CME Autoantibodies to NMDA receptor in patients with chronic forms of epilepsia partialis continua

Y. Takahashi, PhD; H. Mori, PhD; M. Mishina, PhD; M. Watanabe, PhD; T. Fujiwara, PhD; J. Shimomura, PhD; H. Aiba, MD; T. Miyajima, PhD; Y. Saito, MD; A. Nezu, PhD; H. Nishida, PhD; K. Imai, MD; N. Sakaguchi; and N. Kondo, PhD

Abstract—*Background:* Antibody-mediated and cytotoxic T cell-mediated pathogenicity have been implicated as the autoimmune pathophysiologic mechanisms in Rasmussen's encephalitis. *Methods:* The authors investigated autoantibodies against the NMDA glutamate receptor (GluR) ε2 subunit and their epitopes in serum and CSF samples from 15 patients with chronic epilepsia partialis continua (EPC), 17 with West syndrome, 10 with Lennox–Gastaut syndrome, and 11 control subjects. *Results:* In 15 patients with chronic EPC, we detected NMDA-type GluR ε2 autoantibodies in histologically proven Rasmussen's encephalitis (3/3 patients), clinical Rasmussen's encephalitis (6/7 patients), acute encephalitis/encephalopathy (2/3 patients), and nonprogressive EPC (2/2 patients). Serum IgM autoantibodies were found in the early phase of EPC and became negative later in four patients. The autoantibodies were not detected in West syndrome, Lennox–Gastaut syndrome, or controls. Among 10 patients with histologically proven or clinical Rasmussen's encephalitis, epitope analyses showed that the autoantibodies were predominantly against C-terminal epitopes and rarely against N-terminal epitope, with inconsistency in profile during the courses of disease. Epitope recognition spectrum of autoantibodies was broader in CSF than in serum, and the serum or CSF profile showed an increase in number of epitopes as disease progressed in some patients. *Conclusions:* The presence of autoantibodies against NMDA GluR ε2 suggests autoimmune pathologic mechanisms but is not a hallmark of Rasmussen's encephalitis. Patients with Rasmussen's encephalitis may have autoantibodies against several neural molecules, and these autoantibodies may be produced in the CNS after cytotoxic T cell-mediated neuronal damage.

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Epilepsia partialis continua (EPC) is characterized by continuous myoclonic jerks of the extremities, face, etc., usually without impairment of consciousness. The clinical evolution of EPC is variable, depending on the patient's age and underlying brain diseases.[1,2] EPC was classified into two groups: a group with stable neurologic deficit, and another group with slowly progressive neurologic deficit.[3] The latter cases are classified as chronic progressive EPC of childhood in the revised classification of epileptic syndromes,[4] which may be diagnosed as Rasmussen's encephalitis[5-7] upon histologic confirmation. Histologic examination revealed infiltration of T lymphocytes and microglia cells, astrocytosis, and neuronal loss.[6,8,9] As glutamate receptor 3 (GluR3) was reported as an autoantigen in Rasmussen's encepha-

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From the Department of Pediatrics (Drs. Takahashi and Kondo, N. Sakaguchi), Gifu University School of Medicine, and Department of Neurology (Dr. Nishida), Gifu Prefectural Gifu Hospital; Department of Molecular Neurobiology and Pharmacology (Drs. Mori and Mishina), Graduate School of Medicine, University of Tokyo, and Department of Pediatrics (Dr. Saito), Tokyo Women's Medical University; Department of Anatomy (Dr. Watanabe), Hokkaido University School of Medicine; National Epilepsy Center (Drs. Takahashi, Shimomura, and Fujiwara), Shizuoka MIND; Department of Neurology (Dr. Aiba), Shizuoka Children's Hospital; Department of Pediatrics (Dr. Miyajima), Shiga Medical Center for Children; Department of Pediatrics (Dr. Nezu), Urafune Hospital, Yokohama City University School of Medicine; and Department of Pediatrics (Dr. Imai), Faculty of Medicine, Osaka University, Japan.

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Address correspondence and reprint requests to Dr. Y. Takahashi, National Epilepsy Center, Shizuoka MIND, 886 Urushiyama, Shizuoka 420-8688, Japan; e-mail: takahashi-ped@umin.ac.jp

Table 1 *Clinical characteristics of patients*

Patient no.	Onset age		Sex	Causative or underlying factors	Seizure type	Outcome			
	Epilepsy	EPC				Neurologic	Mental	Seizure	At age, y
1	2 d	1.1 y	M	DPT vaccination, status	CPS, EPC	QP	MR+++	Daily	4.5
2	2 mo	7 mo	M	Viral meningitis	CPS, EPC	QP	MR+++	Daily	4
3	2 mo	4 mo	M	Polio vaccination, status	CPS, EPC	QP	MR+++	Disappeared	8
4	3.6 mo	3.7 mo	M	Infection	CPS, EPC	HP	MR+	Daily	4.3
5	3.8 y	3.8 y	M	Head trauma	EPC, CPS	HP	MR+	Daily	9
6	3.8 y	3.8 y	F	Influenza, influenza vaccination	CPS, EPC	QP	MR+	Daily	5.3
7	5.2 y	6.1 y	F	Status	CPS, EPC	HP, HA	Normal	Daily	6.6
8	5.9 y	5.9 y	F	Infection	EPC, SPS	Normal	Normal	Monthly	7
9	6.1 y	6.9 y	M	—	EPC, CPS	HP	MR+	Daily	10 y
10	8.9 y	9.5 y	F	Infection, status	CPS, EPC	HP	MR++	Daily	15 y
11	9 y	20 y	M	Alcohol	CPS, EPC	Normal	Normal	Daily	25 y
12	9.1 y	9.1 y	M	IgA deficiency, encephalitis	CPS, EPC	QP	MR+++	Daily	10 y
13	11.3 y	11.3 y	F	Encephalitis	EPC	Normal	MR+	Disappeared	16 y
14	12.5 y	12.9 y	M	—	CPS, EPC	QP	MR++	Weekly	20 y
15	23 y	31 y	F	—	EPC, CPS	Normal	MR+	Daily	38 y

EPC = epilepsia partialis continua; DPT = diphtheria–pertussis–tetanus; status = status epilepticus; CPS = complex partial seizure; SPS = simple partial seizure; QP = quadriplegia; HP = hemiplegia; HA = hemianopsia; MR = mental retardation.

litis,[10] epilepsies causally related to autoantibodies are highlighted.

The ionotropic GluRs are subdivided into three major subtypes: NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate, and kainate receptors. The NMDA GluR are heterodimers composed of GluR ε (ε1 to ε4) (NMDAR2A to 2D) and GluR ζ1 (NMDAR1) subunits.[11] GluR ε2 is the essential molecule for synaptic plasticity and development.[12] In Rasmussen's encephalitis, not only epileptic seizures but also impairment of higher brain functions (e.g., mental retardation and visual disturbance) occur frequently. Therefore, we examined autoantibodies against NMDA GluR ε2 and their epitopes in sera and CSF in patients with chronic forms of EPC and Rasmussen's encephalitis to estimate the immunologic aspects in the impairment of higher brain functions. We could not examine autoantibodies against the GluR3 subunit because its cDNA was unavailable.

Patients and methods. *Patients.* We examined the serum and CSF samples of patients with chronic EPC (Patients 1 to 15)[13,14] (table 1; also see the supplementary material on the *Neurology* Web site; go to www.neurology.org), West syndrome (17 patients), and Lennox–Gastaut syndrome (10 patients). Fifteen patients with chronic EPC manifested intractable EPC for >1 month, and all except Patients 8 and 11 showed deterioration or neuropsychological impairment. Patients 10 and 11 had negative tests for anti-GluR3 autoantibodies in another hospital. Three patients (Patients 6, 12, and 13) manifested EPC causally related to acute encephalitis/encephalopathy (acute type). Two (Patients 8 and 11) had chronic nonprogressive EPC, good neuropsychological outcome, and normal MRI (nonprogressive type). Three patients (Patients 4, 5, and 7) were histologically diagnosed with Rasmussen's encephalitis (definitive Rasmussen type). Seven patients (Patients 1 to 3, 9, 10, 14, 15) had progressive EPC, neuroimaging findings characteristic of Rasmussen's encephalitis,[15] and neuropsychological impairment (clinical Rasmussen type). In the clinical Rasmussen type, three patients (Patients 1 to 3) with infantile onset manifested quadriplegia shortly after the onset, which was not typical for Rasmussen's encephalitis. Bilateral involvement in patients with Rasmussen's encephalitis was reported in infantile patients[2,16] and adult patients.[16]

Serum samples from 11 healthy children were also examined as controls. Informed consent was obtained from each patient or guardian after oral or written explanation according to the ethical principles of the Declaration of Helsinki.

Establishment of stable transformant cells expressing GluR ε2. Using the tetracycline induction system,[17] cDNA of GluR ε2,[18] pSTneoB,[19] and G418 selection,[20] we established stable NIH3T3 transformant cell lines expressing full-length GluR ε2 (B18) and GluR δ2 subunits (see the supplementary material on the *Neurology* Web site). Identification of three clones with integrated cDNA of GluR ε2 was done by PCR (see figure E-1A on the *Neurology* Web site). Western blot was used to confirm the expression of GluR ε2 protein after 48-hour culture with doxycycline (see figure E-1B on the *Neurology* web site).

Detection of autoantibodies against GluR ε2 in sera and CSF. Supernatants of cell extracts from B18 and the control cell line (A1) cultured for 48 hours with doxycycline (1 μg/mL) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the gels were transferred to nitrocellulose membranes. Each membrane was cut into 20 strips after overnight blocking with the blocking buffer (0.02 *M* Tris-HCl, 0.16 *M* NaCl, 0.05% bovine serum albumin). The strips of B18 and A1 were reacted with sera (diluted 20-fold with blocking buffer) or CSF (diluted 15-fold with blocking buffer) for 48 hours at 4 °C and were stained by alkaline phosphatase-labeled second antibodies (IgG, IgA, or IgM; Jackson ImmunoResearch, West Grove, PA). The presence of autoantibodies against GluR ε2 was judged by a positively stained band with molecular size of about 180 kd, which was found only on the B18 strip and not on the A1 strip (figure 1). As B18 cells cultured without doxycycline showed leaked expression of GluR ε2 (see figure E-1B on the *Neurology* Web site), we used A1 (see the supplementary material on the *Neurology* Web site) as control. The presence of autoantibodies against GluR ε2 was recognized usually in patients' sera diluted <400-fold (data not shown). Because the concentration of GluR ε2 cannot be measured in each supernatant of homogenates of B18 cells, we examined the autoantibodies in 20-fold-diluted sera to reduce false–negative results. Data obtained by 48-hour incubation at the first

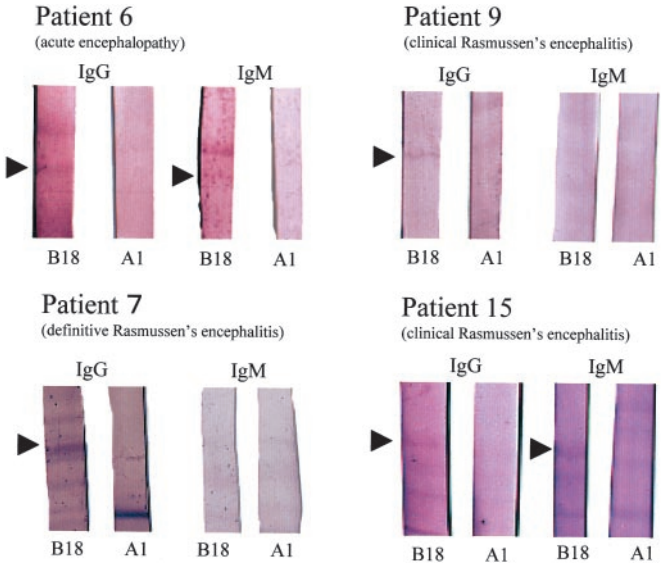

Figure 1. Detection of autoantibodies against whole molecule of NMDA glutamate receptor (GluR) ϵ2 subunit. Data for IgG and IgM autoantibodies are shown. B18 = strip of nitrocellulose membrane containing proteins from supernatants of B18 cell extracts after 48-hour incubation with doxycycline (NIH3T3 cell line, expressing whole GluR ϵ2 proteins); A1 = strip of nitrocellulose membrane containing supernatant of cell extract of A1 after 48-hour incubation with doxycycline (NIH3T3 cell line, expressing only reverse tetracycline transactivator); arrowheads = positive bands of whole molecule of NMDA GluR ϵ2 (about 180 kd), stained by patient's autoantibodies and alkaline phosphatase-labeled second antibodies.

step showed clearer positive bands than those by 24-hour incubation (data not shown).

Preparation of bacterial fusion proteins containing peptides from the GluR ϵ2 subunit. DNA fragments encoding amino acid residues 1 to 48 (an amino-terminal peptide; N), 998 to 1,074 (a carboxyl-terminal peptide; C1), 1,053 to 1,153 (a carboxyl-terminal peptide; C2), and 1,353 to 1,432 (a carboxyl-terminal peptide; C3) were amplified by PCR using appropriate synthetic oligonucleotides. The positions of candidate epitopes were determined considering molecular interactions. The fragments were cloned to the bacterial fusion protein expression plasmid vector pGEX-4T-2 (Pharmacia Biotech AB, Uppsala, Sweden) or pMAL (New England Biolabs, Beverly, MA) for the production of fusion protein with glutathione *S*-transferase (GST) or maltose-binding protein[21] (see figure E-2 on the *Neurology* Web site). The expression plasmids were transformed into *Escherichia coli* (BL21 or TB1), and subsequent induction with isopropyl-β-D-thiogalactopyranoside was performed by routine methods.[22] The transformed *E. coli* were sonicated to obtain supernatants of the bacterial homogenates.

Epitope analyses of autoantibodies against GluR ϵ2 in sera and CSF. Supernatants of bacterial homogenates that contained induced GluR ϵ2 polypeptide fusion proteins synthesized by the above methods were subjected to SDS-PAGE, and the proteins in gels were transferred to nitrocellulose membranes. Each membrane was reacted with serum or CSF (diluted 20- or 15-fold with blocking buffer) for 48 hours at 4 °C and stained by alkaline phosphatase-labeled second antibodies (IgG or IgM). A positive reaction with an epitope was judged by a stained band at the appropriate molecular weight (figure 2).

Results. *Autoantibodies against GluR ϵ2 molecules in sera from patients with chronic EPC.* Autoantibodies against GluR ϵ2 molecule were studied in the sera of 15 patients with chronic EPC (table 2). Thirteen of 15 patients had IgG autoantibodies against GluR ϵ2 molecule during the course of disease, and 8 of 13 patients had both IgG and IgM autoantibodies. None of the patients we examined had IgA autoantibodies against GluR ϵ2. The frequencies of IgG and IgM autoantibodies against GluR ϵ2 in each category of patients were three and one of three in definitive Rasmussen type, six and four of seven in clinical Rasmussen type, two and two of three in acute type, and two and one of two in nonprogressive type, respectively.

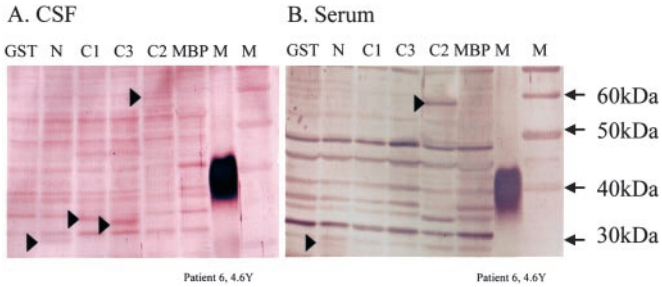

Figure 2. Epitope analysis of autoantibodies against NMDA glutamate receptor (GluR) ϵ2 by immunoblot. (A) Result of epitope analyses using CSF; (B) results using serum from Patient 6. Lane glutathione S*-transferase (GST) = bacterial homogenate transformed by mock GST vectors; lanes N, C1, and C3 = bacterial homogenates containing fusion proteins composed of GST and the corresponding peptide; lane C2 = bacterial homogenate containing fusion proteins composed of maltose-binding protein (MBP) and peptide translated from C2 region; lane MBP = bacterial homogenate transformed by mock MBP vectors; lane M = size marker; triangles = positive bands stained by patient's autoantibodies and alkaline phosphatase-labeled second antibodies. Predicted sizes of positive bands are 30 kd (N), 34 kd (C1), 33 kd (C3), and 58 kd (C2). The large band in lane M is carbonic anhydrase (about 43 kd).*

In Patient 1, autoantibodies against GluR ϵ2 were negative shortly after the onset of epilepsy and evolutionally became positive. In Patient 1, both IgM and IgG autoantibodies were negative at the age of 0.8 years when he started to have catastrophic epilepsy. Then, the autoantibodies appeared at age 0.9 years with persistent catastrophic epilepsy and were followed by the appearance of EPC. Therefore, the early stage of catastrophic epilepsy was associated with absence of autoantibodies against the NMDA GluR ϵ2 subunit. In Patient 5, after IV high-dose γ-globulin infusion therapy (200 mg/kg wt, 5 days), EPC remitted and complex partial seizures decreased to yearly occurrence, accompanied by negative autoantibodies at the age of 5.2 years. At age 7.3 years, EPC recurred and the autoantibodies again became positive. Thus, Patient 5 showed fluctuation of autoantibodies in relation to the severity of clinical symptoms modulated by immunotherapy. Although Patient 3 had no autoantibodies against GluR ϵ2, the test was done at the residual stage[7] when EPC and complex partial seizures had both remitted.

In all four patients who were followed for periods of >2 years (Patients 1, 2, 5, and 9), serum IgM autoantibodies were found in the early phase of EPC and became negative later.

Autoantibodies against cerebellar Purkinje cell-specific GluR δ2 were examined in 11 patients except Patients 4,

Table 2 *Autoantibodies against glutamate receptors*

Patient no.	Age at exam, y	EPC		CPS	Sample	Whole GluR ϵ-2			Epitope analysis			
		Ext	Face			IgG	IgM	IgA	N	C1	C2	C3
1	0.8	−	−	D	Serum	−	−	−	NT	NT	NT	NT
	0.9	−	−	D	Serum	+	+	−	−	−	+	−
	1.3	+	−	D	Serum	+	−	−	+	+	−	+
	2.5	−	−	D	Serum	NT	NT	−	−	−	−	−
	4.1	−	+	D	Serum	+	−	−	−	−	+	−
2	1.1	+	−	D	Serum	+	+	−	−	−	−	−
	1.1	+	−	D	CSF	+	+	−	+	+	+	+
	4.0	+	−	W	Serum	+	−	−	−	−	+	−
3	3.8	−	−	C	Serum	−	−	−	−	−	−	−
4	3.9	+	−	M	Serum	+	−	NT	−	+	+	+
	3.9	+	−	M	CSF	+	−	NT	−	+	+	+
5	3.9	+	−	M	Serum	+	−	−	−	−	−	+
	4.7	+	−	M	Serum	+	+	NT	−	−	−	−
	5.2	−	−	Y	Serum	−	−	NT	−	−	−	−
	7.3	+	−	M	Serum	+	+	NT	−	−	+	−
	8.1	+	+	M	Serum	+	−	NT	−	−	−	−
6	4.6	+	+	D	Serum	+	+	NT	+	−	+	−
	4.6	+	+	D	CSF	NT	NT	NT	+	+	+	+
	4.8	+	+	M	Serum	+	+	NT	−	−	+	−
	4.8	+	+	M	CSF	NT	NT	NT	+	+	+	+
7	6.1	+	−	W	Serum	+	−	NT	−	+	−	+
	6.1	+	−	W	CSF	NT	NT	NT	−	+	+	+
	6.7	+	−	D	Serum	+	−	NT	−	+	−	+
	6.7	+	−	D	CSF	NT	NT	NT	+	+	+	+
8	6	+	−	D	Serum	+	+	NT	−	−	+	−
	6	+	−	D	CSF	NT	NT	NT	+	−	+	+
	6.1	−	−	C	Serum	NT	NT	NT	−	−	+	−
	7.6	+	−	C	Serum	+	−	NT	−	−	+	+
9	7	+	+	C	Serum	+	+	NT	−	−	+	−
	10	−	+	Y	Serum	+	−	NT	NT	NT	NT	NT
10	12	+	−	D	Serum	+	−	NT	−	−	+	−
11	25	−	+	C	Serum	+	−	NT	−	+	+	+
	25	−	+	C	CSF	+	+	NT	+	+	−	+
12	10	+	+	D	Serum	+	+	NT	−	−	+	−
	11	−	−	C	Serum	+	+	NT	−	+	+	+
	11	−	−	C	CSF	NT	NT	NT	+	+	+	+
13	11.5	+	−	C	Serum	−	−	−	NT	NT	NT	NT
14	18.5	−	−	W	Serum	+	−	NT	+	−	+	+
	19	−	−	W	CSF	NT	NT	NT	−	+	−	+
15	38	−	−	D	Serum	+	+	NT	−	+	−	+

Age at exam = age at examination of autoantibodies; EPC = epilepsia partialis continua; Ext = involvement of extremities by EPC; Face = EPC on facial muscles, tongue, or throat; CPS = complex partial seizures; D = daily; W = weekly; M = monthly; Y = yearly; C = controlled; GluR = glutamate receptor; NT = not tested; (+) = presence of autoantibodies; (−) = absence of autoantibodies.

11, 13, and 15, and all the results were negative (data not shown).

No autoantibodies against GluR ϵ2 molecule were detected in the sera of 17 patients with West syndrome, 10 patients with Lennox–Gastaut syndrome, and 11 control subjects.

Autoantibodies against GluR ϵ2 molecules in CSF from patients with chronic EPC. IgG and IgM autoantibodies

against GluR ε2 molecule were positive in 3 and 2 of 3 patients, respectively (see table 2) but were negative in 14 patients with neurologic diseases other than EPC.

Epitope analyses of autoantibodies against GluR ε2 in sera and CSF from patients with chronic EPC. IgG autoantibodies against the putative epitopes (N, C1, C2, and C3) of the GluR ε2 molecule were examined using bacterial fusion proteins containing the corresponding GluR ε2 polypeptides (see figure E-2 on the *Neurology* Web site; also see figure 2). Among 13 patients with IgG autoantibodies against the GluR ε2 molecule, 3 patients had serum autoantibodies to N epitope, 6 had autoantibodies to C1, 11 had autoantibodies to C2, and 9 had autoantibodies to C3 (see table 2). Autoantibodies to C2 epitope predominated, whereas autoantibodies to the extracellular N epitope were rare. The epitope recognition profile was not consistent during the course of disease in almost all patients examined repeatedly (Patients 1, 2, 5 to 8, 12). The CSF of Patients 2, 6, 7, and 12 reacted with all epitopes (N, C1, C2, and C3); CSF of Patients 4, 8, and 11 reacted with three epitopes (except C1); and CSF of Patient 14 reacted with two epitopes (C1 and C3). The spectra of epitope recognition of the autoantibodies were broader in the CSF than in the serum in almost all patients, judging from simultaneously collected samples.

In Patients 2, 7, 8, and 12, IgG autoantibodies in sera and/or CSF reacted with increasing numbers of epitopes as disease progressed. In Patient 1, the number of epitopes increased from one to three, accompanying onset of EPC, and decreased with remission of EPC, whereas the numbers of epitopes showed no temporal increase in Patients 5 and 6.

Discussion. Autoantibodies against whole NMDA GluR ε2 molecule in the serum were detected in 13 of 15 patients with chronic EPC and not in patients with West syndrome, Lennox–Gastaut syndrome, or controls. In patients with chronic EPC, three of three definitive Rasmussen-type patients, six of seven clinical Rasmussen-type patients, two of two nonprogressive-type patients, and two of three acute-type patients had autoantibodies against whole NMDA GluR ε2. The IgM autoantibodies changed from positive to negative in definitive or clinical Rasmussen-type patients. The autoantibodies against NMDA GluR ε2 fluctuated in accordance with disease severity in some patients. We detected no autoantibodies against GluR δ2 in sera at the same dilution as autoantibodies against GluR ε2 in patients with chronic EPC. These data suggest that autoantibodies against NMDA GluR ε2 might be causally related to intractable partial seizures, especially EPC, not to generalized seizures in patients with West syndrome or Lennox–Gastaut syndrome. The presence of autoantibodies against NMDA GluR ε2 might suggest the involvement of autoimmune pathologic mechanisms but is not a hallmark of Rasmussen's encephalitis.

Other than autoantibodies against GluR3 and GluR ε2, autoantibodies against neuronal acetylcholine receptor α_7 subunit[23] and munc-18[24] have been also reported in patients with Rasmussen's encephalitis. Patient 10 in our study had a negative test for anti-GluR3 autoantibodies in another hospital but was found to have autoantibodies against GluR ε2. Furthermore, autoantibodies against GluR3 were found in only 25% of patients with Rasmussen's encephalitis in a recent report[25] and were detected also in neurologic diseases other than Rasmussen's encephalitis[25-27] and controls.[25] We speculate that Rasmussen's encephalitis is not associated with specific autoantibodies, and various autoantibodies against neural molecules might contribute to the pathologic process. Although we could not examine autoantibodies against GluR3, patients with Rasmussen's encephalitis might have several kinds of autoantibodies against neural molecules, including GluR3 and GluR ε2.

In our patients with definitive and clinical Rasmussen's encephalitis, the spectra of epitope recognition of the autoantibodies against GluR ε2 were broader in the CSF than in the serum, judging from simultaneously collected samples. Furthermore, our examination of the autoantibodies was done almost at the same dilution in sera (20-fold) and CSF (15-fold), but the prevalence of the autoantibodies in CSF was as same as in sera. This may suggest relatively higher concentration of the autoantibodies in CSF compared with ordinary infectious diseases. Thus, our data might suggest that serum autoantibodies against GluR ε2 are not generated in peripheral circulation by molecular mimicry following infection but are generated in the CNS.

In Patients 2, 7, 8, and 12, the epitopes recognized by IgG autoantibodies increased with disease progression in sera and/or CSF, and in Patient 1, the number of epitopes increased from one to three, accompanying onset of EPC, and decreased with remission of EPC. These phenomena might suggest "epitope spreading,"[28] which is the mechanism of autoimmune disease mediated by T cells. The predominance of autoantibodies against intracellular C-terminal epitopes of GluR ε2 also suggests the involvement of T cell-mediated autoimmunity in Rasmussen's encephalitis, because T cells, and not B cells, can recognize the cytoplasmic domains of transmembranous molecules.

Mice immunized with the GluR3B peptide produced autoantibodies but exhibited no epilepsy, even by facilitated entry of the autoantibodies into the brain.[29] Furthermore, lymphocytic infiltration of predominantly T cells and sparse B cells were reported in resected tissues of Rasmussen's encephalitis patients,[9] and local CNS immune responses in Rasmussen's encephalitis included local–clonal expansion of T cells responding to discrete antigen epitopes.[30] $CD8^+$ T-cell cytotoxicity was reported to contribute to the pathogenesis of Rasmussen's encephalitis, and neuronal loss resulting from cytotoxic T-cell attack and concomitant release of antigens such as GluR3 were estimated to generate secondary antibody-mediated damage.[31,32] These observations suggest that the primary cause of Rasmussen's encephalitis

is not autoantibodies but T cell-mediated autoimmunity. Also from our data, probable heterogeneity of autoantibodies in Rasmussen's encephalitis, epitope spreading, and predominant intracellular epitopes of autoantibodies against the NMDA GluR ϵ2 suggest an important role of T cell-mediated autoimmunity.

Although the presence of autoantibodies against GluR ϵ2 is not a hallmark of Rasmussen's encephalitis, the autoantibodies are important for the diagnosis of Rasmussen's encephalitis, especially in patients without EPC, which is a typical clinical diagnostic marker of Rasmussen's encephalitis, and suggest the indication of immunosuppressive therapies against cytotoxic T cells.

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