients, 21 PD patients, 20 MCI patients, and 19 IME patients) and 17 healthy controls by 1DE immunoblotting using the human PGAM1 full-length recombinant protein with GST (Figs. 2 and 3). As a result, the positivity rates were 81% (17 of 21) in MS patients, 77% (10 of 13) in NMO patients, 24% (5 of 21) in PD patients, 30% (6 of 20) in MCI patients, 32% (6 of 19) in IME patients, and 24% (4 of 17) in healthy controls. Statistically, the prevalence of the anti-PGAM1 antibody was significantly higher in patients with MS than in patients with PD ($P<0.001$), MCI ($P<0.003$), and IME ($P<0.005$), and in healthy controls ($P<0.002$). The prevalence of the anti-PGAM1 antibody was also significantly higher in patients with NMO than in patients with PD ($P<0.008$), MCI ($P<0.03$), and IME ($P<0.04$), and in healthy controls ($P<0.02$). These findings indicate that the anti-PGAM1 antibody has a stronger correlation with MS and NMO than with PD, MCI, IME, and being healthy.

4. Discussion

We identified PGAM1 as the target antigen of autoantibodies in sera from the MS patients by proteomics-based analysis. Western blotting analysis using the human PGAM1 recombinant protein

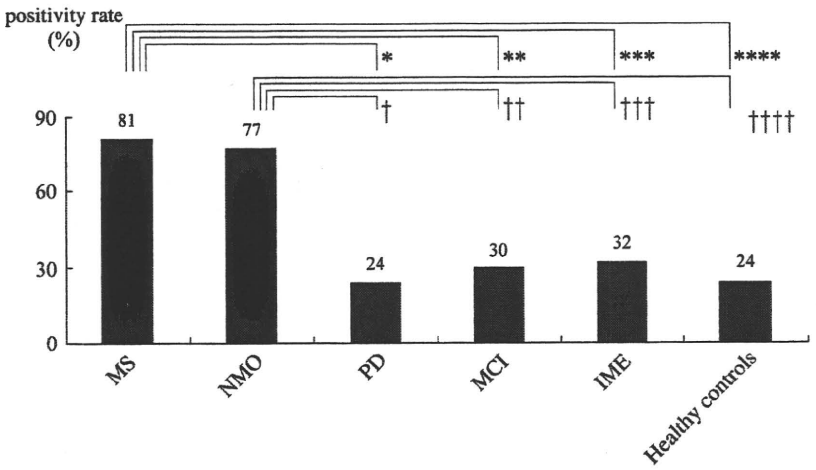

Fig. 3. Prevalence of antibodies against human phosphoglycerate mutase 1 (PGAM1) full-length recombinant protein. MS, patients with multiple sclerosis; NMO, patients with neuromyelitis optica; PD, patients with Parkinson's disease; MCI, patients with multiple cerebral infarctions; IME, patients with infectious meningoencephalitis. *$P<0.001$, **$P<0.003$, ***$P<0.005$, ****$P<0.002$, †$P<0.008$, ††$P<0.03$, †††$P<0.04$, and ††††$P<0.02$.

showed that the prevalence of the anti-PGAM1 antibody is much higher in not only patients with MS, but also those with patients with NMO, than in those with other neurological diseases and in healthy controls. To the best of our knowledge, this is the first study that elucidated the relationships between the anti-PGAM1 antibody and CNS autoimmune diseases. Lu et al. (2008) reported that the prevalence of the anti-PGAM1 antibody is much higher in patients with autoimmune hepatitis (AIH) than in those with other hepatic diseases and in healthy subjects. AIH is a rare liver disease and is characterized by hypergammaglobulinemia even in the absence of cirrhosis, characteristic autoantibodies, and a favorable response to immunosuppressive treatment (Zachou et al., 2004; Zolfino et al., 2002). Although the etiology of this disease is as yet unknown, the presence of several circulating autoantibodies such as the anti-nuclear antibody, anti-smooth muscle antibody, anti-liver kidney microsome type 1 antibody, and anti-liver cytosol type 1 antibody, which are serological markers for diagnostic criteria (Alvarez et al., 1999), suggests the important role of humoral mechanisms in AIH. There are several reports on MS patients with the complication of AIH (Pulicken et al., 2006; Takahashi et al., 2008; Ferrò et al., 2008). de Seze et al. (2005) reported that the prevalence of AIH seems to be about tenfold higher in patients with MS than in the general population. The anti-PGAM1 antibody can be generated in an immunological background common to both autoimmune CNS diseases and AIH.

Phosphoglycerate mutase is a glycolytic enzyme that catalyzes the interconversion of 3- and 2-phosphoglycerate with 2, 3-bisphosphoglycerate as the primer of the reaction (Fothergill-Gilmore and Watson, 1989). In mammalian tissues, PGAM exists in three isozymes, composed of homodimeric and heterodimeric combinations of two different subunits, type M (muscle form, PGAM2) and type B (brain form, PGAM1). The homodimer MM form is mainly expressed in the muscle; the BB form in the brain, kidney and liver; and the heterodimer MB form in the heart (Omenn and Cheung, 1974; Zhang et al., 2001). A previous study showed that PGAM1 is induced after hypoxia, which would occur in patients with cerebral infarction (Takahashi et al., 1998). In this study, the positivity rate of the anti-PGAM1 antibody in patients with MCI is not significantly higher than those in patients with other neurological diseases and in healthy controls. This finding suggests that an immunological background is important for production of the anti-PGAM 1 antibody.

In conclusion, the results of this study suggest that the anti-PGAM1 antibody is not only a marker of AIH but also a nonspecific marker of CNS autoimmune diseases. However, further studies are required to assess the presence of the anti-PGAM1 antibody in a large cohort of patients, including those with other autoimmune-mediated diseases, and controls.

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INTERNAL MEDICINE

□ CASE REPORT □

Serial Monitoring of Basal Metabolic Rate for Therapeutic Evaluation in an Isaacs' Syndrome Patient with Chronic Fluctuating Symptoms

Yuichi Hayashi, Akio Kimura, Norihito Watanabe, Megumi Yamada, Takeo Sakurai, Yuji Tanaka, Isao Hozumi and Takashi Inuzuka

Abstract

A 52-year-old man presented with hyperhydrosis, painful pseudomyotonia and gait disturbance. The condition was diagnosed as Isaacs' syndrome on the basis of characteristic findings noted on an electromyogram. Carbamazepine treatment was only partially and transiently effective. Intravenous immunoglobulin therapy was effective. The basal metabolic rate (BMR) was serially monitored using an automatic integrated system for breath analysis. Serial monitoring of the BMR facilitates therapeutic evaluation in an Isaacs' syndrome patient with chronic fluctuating symptoms.

Key words: Isaacs' syndrome, intravenous immunoglobulin, basal metabolic rate, automatic integrated system for breath analysis, carbamazepine

(Inter Med 49: 475-477, 2010)
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Introduction

Isaacs' syndrome (IS) is characterized by spontaneous and continuous activity of muscle fibers (1). Most cases of IS occur sporadically, and only 38% of all patients with IS test positive for anti-voltage-gated potassium channel (VGKC) antibodies (2).

Isaacs reported that the basal metabolic rate (BMR) is elevated in patients with acute-phase IS, but is normalized with treatment (1). Studies involving the serial monitoring of the BMR of these patients have not been performed because of the complicated procedures involved. We present the case of a patient with sporadic IS without anti-VGKC antibodies over a chronic fluctuating course. We serially monitored the patient's BMR for therapeutic evaluation using an automatic integrated system for breath analysis.

Case Report

A 52-year-old man presented with gait disturbance, painful muscle cramps and hyperhydrosis and was admitted to our hospital in February 2008. He had no specific familial history of disease. He began to suffer from hyperhydrosis when he was in his 30s and from gait disturbance in May 2007. He occasionally experienced dysphagia but recovered from it naturally. His clinical course had fluctuated over several months.

Physical examination revealed that the patient was well nourished. He suffered from mild mental retardation. His blood pressure, heart rate and body temperature were all found to be normal. Neurological examination revealed myokimia, hyperhydrosis, and hypertrophy of the leg muscles. Furthermore, the patient experienced muscle cramps at various times during the day and night. Grip myotonia was not detected; however, the patient's fingers spontaneously flexed after they were extended. His reflexes were slightly exaggerated but the Babinski sign was absent. A photograph of the patient showed flexed upper-limbs, forward-bend posture, standing with legs bending outwards, and hypertrophy of the calf muscles (Fig. 1). He walked with such posture. Painful cramps often occurred during walking. His cranial nerves and sensory perception were normal. He had experienced no epilepsy, hallucination, or insomnia.

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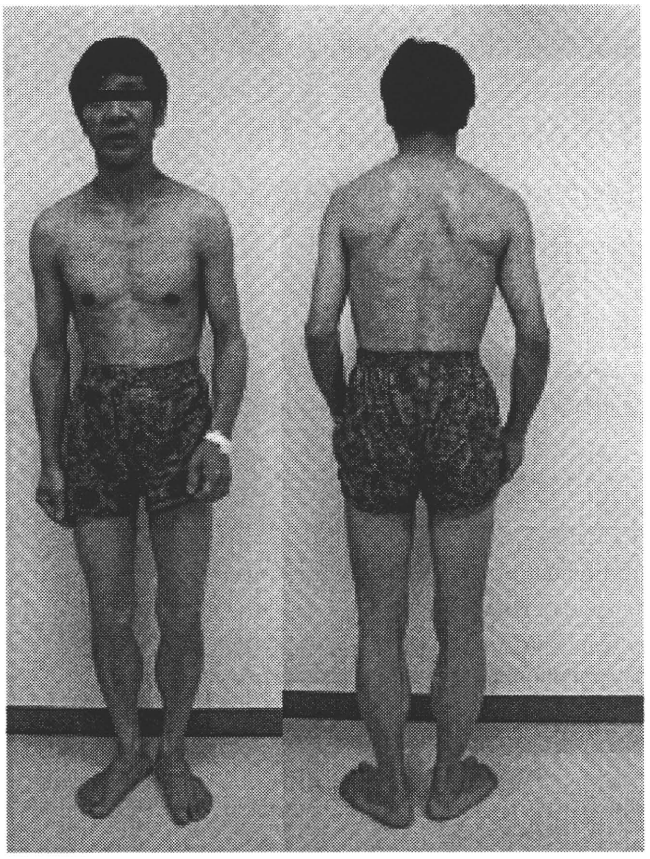

Figure 1. **A photograph of the patient.**

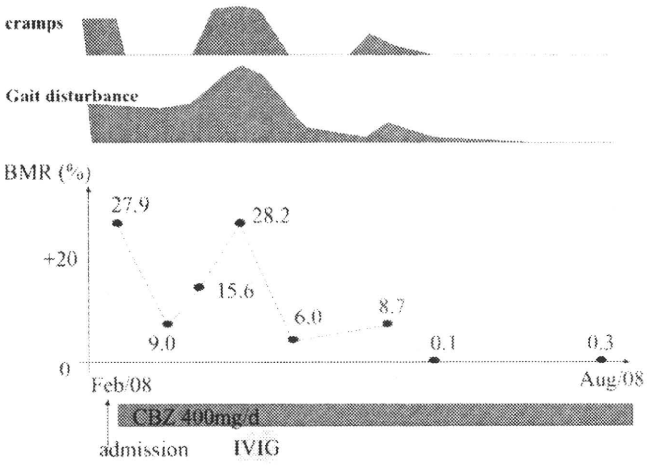

Figure 2. **Serial monitoring of basal metabolic rates. We serially monitored the patient's basal metabolic rate (BMR) using an automatic integrated system for breath analysis (FUDAC-77), and found that the clinical symptoms fluctuated in tandem with the BMR. BMR: basal metabolic rate, CBZ: carbamazepine, IVIG: intravenous immunoglobulin (0.4 g/kg/d for 5 d)**

Laboratory tests revealed that all the parameters, including the serum creatine kinase (CK) level and the thyroid hormone levels, were within the normal limits. Antibodies against VGKC and glutamic acid decarboxylase antibody were not detected in the serum.

An electromyogram (EMG) of the right biceps brachii, quadriceps and tibialis anterior muscles did not show myotonic discharge. Randomized doublet or triplet muscle waves were observed in the myokimic muscles of the left calf. A surface EMG study showed spontaneous and continuous motor-unit activity in the right biceps and the rectal abdominal muscles and the presence of M-wave afterdischarges in the upper extremities. Nerve conduction study was normal in the upper extremities, but it could not be assessed in the tibial nerves because of muscle cramps. Brain MRI and electroencephalography (EEG) showed no obvious abnormal findings. CT of the muscles showed hypertrophy of the calf muscles.

The BMR was measured at 9 a.m. while the patient was at rest and before breakfast, using an automatic integrated system for breath analysis (FUDAC-77, Fukuda Denshi, Tokyo, Japan). The BMR was 27.9% higher than the upper limit of the normal range for men in their 50s.

IS was diagnosed on the basis of the characteristic findings noted on the EMG, and the patient was administered oral carbamazepine (CBZ: 400 mg/d). The frequency of muscle cramps was reduced with the treatment. Furthermore, the BMR was reduced to 9.0% higher than the upper limit of the normal range. However, the gait disturbance did not improve. Shortly thereafter, the patient's symptoms deteriorated once again, and despite CBZ treatment, he frequently experienced muscle cramps all over his body, both during the day and at night. As was expected with the exacerbation of the symptoms, the BMR was increased to 28.2% higher than the upper limit of the normal range.

We initiated intravenous immunoglobulin (IVIG) therapy (0.4 g/kg/d for 5d). The patient's symptoms improved with IVIG, and the BMR was normalized and maintained for at least 6 months (Fig. 2).

Discussion

We present the case of a patient with chronic fluctuating symptoms of IS, not accompanied by any hormonal disease. The BMR was serially assessed, and it was found that the patient's clinical symptoms fluctuated in tandem with the BMR (Fig. 2).

The BMR is associated with many factors: age, sex, race, and thyroid hormone level (3). The major factors causing an increase in the BMR are hormonal disease and physiological factors: pregnancy, diet, a high environmental temperature, exercise, or a state of excitement (3). When we measure the BMR, the observed value reflects the result of total oxygen consumption of the whole body. The oxygen consumption of the brain and muscles at rest is estimated to be 23 and 20% of that of the whole body, respectively. The oxygen consumption of muscles at exercise reaches maximally 60 times that at rest (4). In general, the BMR test is not used as an indicator of chronic muscle activity; however, secondary chronic muscle activity due to an underlying disease may cause fluctuation in the BMR.

It is reported that IS is sometimes accompanied with dis-

turbance of the central nervous system (CNS), such as Morvan syndrome (5) or limbic encephalitis (6). However, the findings of brain MRI and EEG in the present case suggested no accompaniment of such a CNS disease. The increasing value of BMR in our case mainly reflected the oxygen consumption of muscles, not that of the brain.

In 1961, Isaacs first reported the cases of patients with acute-phase IS, whose BMR was elevated because of continuous or spontaneous muscle fiber activity, but was normalized with treatment (1). However, at that time, serial monitoring of the BMR was not performed because the methods available were complicated.

A closed-circuit respiratory device has been used to calculate the BMR (3). In the method that has traditionally been used to determine the BMR, the patient is required to breathe through the mouth into the analyzer for 6 minutes while at rest and before breakfast, and a skilled medical technologist analyzes the resting end-respiratory volumes for 6 minutes and manually draws a straight line to calculate the BMR.

Recently, an automatic integrated system for breath analysis (FUDAC-77) has been developed; this device automatically calculates the correct BMR by application of the method of least squares (a straight line experiment) (7), and remarkably facilitates its monitoring.

Anti-convulsion drug treatment (1), IVIG therapy (8), and plasma exchange (9) are reported to be effective modalities for IS patients; however, no study thus far has performed an objective therapeutic evaluation of the parameters that reflect the symptoms, such as the BMR. The condition of IS patients is reflected in real time in the BMR.

In the present case, CBZ treatment reduced spontaneous muscle activities, i.e., painful cramps. The BMR was reduced in tandem. However, the gait disturbance and posture did not improve because of completely uncontrolled continuous muscle fiber activities. Spontaneous and continuous muscle fiber activities were finally controlled by IVIG treatment.

Improvement shown by patients treated with immunomodulatory treatments is observed neurophysiologically, but quantitative assessment is also necessary. Serial BMR monitoring is a well-tolerated, quantitative assessment for IS patients with such a fluctuating course.

Acknowledgement

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INTERNAL MEDICINE

□ PICTURES IN CLINICAL MEDICINE □

Markedly Ring-enhanced Optic Nerves Due to Metastasis of Signet-ring Cell Gastric Carcinoma

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Key words: MRI, signet-ring cell carcinoma, optic nerve, cerebrospinal fluid, optic neuropathy, leptomeningeal carcinomatosis

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Picture 1. Coronal fat-suppressed MRI with gadolinium showed a marked ring enhancement of the surrounding optic nerves (arrows).

Signet-ring cell carcinoma frequently causes leptomeningeal carcinomatosis, one cause of optic neuropathy in elderly patients. A 77-year-old woman had shown progressive bilateral blindness for one month without any other symptoms. Coronal fat-suppressed MRI with gadolinium showed a marked ring enhancement of the surrounding optic nerves (Picture 1). CSF cytodiagnosis and histopathological examination of the gastric biopsy samples revealed signet-ring cell carcinoma. The patient was diagnosed with leptomeningeal carcinomatosis due to gastric cancer. She died 2 weeks after the diagnosis. Coronal fat-suppressed MRI with gadolinium is useful for the differential diagnosis of optic neuropathy, especially leptomeningeal carcinomatosis (1, 2).

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Antibodies to the $GABA_B$ receptor in limbic encephalitis with seizures: case series and characterisation of the antigen

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Summary

Background **Some encephalitides or seizure disorders once thought idiopathic now seem to be immune mediated. We aimed to describe the clinical features of one such disorder and to identify the autoantigen involved.**

Methods **15 patients who were suspected to have paraneoplastic or immune-mediated limbic encephalitis were clinically assessed. Confocal microscopy, immunoprecipitation, and mass spectrometry were used to characterise the autoantigen. An assay of HEK293 cells transfected with rodent $GABA_{B1}$ or $GABA_{B2}$ receptor subunits was used as a serological test. 91 patients with encephalitis suspected to be paraneoplastic or immune mediated and 13 individuals with syndromes associated with antibodies to glutamic acid decarboxylase 65 were used as controls.**

Findings **All patients presented with early or prominent seizures; other symptoms, MRI, and electroencephalography findings were consistent with predominant limbic dysfunction. All patients had antibodies (mainly IgG1) against a neuronal cell-surface antigen; in three patients antibodies were detected only in CSF. Immunoprecipitation and mass spectrometry showed that the antibodies recognise the B1 subunit of the $GABA_B$ receptor, an inhibitory receptor that has been associated with seizures and memory dysfunction when disrupted. Confocal microscopy showed colocalisation of the antibody with $GABA_B$ receptors. Seven of 15 patients had tumours, five of which were small-cell lung cancer, and seven patients had non-neuronal autoantibodies. Although nine of ten patients who received immunotherapy and cancer treatment (when a tumour was found) showed neurological improvement, none of the four patients who were not similarly treated improved ($p=0\cdot005$). Low levels of $GABA_{B1}$ receptor antibodies were identified in two of 104 controls ($p<0\cdot0001$).**

Interpretation **$GABA_B$ receptor autoimmune encephalitis is a potentially treatable disorder characterised by seizures and, in some patients, associated with small-cell lung cancer and with other autoantibodies.**

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Introduction

Synaptic plasticity is an essential property of neurons that is involved in memory, learning, and cognition. Plasticity depends on the interactions of ion channels and synaptic receptors, including excitatory glutamate NMDA receptors and AMPA receptors, and inhibitory $GABA_B$ receptors.[1,2] In animal models, pharmacological or genetic disruption of these receptors result in seizures and changes in memory, learning, and behaviour.[3–6] Immune responses against these receptors would therefore be expected to result in similar symptoms. Indeed, two disorders, one associated with antibodies to extracellular epitopes of the NR1 subunit of NMDA receptors[7] and the other associated with antibodies to GluR1/2 subunits of AMPA receptors,[8] have recently been identified. These disorders result in encephalitis with prominent psychiatric, behavioural, and memory problems, often accompanied by seizures. The antibodies implicated in these two autoimmune disorders cause a decrease in the amounts of the target receptor in cultured neurons, suggesting the antibodies are pathogenic. Patients with these syndromes often respond to treatment, and in some patients the immune response occurs as a paraneoplastic event. These findings, as well as the prevalence of some of these disorders (eg, anti-NMDA receptor encephalitis[7,9,10]), have raised the possibility that other syndromes in which memory and behaviour are impaired and seizures are common could also be immune mediated. In some of these syndromes an immune-mediated pathogenesis is suggested by the clinical response to immunotherapy, the CSF and MRI findings suggesting limbic encephalitis, and the detection of antibodies to unknown neuronal cell-surface antigens. We aimed to identify the autoantigen involved in a new disorder that has most of these suggestive features.

Methods

Study population

Between January, 2006, and June, 2009, we studied 410 patients with encephalitis suspected to be paraneoplastic or immune mediated. These patients were seen by the authors or by clinicians at other institutions and the patients' sera and CSF were sent for analysis of novel autoantibodies to the Center for Paraneoplastic Disorders at the University of Pennsylvania (PA, USA). We identified autoantibodies in the serum or CSF of 357 patients, including 275 patients with antibodies to NMDA receptors (including 75 patients previously reported[7]), 27 with antibodies to voltage-gated potassium

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channels, 19 with antibodies to glutamic acid decarboxylase 65 (GAD65), 15 with antibodies to AMPA receptors (including ten patients previously reported[8]), 11 with anti-Ma2 antibodies, eight with anti-HuD antibodies, and two with anti-CRMP5 antibodies (patients each had only one of these antibodies). Of the remaining 53 patients, 15 had serum or CSF antibodies with reactivity against neuronal cell-surface antigens predominantly in the neuropil of sectioned rat brain. Because of the serum and CSF findings and the response to immunotherapy and cancer treatment of the first of these patients to be clinically and immunologically studied (the index patient), we focused on these 15 patients. Clinical information about the patients was obtained by the investigators or provided by referring physicians. Patients were said to have neurologically improved if they were able to function independently or with little assistance when they returned home. Control samples were CSF or serum from 104 patients, including 91 randomly selected by use of an online random integer generator from the 410 individuals with encephalitis and 13 who had syndromes associated with GAD65 antibodies and who were not included in the group of 410 patients. These 13 patients were seen either by the study investigators or their serum, CSF, and clinical information were sent from other institutions to the primary investigator (JD) for study of disorders of unknown cause.

Studies were approved by the University of Pennsylvania Institutional Review Board, and written informed consent was obtained from all patients or their representatives.

Procedures

To establish whether serum or CSF contained antibodies to neural tissue, sagittal sections were taken from the brains of adult female Wistar rats; brains had been immersed in 4% paraformaldehyde at 4°C for 2 h, cryoprotected with 40% sucrose for 24 h, and snap frozen in chilled isopentane. Paraffin-embedded tumour tissue from patients was deparaffinised and the antigens retrieved.[11] 7 μm thick frozen (or 4 μm paraffin) tissue sections were incubated with 0·3% hydrogen peroxide for 20 min, with 10% goat serum in PBS for 1 h, and with patients' or control individuals' serum (1:250) or CSF (1:10) or a guineapig polyclonal antibody against an intracellular epitope of the $GABA_{B1}$ receptor (1:200; AB2256, Millipore, Billerica, MA, USA) at 4°C overnight. After using the appropriate secondary antibodies (all 1:2000, diluted in PBS with 5% goat serum), labelling was developed with the avidin–biotin–peroxidase method. Results were photographed under a fluorescence microscope using Zeiss Axiovision software (Zeiss, Thornwood, NY, USA).

Immunohistochemistry with human tissue (small-cell lung cancer) was done by use of IgG purified from patients' or control individuals' sera and labelled with biotin.[12] No secondary antibody was needed, thus avoiding background labelling caused by other human IgG in the tissue.

To identify the antigen and its localisation on cells in vitro, rat hippocampal neuronal cultures were prepared as reported previously.[13] Live neurons grown on coverslips were incubated for 1 h at 37°C with patient or control serum (final dilution 1:200) or CSF (1:10). After removing the media and washing with PBS, neurons were fixed with 4% paraformaldehyde and were made permeable with 0·1% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA). Neurons were single or double immunolabelled with a guineapig polyclonal $GABA_{B1}$ receptor antibody (1:200), followed by the corresponding Alexa Fluor secondary antibodies (1:2000; Molecular Probes, Invitrogen, Eugene, OR, USA). Results were photographed as detailed above.

Rat hippocampal neurons were grown in 100 mm wells (10^6 neurons per well) and incubated at 37°C with filtered serum (1:500) for 1 h. Neurons were then washed with PBS, lysed with buffer (sodium chloride 150 mM, EDTA [edetic acid] 1 mM, tris(hydroxymethyl) aminomethane [Tris]-hydrochloric acid 100 mM, deoxycholate acid 0·5%, 1% Triton X-100, pH 7·5) containing protease inhibitors (P8340; Sigma-Aldrich), and centrifuged at $16·1 \times 10^3$ gravities for 20 min at 4°C. The supernatant was retained and incubated with protein A/G agarose beads (20423; Pierce, Rockford, IL, USA) overnight at 4°C, centrifuged, and the pellet containing the beads with patients' antibodies bound to the target cell-surface antigen was washed with PBS, aliquoted, and kept at –80°C. A 25 μL aliquot of this pellet was resuspended in Laemmli buffer, boiled for 10 min, separated in 4–15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins visualised with EZBlue gel staining (G1041; Sigma-Aldrich). Protein bands from the gels were cut and sent for mass spectrometry to the Proteomics Core Facility of the Genomics Institute at the Abramson Cancer Center (University of Pennsylvania, PA, USA). Protein bands were trypsin digested and analysed with a nanoLC/nanospray/LTQ mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA) as reported previously.[14] Briefly, a 3 μL trypsin-digested sample was injected with autosampler (Eksigent, Dublin, CA, USA). The digested samples were separated on a 10 cm C18 column, using nanoLC (Eksigent) with a 200 μL/min flow rate, and a 45 min gradient. Online nanospray was used to spray the separated peptides into a linear trap quadrupole, and raw data were obtained with Xcalibur software (Thermo Scientific, Waltham, MA, USA). The raw data files were searched against the National Center for Biotechnology Information and Swiss-Prot (Swiss Institute of Bioinformatics, Basel, Switzerland) databases with Mascot (Matrix Science, Boston, MA, USA). The cutoff score for definite protein identification was 70 or more.

After characterisation of the antigen, frozen samples of the pellets were separated in SDS-PAGE, transferred to nitrocellulose (162-0115; Bio-Rad, Hercules, CA, USA),

and blotted with the polyclonal antibodies against $GABA_{B1}$ (1:2000) or $GABA_{B2}$ (1:1000) receptor subunits. The reactivity was developed by use of biotinylated anti-guineapig IgG made in goat (1:2000; Vector Laboratories, Burlingame, CA, USA) and the avidin–biotin–peroxidase diaminobenzidine method.

To determine the sensitivity and specificity of patients' antibodies for the $GABA_B$ receptor, we used a semi-quantitative confocal microscopy analysis similar to that used for other synaptic receptors.[7,8] Live rat hippocampal neurons cultured for 14–21 days in vitro were incubated with patients' CSF (1:30 dilution in Neurobasal B27 medium; GIBCO, Invitrogen, Carlsbad, CA, USA) for 24 h, washed in PBS, fixed in paraformaldehyde (4% paraformaldehyde, 4% sucrose in PBS) for 5 min, made permeable with 0·25% Triton X-100 for 10 min, and blocked with 5% normal goat serum for 1 h. Neurons were incubated with a guineapig polyclonal antibody against an intracellular epitope of the $GABA_B$ receptor (1:1000; Invitrogen) and a mouse monoclonal antibody against the presynaptic marker Bassoon (1:200; Stressgen, Victoria, BC, Canada), washed, and incubated with the appropriate fluorescent-conjugated secondary antibodies (1:1000, Molecular Probes).

A laser-scanning confocal microscope (Leica TCS SP2; Leica, Deerfield, IL, USA) was used to obtain images. For each image, laser light levels and detector gain and offset were adjusted so that no pixel values were saturated. Images were automatically segmented with an iterative thresholding approach that finds maxima of fluorescence intensity,[15] and areas of interest containing dendrites were selected, and the number of individual clusters along dendrites was quantified by use of ImageJ interactive software (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA) as described previously.[7,8] The colocalisation of clusters

	Sex	Age (years)	Tumour by imaging or pathology	Presenting symptoms	Other clinical and immunological features
Patient					
1	Female	60	SCLC	Subacute onset of complex partial seizures, confusion, memory impairment	SIADH
2	Male	66	SCLC	Subacute onset of seizures, confusion, memory deficit, behavioural problems	N-type VGCC antibodies
3	Female	53	SCLC	Rapidly progressive memory deficits, abnormal sleeping habits, followed by frequent seizures (focal, secondarily generalised), confusion, decline in mental status leading to coma	Pruritic rash with initial weakness
4	Male	75	Mediastinal adenopathy	Subacute onset of seizures, confusion, memory deficit, psychosis, encephalitis; died soon after presentation, before definitive diagnosis or treatment	Poor respiratory status, refused intubation
5	Male	68	Neuroendocrine tumour of the lung	Subacute onset of seizures, status epilepticus, confusion, memory deficit	..
6	Female	43	CT and FDG/PET negative	Subacute onset of secondarily generalised tonic-clonic seizures, confusion, bizarre behaviours, delusions, paranoia, memory impairment	N-type VGCC antibodies
7	Male	69	CT and FDG/PET negative	Subacute onset of seizures, status epilepticus, severe encephalopathy, severe memory deficit, confusion	History of bipolar disorder
8	Female	24	CT and FDG/PET negative	Subacute onset of seizures, status epilepticus, confusion, memory deficit, fever; required intubation and ventilation owing to poor level of consciousness and airway protection	N-type VGCC antibodies
9	Male	63	CT and FDG/PET negative	Subacute onset of seizures, confusion, memory deficit, paranoia, psychosis, gustatory hallucinations	TPO and GAD65 antibodies; hypothyroidism and type 2 diabetes mellitus
10	Female	45	Benign ovarian mass	Subacute onset of complex partial and generalised seizures, confusion, short-term memory deficits	TPO and thyroglobulin antibodies in serum (not in CSF); no endocrinopathy
11	Female	62	CT chest, abdomen, pelvis negative	Subacute onset of generalised seizures, confusion, memory deficit, decreased level of consciousness, fluent aphasia, abnormal orolingual movements	..
12	Male	29	CT and FDG/PET negative	Subacute onset of temporal lobe and generalised tonic-clonic seizures, confusion, memory deficits; no cognitive deterioration	Childhood seizures
13	Female	30	CT and FDG/PET negative	3-month history of severe memory deficit, confusion, followed by seizures (generalised, subclinical)	GAD65 antibodies without endocrinopathy
14	Male	69	SCLC	Subacute onset of generalised tonic-clonic seizures, worsened short-term memory deficit, confusion	Mild short-term memory deficit from past history of subarachnoid haemorrhage
15	Male	70	SCLC	Subacute onset of seizures (partial motor and generalised); severe short-term memory loss, confusion, confabulation, visual hallucinations, disorientation, agitation	GAD65, TPO, and SOX1 antibodies; no endocrinopathy
Control					
1	Female	63	CT and FDG/PET negative	1 year progression of cerebellar ataxia; normal mental status, no seizures, no muscle spasms or stiffness	GAD65 antibodies, adult-onset insulin-dependent diabetes mellitus
2	Female	61	CT and FDG/PET negative	6 week history of gait disturbance, lower extremity myoclonus and stiffness; dysphagia, dysarthria, nystagmus, left gaze palsy. No seizures or cognitive symptoms	GAD65, TPO, and thyroglobulin antibodies (mild thyroid dysfunction)

SCLC=small-cell lung cancer. SIADH=syndrome of inappropriate antidiuretic hormone. VGCC=voltage-gated calcium channel. FDG=fluorodeoxyglucose. TPO=thyroid peroxidase. GAD65=glutamic acid decarboxylase 65. SOX1=sex determining region Y-box 1.

Table 1: **Demographic features and symptoms**

labelled with patients' antibodies, commercial $GABA_B$ antibodies, and Bassoon was quantified using a software macro (written by EH) in ImageJ.

Owing to the reactivity of patients' antibodies with rat tissue and hippocampal neuronal cultures, and the homology between human and rat $GABA_B$ receptor sequences (the B1 receptor subunit has 91·3% cDNA sequence identity and 98·6% amino acid sequence identity in the two species),[16] HEK293 cells were transfected with plasmids containing rodent $GABA_{B1}$ or $GABA_{B2}$ or plasmids without an insert (control), by use of a method previously reported.[7] In other experiments, cells were transfected with $GABA_{B1}$ and $GABA_{B2}$ in equimolar ratios. Cells were then grown for 24 h before assessment. Transfected cells were fixed in 4% paraformaldehyde, made permeable with 0·1% Triton X-100, and then incubated overnight at 4°C with patients' serum (1:200) or CSF (100%) and the guineapig polyclonal $GABA_{B1}$ receptor antibody (1:20000) or a polyclonal $GABA_{B2}$ receptor antibody (1:10000, generated by SJM), washed in PBS, and incubated with the appropriate Alexa Fluor secondary antibodies (1:2000). Results were photographed as before.

Antibody titres were obtained by use of HEK293 cells expressing $GABA_{B1/B2}$ incubated with serial dilutions of serum and CSF, starting at 1:1 dilution. Patients' antibody IgG subtypes in serum or CSF were identified by use of the HEK293 transfected cells and secondary anti-human

	MRI	CSF	Serum antibody titres*	CSF antibody titres*	Chronological list of treatments	Outcome (duration of follow-up)
Patient						
1	FLAIR/T2 increased signal in medial temporal lobes	9 WBC per μL; protein 350 mg/L; no OCBs	640	160	IVIg, corticosteroids, chemotherapy	Substantial improvement; mild residual short-term memory deficit; lives independently; seizure free (12 months)
2	Normal	Normal	1280	..	Corticosteroids, IVIg, chemotherapy	Substantial improvement; died of metastatic disease (15 months)
3	FLAIR/T2 increased signal in medial temporal lobes	..	160	..	Tumour removal (lobectomy), IVIg	Partial improvement after tumour removal and IVIg (4 months); lost to follow-up
4	Normal	..	2560	640	None	Died soon after presentation of rapidly progressive respiratory failure
5	FLAIR/T2 increased signal in medial temporal lobes	..	1280	..	Supportive	Died 6 months after symptom presentation; $GABA_B$ antibodies detected after patient's death in archived serum
6	FLAIR/T2 increased signal in small area of corpus callosum	95 WBC per μL; protein 1040 mg/L; increased IgG index	..	640	Corticosteroids, mycophenylate mofetil	Substantial improvement; lives independently; seizure free (9 months)
7	FLAIR/T2 increased signal in left medial temporal lobe	..	..	640	Corticosteroids, plasma exchange	Initial substantial response to corticosteroids; relapsed 1 month later; died after 5 months in ICU with refractory seizures, status epilepticus, and systemic complications; $GABA_B$ antibodies detected after patient's death in archived serum
8	FLAIR/T2 increased signal in medial temporal lobes	19 WBC per μL; protein 460 mg/L	5120	2560	Corticosteroids, plasma exchange	Substantial improvement; mild residual short-term memory deficit; seizure free (3 months)
9	FLAIR/T2 increased signal in medial temporal lobes	75 WBC per μL; protein 260 mg/L; OCBs present	Negative	4	Corticosteroids	Full recovery (41 months)
10	FLAIR/T2 increased signal in medial temporal lobes	81 WBC per μL; protein 300 mg/L	10240	..	Corticosteroids	Substantial improvement. Residual short-term memory deficit. Lives independently. Seizure free (72 months)
11	Normal	20 WBC per μL; protein 220 mg/L	40	40	Corticosteroids	Full recovery (6 months)
12	FLAIR/T2 increased signal in left medial temporal lobe and insula	950 WBC per μL; OCBs present	Negative	10	Symptomatic	Temporal lobe biopsy 20 months after symptom presentation showing reactive astrocytosis, without inflammation; no follow-up available after biopsy
13	FLAIR/T2 increased signal in medial temporal lobes	4 WBC per μL; protein 1090 mg/L; 6 OCBs	Negative	4	Corticosteroids	Full recovery, except for infrequent brief episodes of visual hallucinations (10 months)
14	FLAIR/T2 increased signal in left medial temporal lobe	Traumatic; negative cytology	..	80	Chemotherapy	Residual short-term memory deficit; seizures controlled; died of sepsis (3 months)
15	Normal	0 WBC per μL; protein 950 mg/L	..	640	IVIg, corticosteroids, chemotherapy	Seizures responded to antiepileptics; memory deficit persisted; died of cancer-related treatment (2 months)
Control						
1	Normal	3 WBC per μL; protein 780 mg/L; 1 OCB	Negative	2	IVIg	No seizures or cognitive deficits; limited response of cerebellar ataxia to IVIg (12 months)
2	Normal	2 WBC per μL; protein 520 mg/L; OCBs present	Negative	2	IVIg, corticosteroids	No seizures or cognitive deficits; full recovery after steroids and IVIg (12 months)

*Titres defined as the reciprocal of the maximal dilution that gave positive immunostaining. FLAIR=fluid-attenuated inversion recovery. WBC=white blood cells (normal <4 per μL). OCB=oligoclonal band. IVIg=intravenous immunoglobulin. ICU=intensive care unit.

Table 2: **Diagnostic tests, treatment, and outcome**

antibodies specific for IgG1, IgG2, IgG3, or IgG4 (all 1:50; Sigma-Aldrich) as reported.[17]

Statistical analysis

The association between $GABA_B$ receptor antibodies and other autoantibodies (GAD65, N-type voltage-gated calcium channel, thyroid peroxidase, thyroglobulin, or SOX1) and that between neurological improvement and cancer treatment or immunotherapy were analysed with Fisher's two-sided exact test. The colocalisation of patients' antibodies with the polyclonal $GABA_B$ receptor antibodies or antibodies to the synaptic marker Bassoon was analysed with the Student's *t* test.

Role of the funding source

The study sponsor had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

In May, 2008, a 60-year-old woman with a long history of smoking was admitted to hospital with confusion, memory problems, and new-onset generalised tonic-clonic and partial complex seizures refractory to treatment (index patient; patient 1). At examination, she was confused about the time and where she was and had poor concentration and short-term memory (table 1). Although she had saccadic pursuits with lateral gaze, no cranial nerve abnormalities were noted. Strength, sensation, reflexes, and coordination were normal. MRI of the brain showed increased fluid-attenuated inversion recovery (FLAIR) signal in the medial temporal lobe of both hemispheres, compatible with limbic encephalitis (table 2, figure 1A). Diffuse slowing and bilateral periodic lateralised epileptiform discharges were seen on encephalography (EEG). In the CSF there were nine white blood cells per μL, total protein concentration was 350 mg/L, and glucose concentration was 3·94 mmol/L; there were no oligoclonal bands and cytological findings were normal. PCR for herpes simplex virus, West Nile virus, and St Louis encephalitis were negative. The patient had hyponatraemia (119 mEq/L) caused by syndrome of inappropriate antidiuretic hormone secretion. Combined CT and fluorodeoxyglucose-PET showed mediastinal lymphadenopathy, which was proven by biopsy to be small-cell lung cancer. The patient was treated with antiepileptic drugs (levetiracetam, valproic acid, and phenytoin) and immunotherapy (intravenous immunoglobulins and corticosteroids), immediately followed by chemotherapy with cisplatin and etoposide. The patient's short-term memory and cognition improved, and seizures resolved. After chemotherapy the patient had standard prophylactic whole-brain radiation therapy. Brain MRI 1 month after symptom presentation showed improvement of the abnormal FLAIR signal (figure 1B); MRI at 3 months and 9 months were unchanged except for progressive general atrophy, probably secondary to radiation (figure 1C, D). 1 year after symptom presentation, the patient had only mild deficits in memory and cognition and lived independently.

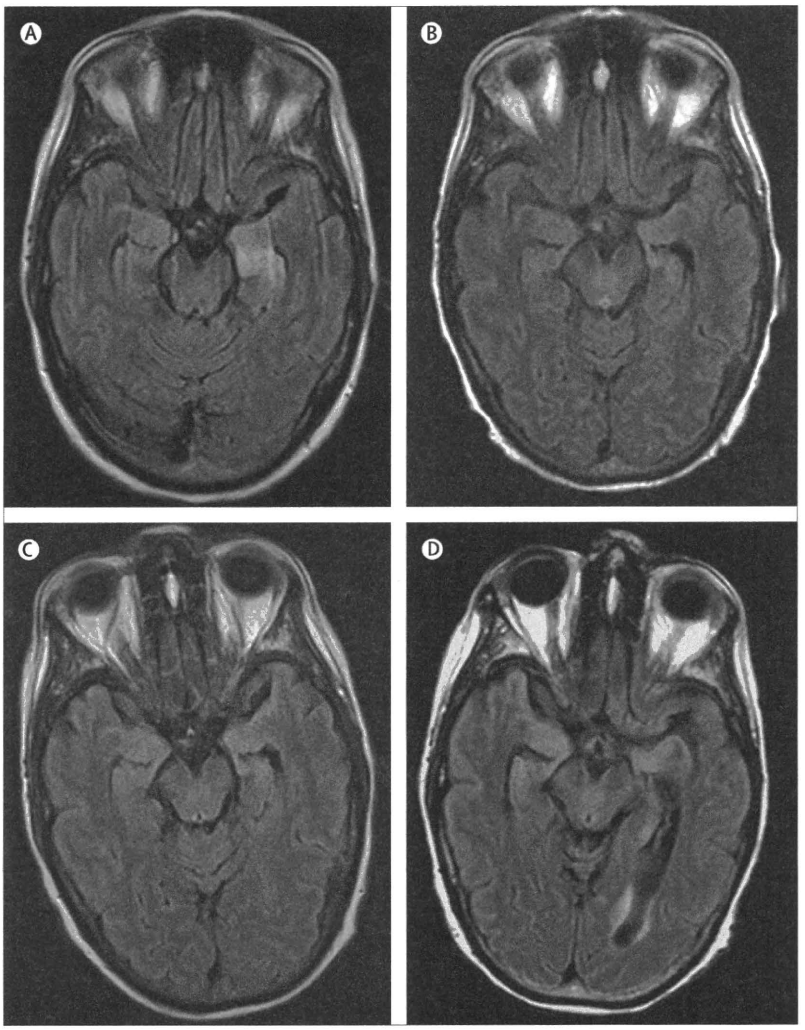

Figure 1: **MRI of a patient with $GABA_B$ receptor antibodies and limbic encephalitis**
Axial fluid-attenuated inversion recovery (FLAIR) MRI from patient 1 at presentation (A) showed increased signal in the medial temporal lobes, which was more pronounced on the left. Repeat study at 1 month (B) showed improvement of the FLAIR signal that remained stable at 3 months and 9 months (C, D), with development of mild generalised atrophy (the patient received standard whole-brain radiation therapy as prophylaxis for small-cell lung cancer metastases).

Sera and CSF from the index patient and the 14 other patients (patients 1–15) showed a pattern of reactivity with the neuropil of rat brain (figure 2) that was different from that reported with antibodies against NR1 subunits of the NMDA receptor, GluR1/2 subunits of the AMPA receptor, or voltage-gated potassium channels.[7,8,18] When non-fixed and non-permeabilised cultures of rat hippocampal neurons were incubated with patients' serum or CSF, intense reactivity with the cell surface was seen (figure 3A). Similar studies with serum or CSF from

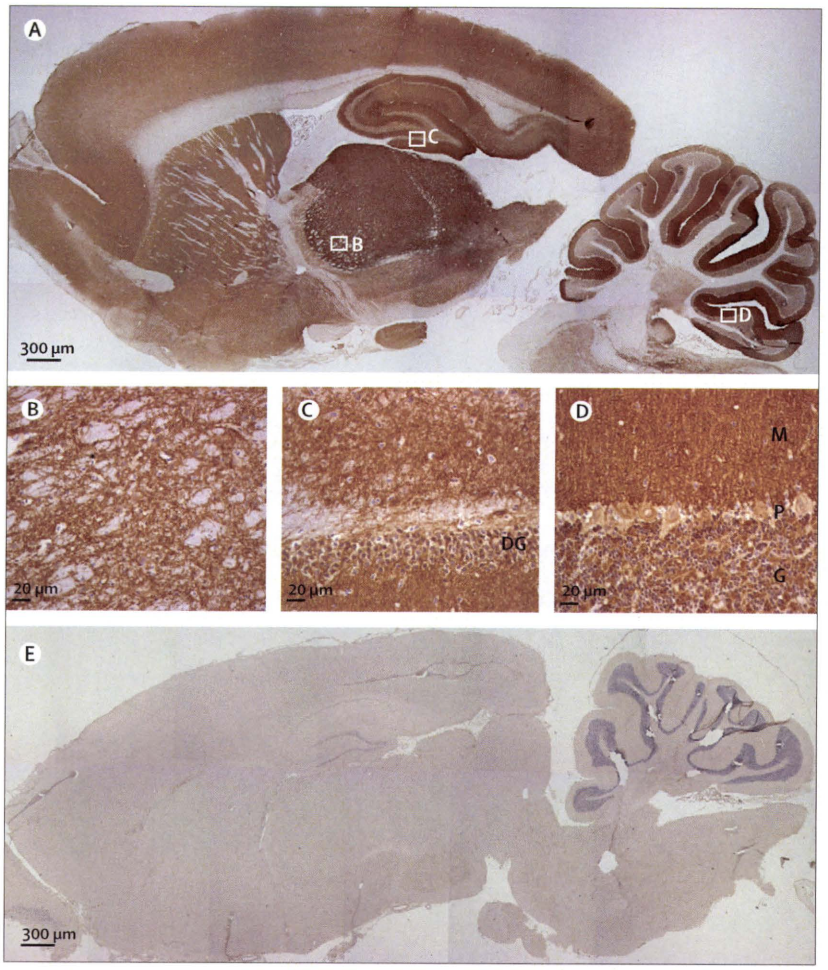

Figure 2: **Immunolabelling of rat brain with patients' antibodies**
Sagittal section of rat brain immunolabelled with CSF of a patient with limbic encephalitis (A) and a control individual (E). Note the extensive staining in A of the neuropil of thalamus (B), hippocampus (C), cerebellum (D), and cerebral cortex. DG=dentate gyrus. M=molecular layer. P=Purkinje cell layer. G=granular cell layer. Avidin–biotin–peroxidase method; sections counterstained with haematoxylin.

control individuals showed no reactivity with rat brain tissue (figure 2E) or cultures of neurons (figure 3B).

The $GABA_B$ receptor was identified as the target antigen by immunoprecipitation of the antigen with patients' serum samples and peptide sequence recognition ($GABA_{B1}$ and $GABA_{B2}$) by mass spectrometry (webappendix). Immunoprecipitates were obtained using the serum from four patients. Electrophoresis of the immunoprecipitates showed similar protein bands at about 90 kDa and 105 kDa (Figure 3C). The indicated protein bands contained sequences derived from $GABA_{B1}$ and $GABA_{B2}$ (protein scores for B1: 160, 225, 342, and 178; protein scores for B2: 1094, 1784, 1946, and 2653).

See Online for webappendix

The results were confirmed by immunoblotting the immunoprecipitates with antibodies specific for $GABA_{B1}$ and $GABA_{B2}$. Immunoblot analysis confirmed that the band at about 105 kDa was recognised by anti-$GABA_{B1}$ and anti-$GABA_{B2}$ antibodies, and the band at about 90 kDa was recognised by anti-$GABA_{B1}$ antibodies (figure 3D).

Colocalisation of patients' antibodies with the $GABA_B$ receptor and the synaptic and extrasynaptic location of the target receptors were noted on confocal microscopy. The colocalisation of patients' antibody clusters with $GABA_B$ receptor clusters (figure 4) was quantified for the dendrites of 23 neurons on four separate coverslips. 103% (SE 0·8%) of the clusters labelled with antibodies from patients colocalised with clusters labelled by the guineapig polyclonal $GABA_B$ receptor antibody, and 107% (SE 0·7%) of guineapig antibody-labelled clusters colocalised with those labelled by patients' antibodies (numbers slightly higher than 100% occur because of overlapping of a few clusters labelled by patient antibodies with two guineapig antibody-labelled clusters and vice versa). These results suggest that all patients' antineuronal cell-surface antibodies target the $GABA_B$ receptors and that almost all neuronal $GABA_B$ receptors are labelled by patients' antibodies. 62% (SE 1·3%) of $GABA_B$ receptor clusters labelled by patients' antibodies were also labelled by Bassoon, significantly fewer than those also labelled by guineapig $GABA_B$ receptor antibodies (Student's t test, $p<0·0001$), suggesting that patient antibodies bind both synaptic and extrasynaptic $GABA_B$ receptors.

The location of the epitope in $GABA_{B1}$ was identified with HEK293 cells transfected with $GABA_{B1}$, $GABA_{B2}$, or both $GABA_B$ receptor subunits. All 15 patients had serum or CSF antibodies that reacted with $GABA_{B1}$ (figure 5), and one had additional reactivity with the $GABA_{B2}$ subunit (data not shown). Similar studies with the 104 control individuals showed that two patients, both with syndromes attributed to GAD65 autoimmunity, had $GABA_{B1}$ receptor antibodies at low titres (CSF 1:2, serum negative), which did not bind at detectable levels to sections of rat brain (Fisher's exact test, $p<0·0001$, data not shown).

Samples from the six patients for whom sufficient serum or CSF was available were analysed for antibody IgG subtypes. All six patients had IgG1 $GABA_{B1}$ antibodies, two had additional IgG3, and one had IgG2 antibodies.

Table 1 shows demographic features and symptoms of the 15 patients and the two control individuals who had antibodies to GAD65. Among the 15 patients, median age was 62 years (range 24–75); eight were men. All patients had seizures, confusion, and memory deficits. In 13 patients the seizures were the presenting symptom; in two (patients 3 and 13) memory deficit and confusion were the presenting symptoms. After further clinical assessment most seizures appeared to have a temporal-lobe onset with secondary generalisation, and three patients had status epilepticus.

Ten patients had unilateral or bilateral increases in medial temporal lobe FLAIR/T2 signal consistent with limbic encephalitis, and one had a small area of increased FLAIR signal in the corpus callosum (table 2). Four patients had normal brain MRI.

CSF was abnormal in nine of ten patients for whom data were available. The most common CSF abnormality was lymphocytic pleocytosis in eight patients. EEG results were available from 12 patients: nine had temporal-lobe seizures, epileptiform discharges, or temporal-lobe slowing; two had generalised slowing; and one had no abnormalities. Several types of seizures were noted on EEG, including complex partial seizures (often of temporal-lobe onset), status epilepticus, and subclinical seizures.

The two control individuals with low titre $GABA_{B1}$ antibodies developed different syndromes in association with high titre GAD65 antibodies in serum and CSF. Neither of these two patients developed seizures or limbic dysfunction (table 1). One had progressive cerebellar ataxia, and the other had gait instability, muscle stiffness, rigidity, myoclonus, and dysarthria, categorised as progressive encephalomyelitis with rigidity and myoclonus.

In addition to $GABA_B$ antibodies, seven of 15 patients had antibodies to one or more of the following: GAD65 (3 patients), thyroid peroxidase (3 patients), N-type voltage-gated calcium channels (3 patients), and SOX1 (1 patient). Only one of the three patients with GAD65 antibodies had endocrinopathy, and one of the three patients with voltage-gated calcium channel antibodies had small-cell lung cancer (table 1). The patient with SOX1 antibodies had small-cell lung cancer.

Seven patients had tumours (table 1), detected at the time of neurological symptom presentation. Of these patients, five had small-cell lung cancer, one had a lung tumour of neuroendocrine origin, and one had mediastinal adenopathy. No other systemic tumours were identified. Because most lung tumours were diagnosed by use of needle biopsy, no tissue was available for analysis of $GABA_B$ receptor expression. However, three of four small-cell lung cancers from control individuals without antibodies or encephalitis (archived tissue from the Division of Anatomic Pathology, University of Pennsylvania) showed reactivity with a guineapig polyclonal antibody to $GABA_{B1}$ receptor and patients' biotinylated IgG, suggesting that these receptors are expressed by small-cell lung cancer (webappendix).

Five of the patients were young (median age 30 years, range 24–45), were non-smokers, and had negative cancer screening including CT/fluorodeoxyglucose-PET, and two of these patients had long-term follow-up (41 and 72 months), making the presence of cancer unlikely.

Nine of 15 patients had a neurological response to immunotherapy (six) or treatment of the tumour as well as immunotherapy (three). The median follow-up of these nine patients was 10 months (range 3–72 months). One patient (patient 2) later died of tumour progression (15 months) and one (patient 3) was lost to follow-up at 4 months. Six patients did not have sustained neurological improvement: three patients (patients 4, 14, and 15) died from tumour or chemotherapy-related complications soon after presentation of the disorder, two were diagnosed with $GABA_B$ receptor antibodies after death (patients 5 and 7), and one was lost to follow-up (patient 12). Of the latter three, only patient 7 was thought to have an autoimmune disorder, and therefore this patient received corticosteroids and plasma exchange; the other two patients did not receive immunotherapy. Overall, after excluding one non-assessable patient (patient 12) nine of the ten patients who received immunotherapy and cancer treatment (when a tumour was found) showed neurological improvement, while none of the four patients (patients 4, 5, 14, and 15) who did not receive immunotherapy or whose tumour treatment was not completed showed improvement (Fisher's exact test p=0·005).

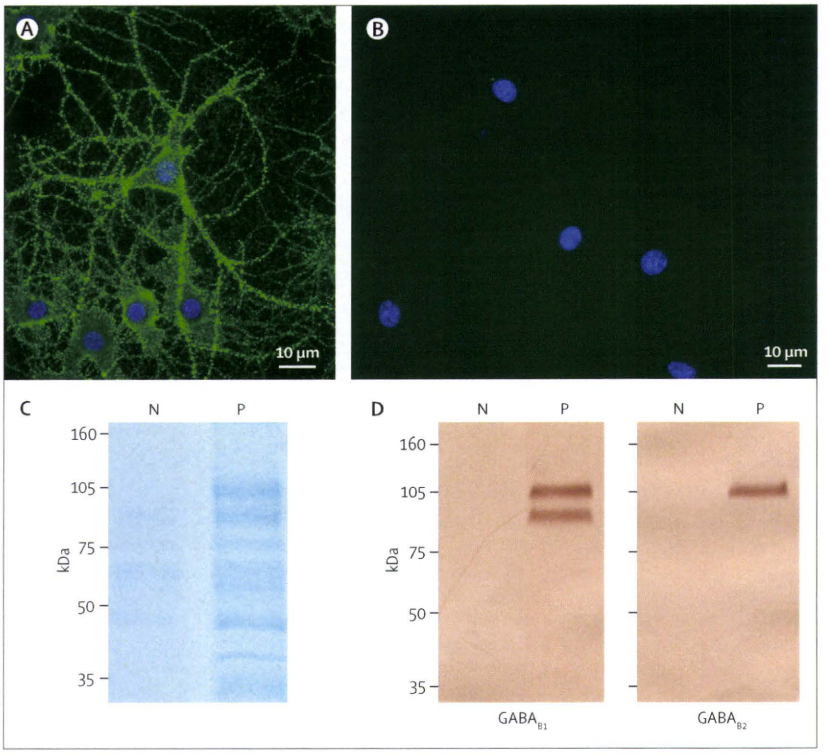

Figure 3: **Culture of rat hippocampal neurons incubated (live, non-permeabilised) with the CSF of a patient with limbic encephalitis and a control individual**

Note the intense punctate reactivity of patient's antibodies with cell surface antigens (A) and the absence of reactivity in the control (B); nuclei of neurons stained with 4',6-diamidino-2-phenylindole (DAPI). The surface antigens were precipitated using the antibodies within the patient's serum, and then electrophoretically separated and visualised with EZBlue (C). Patient's antibodies (P) precipitated two main protein bands at about 105 kDa and 90 kDa; these bands are not seen in the precipitate using serum from a control individual (N). Sequencing of the 105 kDa band by use of mass spectrometry showed it contained the B1 and B2 subunits of the $GABA_B$ receptor (webappendix). The 90 kDa and other smaller bands were proteolytic fragments and patient's IgG products. Subsequent transfer of the gel to nitrocellulose and immunoblotting with antibodies specific for each of the $GABA_B$ (D) subunits confirmed that patient's antibodies precipitated the B1 and B2 subunits (105 kDa) and that the 90 kDa band was a proteolytic fragment of B1.

Discussion

15 patients had autoimmune encephalitis associated with antibodies to extracellular epitopes of the $GABA_B$ receptor

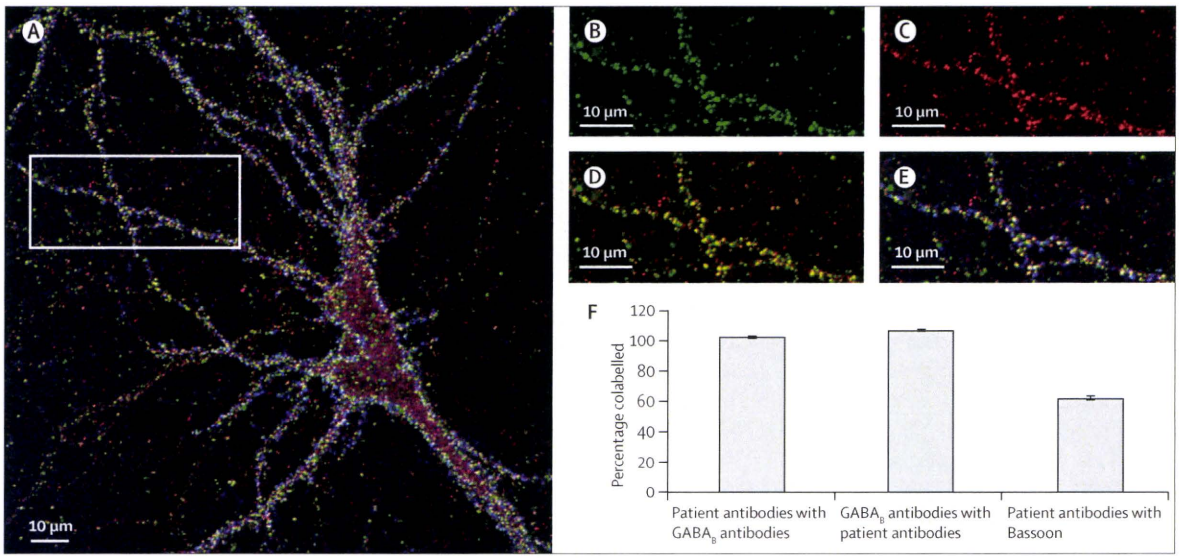

Figure 4: **Confocal image of a cultured triple labelled embryonic rat hippocampal neuron**
Patient's antibodies are in green, a guineapig polyclonal antibody against an intracellular epitope of the GABA$_{B1}$ receptor is in red, and an antibody to the presynaptic marker Bassoon is in blue (A). Area of dendrite from the same neuron showing patient's antibody staining (B), guineapig polyclonal GABA$_{B1}$ receptor antibody staining (C), both patient and guineapig antibody staining (D), and triple staining (E). The colocalisation of labelling of the dendrites of 23 neurons was quantified (F). This suggests that patients' antibodies bind both synaptic and extrasynaptic GABA$_B$ receptors.

and nine responded to treatment. On the basis of clinical, MRI, and EEG findings, the brain regions most affected are the hippocampi and temporal lobes. Thus, it is not surprising that the resulting syndrome is similar to other types of limbic encephalitis (eg, encephalitis associated with antibodies against AMPA receptors or voltage-gated potassium channels), although some clinical and immunological features might suggest GABA$_B$ receptor autoimmunity. We have reported development of seizures in all patients, the association with lung cancer in seven patients (five pathologically confirmed as small-cell lung cancer), and the presence of autoantibodies of unclear relation to this type of limbic encephalitis in seven patients. Disruption of GABA$_B$ receptors by patients' antibodies is a possible explanation for the symptoms because pharmacological[19–21] and genetic[3,4] changes to these receptors in rodents result in phenotypes similar to limbic encephalitis, including prominent seizures, memory deficits, increased anxiety, and mood dysregulation.[22] Moreover, in human beings, some GABA$_B$ receptor polymorphisms are associated with temporal-lobe epilepsy.[23]

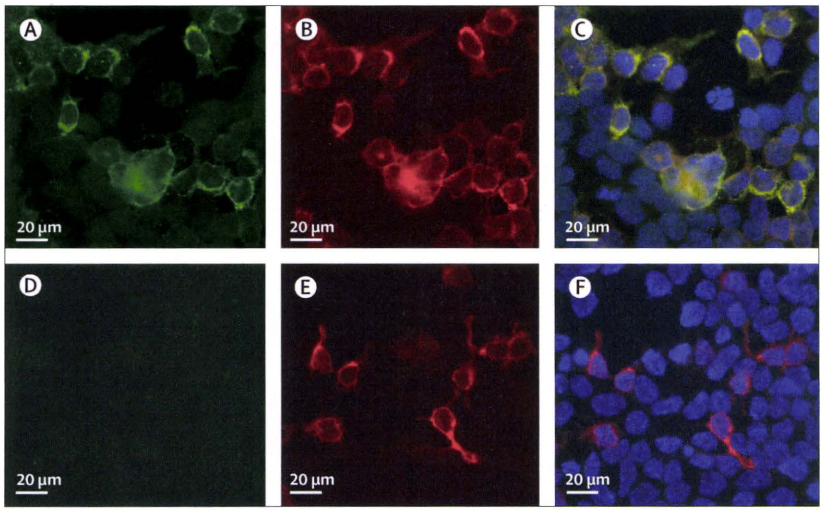

Figure 5: **Detection of antibodies to the GABA$_{B1}$ subunit using a HEK293 cell-based assay**
HEK293 cells transfected with the GABA$_{B1}$ receptor subunit show reactivity with CSF from a patient with limbic encephalitis (A) and a polyclonal antibody against the B1 subunit of the GABA$_B$ receptor (B); both reactivities are merged in C. Similarly transfected cells do not react with CSF from a control individual (D) but do show reactivity with a polyclonal antibody against the B1 subunit of the GABA$_B$ receptor (E); reactivities merged in F. Immunofluorescent method.

GABA$_B$ receptors are G-protein-coupled receptors composed of two subunits, GABA$_{B1}$ and GABA$_{B2}$.[19,24] GABA$_B$ receptors mediate presynaptic inhibition by at least two mechanisms: the activation of G-protein-coupled-inward rectifying potassium channels and the inhibition of calcium channels.[25] These receptors also attenuate presynaptic firing frequencies.[26] Postsynaptic GABA$_B$ receptors mediate inhibition by similar mechanisms[27] and by inducing a slow inhibitory postsynaptic potential.[28] GABA$_B$ receptors limit the duration of network high-activity states, preventing excessive neuronal synchronisation, and allowing new stimuli to break synchronous activity.[29,30] GABA$_B$ receptors are widely distributed in the brain and spinal cord, but the highest levels of GABA$_B$ receptors are found in the hippocampus, thalamus, and cerebellum.[31] In the current study, the corresponding areas of rat brain were more intensely immunolabelled by patients' antibodies. The main antigen recognised by the patients' antibodies, the GABA$_{B1}$ subunit, is necessary for GABA binding and receptor function, whereas the GABA$_{B2}$ subunit is

required for localisation of the receptor to appropriate areas of the cell membrane and G-protein coupling.[32,33]

By use of a HEK293 cell-based assay we showed that the sera or CSF of all 15 patients had antibodies that reacted with $GABA_{B1}$, with additional reactivity to $GABA_{B2}$ in one patient. These findings suggest that HEK293 cells expressing $GABA_{B1/B2}$ or $GABA_{B1}$ could be used as a diagnostic test.

A third of patients with encephalitis and $GABA_B$ receptor antibodies had pathologically confirmed small-cell lung cancer (age range 53–70 years, all smokers). The involvement of this type of tumour in paraneoplastic disorders and its ability to express synaptic proteins, including $GABA_B$ receptors, suggests that it might trigger the immune response against these receptors. In a subgroup of patients with limbic encephalitis and small-cell lung cancer previously thought to be without antibodies or attributed to antibodies against intracellular antigens, $GABA_B$ receptor autoimmunity is probably involved,[34] particularly in patients who improved after treatment of the tumour or immunotherapy.[35,36] Moreover, $GABA_B$ receptor autoimmune encephalitis also seems to develop without cancer association. In this respect, $GABA_B$ receptor autoimmune encephalitis is similar to other synaptic autoimmunities of the CNS (those involving antibodies to NMDA receptors or AMPA receptors)[8–10] or peripheral nervous system (those involving antibodies to acetylcholine receptors or P/Q-type voltage-gated calcium channels) that can develop with or without cancer.[37] As occurs in some of these disorders,[8] almost half of the patients with $GABA_B$ receptor autoimmune encephalitis (including five without tumours) had additional autoantibodies (to TPO, GAD65, SOX1, or N-type voltage-gated calcium channels), suggesting autoimmunity. The overlap with antibodies to GAD65 (an intracellular antigen) suggests that some patients with limbic encephalitis attributed to GAD65 autoimmunity might have $GABA_B$ receptor antibodies as a more likely cause of the symptoms.[38,39] As more relevant cell-surface or synaptic autoantigens are identified, subsets of disorders with unclear definitions, such as steroid-responsive encephalitis or Hashimoto's encephalitis without thyroid peroxidase antibodies in the CSF, will probably be reclassified.

The small number of patients with $GABA_B$ receptor antibodies and the retrospective identification of patients prevented us from assessing the contribution of cancer treatment, immunotherapy, or both, to neurological improvement. Moreover, we were unable to correlate antibody titres with clinical outcome because we did not have serial serum or CSF samples. As this disorder becomes more widely recognised, additional symptoms are likely to be identified. On the basis of the distribution of $GABA_B$ receptors in the brain, one would expect that some patients might develop encephalitis or seizure disorders with less focal limbic dysfunction. This could be tested using HEK293 cells that express $GABA_{B1/B2}$ or $GABA_{B1}$, as described in this paper. By the time antibodies are detected the serum titres can be very low, and we suggest examining both serum and CSF. Identification of these antibodies should prompt the search for a small-cell lung cancer. Recognition of this disorder is important because it is potentially responsive to immunotherapy and treatment of the tumour. The binding of patients' antibodies to the $GABA_B$ receptor in live rat neurons, and the similarity of the syndrome to experimental phenotypes in which this receptor does not function properly, suggest the antibodies are pathogenic. Although $GABA_{B1}$ receptor antibodies are mainly IgG1 and thus able to activate complement, the role of complement-mediated cytotoxicity is questionable in this potentially reversible disorder in which neurons are the main targets. Future studies should focus on the disease mechanism and effects of the antibodies.

Contributors

RC, JR, DF, MBS, WG, AK, KO, TI, MG, FG, and JD designed the study and clinically assessed the patients. EL, ML, XP, EH, and MG did the laboratory studies and prepared the figures. EL, SJM, RB-G, and JD were involved in study design, data analysis, and writing of the report.

Conflicts of interest

RC has received honoraria from Boehringer-Ingelheim and Orion Pharma for projects unrelated to the current study. SJM has received reimbursement for travel and accommodation expenses as well as funding support from Pfizer. JD has received royalties from a patent related to Ma2 autoantibody test and has filed patent applications for NMDA and $GABA_B$ receptor autoantibody tests. JD has received funding from Euroimmun for projects unrelated to the current study. All other authors have no conflicts of interest.

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トピックス

III. 最近の話題
2. 膠原病における新たな抗神経抗体の検索

木村　暁夫　　犬塚　　貴

要　旨

神経障害を伴う膠原病患者において自己免疫異常を背景として出現した抗神経抗体は神経障害に密接な関りをもつ可能性がある．したがって特異的な抗神経抗体を同定することは，その病態解明に重要である．また，時に神経系の日和見感染症や精神疾患との鑑別が問題となるこれら神経症状の，適切な診断と治療効果のメルクマールの確立につながると考えられる．我々はプロテオミクス解析の手法を用いて，神経障害を伴う膠原病に特異的と考えられた新たな抗神経抗体をいくつか同定してきたので報告する．

〔日内会誌　99：1865～1870，2010〕

Key words：膠原病，神経障害，抗神経抗体，二次元免疫ブロット，プロテオミクス

1. 膠原病患者の神経障害と自己抗体

膠原病患者に合併する神経障害と抗神経抗体を含めた自己抗体との関連性を報告した論文はこれまでに多数認められるが，その多くが全身性エリテマトーデス（systemic lupus erythematosus：SLE）[1]に関するものであり，次いでSjögren症候群[2]に関するものである．最近のレビューによると，これまでに精神神経症状を合併したSLEすなわちneuropsychiatric systemic lupus erythematosus（NPSLE）患者において，その血清ないし髄液中より約20種類の自己抗体が報告されている[1]．特に以前より血清ないし髄液中の抗リボソームP抗体がループス精神病と有意な相関があることが指摘されており[3]，近年同抗体の認識抗原がneuronal surface P antigen（NSAP）と名づけられた神経細胞表面に存在する蛋白であることが証明された[4]．一方，SLE患者に存在する抗二本鎖DNA抗体がN-methyl-D-aspartate（NMDA）型抗グルタミン酸受容体の一部（NR2AおよびNR2B）と交差反応性を示し，さらにはアポトーシス経路を介して神経細胞死をもたらすとする報告もある[5]．この報告に基づき，SLE患者血清中の抗NR2A抗体の存在とうつ症状および短期記憶障害との関連性を指摘する報告や，NMDAレセプターに対する抗体を発現するマウスを作成し，lipopolysaccharide（LPS）により血液脳関門を破綻させることで抗体が海馬の神経細胞と結合し，認知機能障害の原因となる神経細胞死に至ったとする報告もある[6]．更にこの報告ではLPSを投与する以前にNMDAレセプターのアンタゴニストであるmemantineを加えることで神経細胞障害を防ぐことができたとして，今後の新たな治療法としての可能性を指摘している．しかし，これらの報告

きむら　あきお，いぬづか　たかし：岐阜大学大学院神経内科・老年学分野

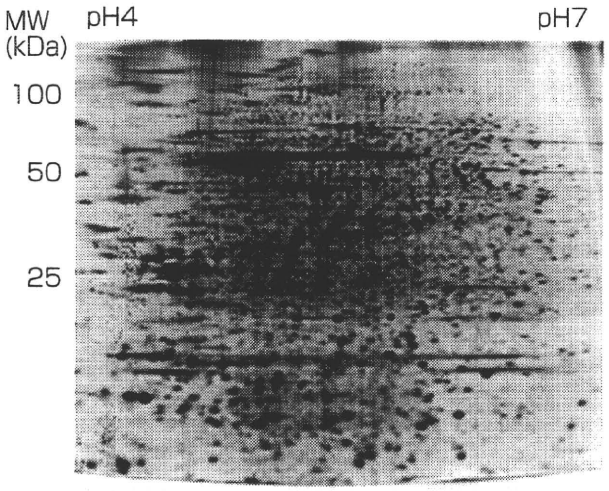

図1. ラット大脳ホモジネートをサンプルとした二次元電気泳動後のゲル画像
蛍光色素（SYPRO® Ruby : Invitrogen）により染色した全蛋白スポット画像を示す

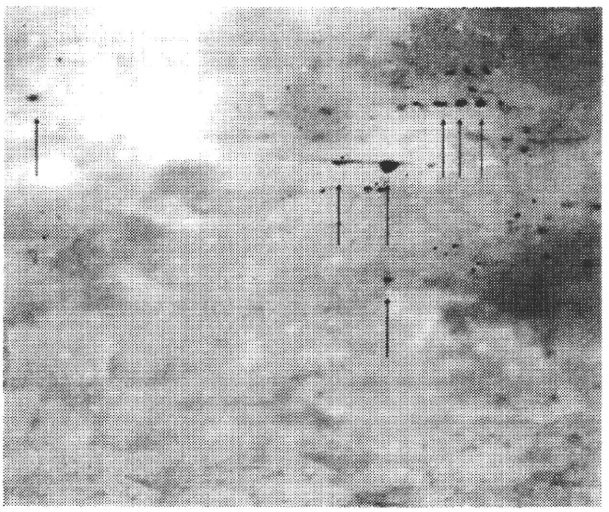

図2. 二次元免疫ブロット後のPVDFメンブレン画像
200倍に希釈した辺縁系脳炎患者の髄液を用い，二次抗体として2,000倍希釈の抗ヒトIgGAM抗体を用いて，抗原抗体反応を施行した．矢印に抗体反応スポットを示す．

も含めNPSLEに特異的といえる自己抗体は未だ確立していない．その理由の一つとしてNPSLE患者にみられる精神神経症状は多彩であることがあげられる．アメリカリウマチ学会（ACR）は1999年に，SLEの精神神経病変を議論するための共通の基盤を作るために，SLEの精神神経症状の新たな分類を提唱した．この分類では，19にわたる精神神経症状を大きく中枢神経病変と末梢神経病変に分け，さらに前者を「神経症状」（neurologic syndromes）と「精神症状」（diffuse psychiatric/neuropshychological syndromes）に大別している．このようなNPSLE患者に合併する多彩な神経障害の病態背景には，多様な機序が存在することが推測され，関与しうる自己抗体も多種類存在する可能性が予想される．今後，NPSLE患者において検出された自己抗体の特異性を議論するにあたり，ACRの分類に基づいた精神神経症状との関連性を詳細に検討する必要があると考えられる．

Sjögren症候群に関する報告では，以前より感覚優位の末梢神経障害の患者で脊髄後根神経節細胞に対する自己抗体を検出したとする報告がある[2]．また，四肢筋力低下・小脳失調を呈した症例で脊髄運動ニューロン・大脳皮質ニューロン・小脳Purkinje細胞に免疫反応性を有する約34 kDaの抗神経抗体を検出したとする報告[7]や，下位運動ニューロン徴候を呈した症例で大脳もしくは脊髄に存在する約50 kDaの蛋白に対する抗神経抗体を検出したとする報告などがある[8,9]．しかし，これらの報告はいずれも症例報告にとどまり，検出された抗神経抗体の病的意義は不明である．今後の症例の蓄積と抗原蛋白の同定ならびに特異性の検討が重要であると思われる．

2. 抗神経抗体の検索

我々は抗神経抗体の検出方法として，二次元電気泳動後にウエスタンブロットを施行する二次元免疫ブロット法を用いている．二次元電気泳動とは一次元目として固定化pH勾配ストリップゲルを使用した等電点電気泳動を行い，さらに二次元目として等電点電気泳動終了後のストリップゲルをポリアクリルアミドゲルにのせて，sodium dodecyl sulfate-polyacrylamide gel electrophoresis（SDS-PAGE）を行うものである．二次元電気泳動を行うことでサンプル中に含まれ

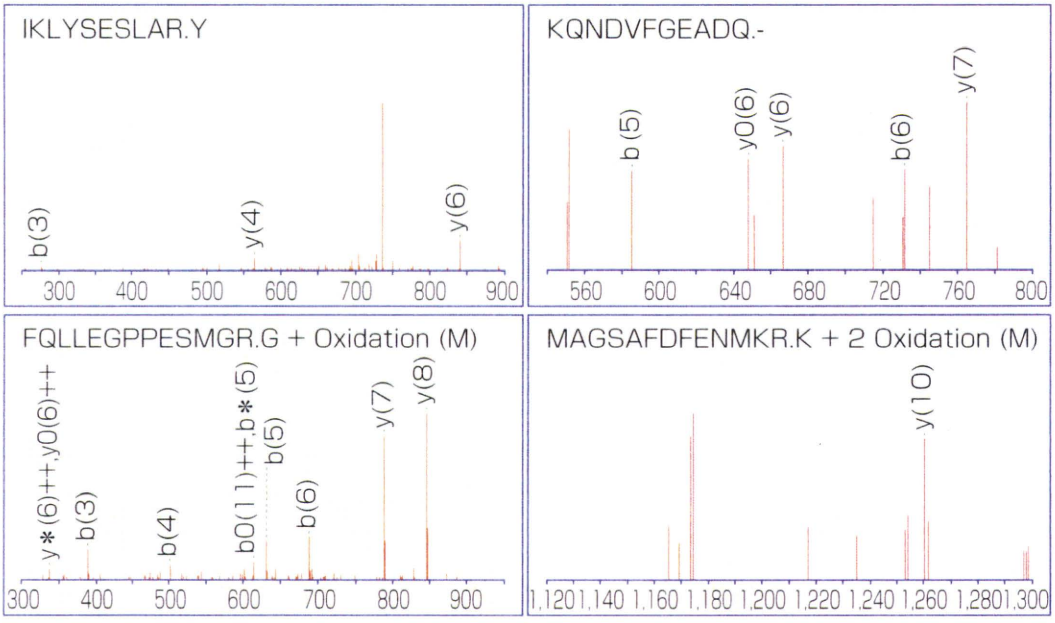

図 3. 質量分析の結果得られたペプチドの MS/MS スペクトルとアミノ酸配列
αGDI の 4 つのペプチドに相当するスペクトルが検出された.

る蛋白質は個々のもつ電荷と分子量の違いによりゲル上に多数のスポットとして展開される. 我々の施設では抗神経抗体の検出のためラット大脳ホモジネートを抗原サンプルとして用いているが, この二次元電気泳動によりラット大脳サンプル中に含まれる最大約 1,400 個の蛋白スポットを再現性よく分離することが可能である (図 1). この方法の長所として抗原となる蛋白質の糖鎖などの側鎖構造がそのまま保たれる点があげられる. 一方, 短所としてSDS-PAGEにより蛋白質の立体構造が失われること, 高分子量蛋白や塩基性蛋白の分離が比較的困難であることがあげられる. 次に二次元電気泳動終了後のポリアクリルアミドゲル内の蛋白をpolyvinylidene difluoride (PVDF) メンブレンにブロットし, ブロット後のメンブレンを用いて抗原抗体反応を行うことにより網羅的に患者血清・髄液中に存在する抗神経抗体を検出している (図 2).

3. 抗神経抗体認識抗原蛋白の同定の実際

初めにラット大脳サンプルを同時に 2 枚のゲルを用いて二次元電気泳動で展開し, 蛍光色素を用いて全蛋白染色する. 一枚のゲルはスポット切り出し用に保存し, 他方のゲルはスキャナーで全蛋白染色後の画像を取りこんだ後, 免疫ブロットを行う. この時, 抗原抗体反応を施行する前のメンブレンの全蛋白染色画像 (全蛋白染色したゲル上の蛋白は色素を保持したままメンブレンに移行する) を画像に取り込み, さらに抗原抗体反応施行後に検出試薬を用いて抗体反応スポットを検出し画像として別に保存する. 最後に抗体反応スポットと抗原抗体反応施行前のメンブレンおよびゲル上の全蛋白染色スポットを画像解析ソフトを用いてマッチングする. 次に一致したスポットにつきin gel消化を行い, 液体クロマトグラフィー (liquid chromatography : LC) とタンデム質量分析 (tandem mass spectrometry : MS/MS) 装置を組み合わせたLC-MS/MSシステムによりペプチドのアミノ酸配列を解析し (図 3), さらにそこから得られたデータを, 検索サーバーを介しデータベース検索により抗神経抗体の認識抗原蛋白を同定している (図 4).

Protein View
Match to : GDIA_RAT Score : 130
Rab GDP dissociation inhibitor alpha - Rattus norvegicus (Rat)
Nominal mass (Mr) : 51074 ; Calculated pI value : 5.00
NCBI BLAST search of GDIA_RAT against nr
Unformatted sequence string for pasting into other applications
Taxonomy : Rattus norvegicus
Fixed modifications : Carbamidomethyl(C)
Variable modifications : Carbamyl (N-term), Oxidation (M)
Cleavage by Trypsin : cuts C-term side of KR unless next residue is P
Sequence Coverage : 10%
Matched peptides shown in Bold Red
1 MDEEYDVIVL GTGLTECILS GIMSVNGKKV LHMDRNPYYG GESSSITPLE
51 ELYKRFQLLE GPPESMGRGR DWNVDLIPKF LMANGQLVKM LLYTEVTRYL
101 DFKVVEGSFV YKGGKIYKVP STETEALASN LMGMFEKRRF RKFLVFVANF
151 DENDPKTFEG VDPQTTSMRD VYRKFDLGQD VIDFTGHALA LYRTDDYLDQ
201 PCLETINRIK LYSESLARYG KSPYLYPLYG LGELPQGFAR LSAIYGGTYM
251 LNKPVDDIIM ENGKVVGVKS EGEVARCKQL ICDPSYIPDR VRKAGQVIRI
301 ICILSHPIKN TNDANSCQII IPQNQVNRKS DIYVCMISYA HNVAAQGKYI
351 AIASTTVETA EPEKEVEPAL ELLEPIDQKF VAISDLYEPI DDGSESQVFC
401 SCSYDATTHF ETTCNDIKDI YKRMAGSAFD FENMKRKQND VFGEADQ

図4. 検索サーバーを利用した蛋白質の同定
Mascot Search Serverに図3の質量分析データを送ってデータベース検索をした結果αGDIが同定された.

4. NPSLE患者と抗神経抗体

我々は，これまで上記プロテオミクス解析の手法を用いてNPSLE患者の血清中よりいくつかの抗神経抗体を同定し報告した[10]．二次元免疫ブロットにより12例の健常者血清に反応せず7例のNPSLE患者の血清にのみ反応した9つのスポットを選択し，このうちの7つのスポットから6つの抗原蛋白を同定した．これらはstress-70 protein, Rab GDP dissociation inhibitor alpha（αGDI），isocitrate dehydrogenase［NAD］subunit alpha, L-lactate dehydrogenase B chain, F-actin-capping protein subunit alpha-2, Rab GDP dissociation inhibitor beta（GDI-2）であった．これらの抗原蛋白のうちαGDIは，神経シナプスに局在し小胞輸送に必要なG蛋白Rab3aのリサイクリングに関与することが知られている．さらにαGDIをコードするGDI-1遺伝子は，精神発達遅滞を唯一の臨床症状とする非特異的X連鎖精神遅滞の原因遺伝子の1つであり，そのノックアウトマウスでは，短期記憶障害，攻撃性の低下，社会行動の変化を示すことが知られている[11]．これらの報告を踏まえ抗αGDI抗体が神経シナプスにおける伝達障害に関与する可能性を考慮し，同抗体の特異性につき検討した．

5. NPSLEと抗αGDI抗体

ヒトαGDIリコンビナント蛋白を用い，NPSLE患者および対照の血清を一次抗体として一次元免疫ブロットを施行した．検索対象はNPSLE患者18例，精神神経症状を合併しないSLE患者19例，多発性硬化症患者12例，感染性髄膜脳炎患者13例，多発性ニューロパチー患者10例，統合失調症患者5例，躁うつ病患者5例，健常者12例であった．結果，NPSLE患者4例と神経症状を合併しないSLE患者1例の計5例で同抗体が陽性でありその他の患者および健常者では全て陰性であった．抗体陽性であった4例のNPSLE患者は臨床的に，ACRの精神神経症状の分類に基づくpsychosisを合併したため，NPSLE全患者18例をpsychosisの有無で2つに分類し改めて抗