we plan to elucidate the interactions between the redox status of coenzyme Q-10 in plasma and other tissues in ALS animal models to confirm our results obtained.

In summary, we demonstrated a significant increase of plasma %CoQ-10 in sALS patients, suggesting systemic oxidative stress in the pathogenesis of the disease.

Acknowledgments

We thank blood donors and Drs. Kazuyuki Mizushima, Yoshio Ikeda, Yukiko Hashimoto, Takayuki Yanagisawa and Masaki Ikeda for their contributions in collecting clinical materials. We thank Dr. Walter C. Dunlap for his valuable comments in the preparation of this manuscript.

References

[1] Agar J, Durham H. Relevance of oxidative injury in the pathogenesis of motor neuron diseases. Amyotroph Lateral Scler Other Mot Neuron Disord 2003;4:232–42.

[2] Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, et al. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 1993;362:59–62.

[3] Ferrante RJ, Browne SE, Shinobu LA, Bowling AC, Baik MJ, MacGarvey U, et al. Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. J Neurochem 1997;69:2064–74.

[4] Przedborski S, Donaldson D, Jakowec M, Kish SJ, Guttman M, Rosoklija G, et al. Brain superoxide dismutase, catalase and glutathione peroxidase activities in amyotrophic lateral sclerosis. Ann Neurol 1996;39:158–65.

[5] Smith RG, Henry YK, Mattson MP, Appel SH. Presence of 4-hydroxynonenal in cerebrospinal fluid of patients with sporadic amyotrophic lateral sclerosis. Ann Neurol 1998;44:696–9.

[6] Tohgi H, Abe T, Yamazaki K, Murata T, Ishizaki E, Isobe C. Remarkable increase in cerebrospinal fluid 3-nitrotyrosine in patients with sporadic amyotrophic lateral sclerosis. Ann Neurol 1999;46: 129–31.

[7] Oteiza PI, Uchitel OD, Carrasquedo F, Dubrovski AL, Roma JC, Fraga CG. Evaluation of antioxidants, proteins, and lipid oxidation products in blood from sporadic amyotrophic lateral sclerosis patients. Neurochem Res 1997;22:535–9.

[8] Bonnefont-Rousselot D, Lacomblez L, Jaudon M, Lepage S, Salachas F, Bensimon G, et al. Blood oxidative stress in amyotrophic lateral sclerosis. J Neurol Sci 2000;178:57–62.

[9] Bogdanov M, Brown Jr RH, Matson W, Smart R, Hayden D, O'Donnell H, et al. Increased oxidative damage to DNA in ALS patients. Free Radic Biol Med 2000;29:652–8.

[10] Iwasaki Y, Ikeda K, Kinoshita M. Vitamin A and E levels are normal in amyotrophic lateral sclerosis. J Neurol Sci 1995;132:193–4.

[11] Paraskevas GP, Kapaki E, Libitaki G, Zournas C, Segditsa I, Papageorgiou C. Ascorbate in healthy subjects, amyotrophic lateral sclerosis and Alzheimer's disease. Acta Neurol Scand 1997;96:88–90.

[12] Molina JA, de Bustos F, Jimenez-Jimenez FJ, Gomez-Escalonilla C, Garcia-Redondo A, Esteban J, et al. Serum levels of coenzyme Q10 in patients with amyotrophic lateral sclerosis. J Neural Transm 2000;107:1021–6.

[13] Molina JA, de Bustos F, Jimenez-Jimenez FJ, Esteban J, Guerrero-Sola A, Zurdo M, et al. Serum levels of beta-carotene, alpha-carotene, and vitamin A in patients with amyotrophic lateral sclerosis. Acta Neurol Scand 1999;99:315–7.

[14] Cohen O, Kohen R, Lavon E, Abramsky O, Steiner I. Serum Cu/Zn superoxide dismutase activity is reduced in sporadic amyotrophic lateral sclerosis. J Neurol Sci 1996;143:118–20.

[15] Moumen R, Nouvelot A, Duval D, Lechevalier B, Viader F. Plasma superoxide dismutase and glutathione peroxidase activity in sporadic amyotrophic lateral sclerosis. J Neurol Sci 1997;151:35–9.

[16] Yamashita S, Yamamoto Y. Simultaneous detection of ubiquinol and ubiquinone in human plasma as a marker of oxidative stress. Anal Biochem 1997;250:66–73.

[17] Yamamoto Y, Yamashita S, Fujisawa A, Kokura S, Yoshikawa T. Oxidative stress in patients with hepatitis, cirrhosis and hepatoma evaluated by plasma antioxidants. Biochem Biophys Res Commun 1998;247:166–70.

[18] Hara K, Yamashita S, Fujisawa A, Ishiwa S, Ogawa T, Yamamoto Y. Oxidative stress in newborn infants with and without asphyxia as measured by plasma antioxidants and free fatty acid. Biochem Biophys Res Commun 1999;257:244–8.

[19] Sohmiya M, Tanaka M, Tak NW, Yanagisawa M, Tanino Y, Suzuki Y, et al. Redox status of plasma coenzyme Q10 indicates elevated systemic oxidative stress in Parkinson's disease. J Neurol Sci 2004;232:161–6.

[20] Miller RG, Munsat TL, Swash M, Brooks BR. Consensus guidelines for the design and implementation of clinical trials in ALS. J Neurol Sci 1999;169:2–12.

[21] Jablecki CK, Berry C, Leach J. Survival prediction in amyotrophic lateral sclerosis. Muscle Nerve 1989;12:833–41.

[22] Yamamoto Y, Ames BN. Detection of lipid hydroperoxides and hydrogen peroxide at picomole levels by an HPLC and isoluminol chemiluminescence assay. Free Radic Biol Med 1987;3:359–61.

[23] Yamamoto Y, Kawamura M, Tatsuno K, Yamashita S, Niki E, Naito C. Formation of lipid hydroperoxides in the cupric ion-induced oxidation of plasma and low density lipoprotein. In: Davies KJA, editor. Oxidative damage and repair, 1st ed. London: Pergamon Press, 1991. p. 287–91.

A Case of Serogroup A Meningococcal Meningitis: A Case Possibly Imported from China

Key words: meningitis, *Neisseria meningitides*, multilocus sequence typing (MLST), China

A 47-year-old man was hospitalized with high fever, headache, vomiting, and agitated state in March 2003. He had returned home from a brief trip to Beijing, China seven days earlier. Physical examination revealed his peripheral temperature to be 40.4°C, heart rate 105 beats per minute and blood pressure 105/55 mmHg. Purpuric lesions were noticed and his neck was extremely stiff. The laboratory investigations included white-cell count 15,600/μl and C-reactive protein 21.8 mg/dl. The cerebrospinal-fluid (CSF) was turbid yellowish white, and cerebrospinal pressure was more than 400 mmH_2O. The cell count was 1,450 cells per mm^3 with 83% lymphocytes and 17% polymorphonuclear leukocytes. CSF protein was 463 mg/dl, and glucose was 0 mg/dl (blood glucose 224 mg/dl). Under the clinical diagnosis of meningococcal meningitis, aggressive treatment was started with glycerol, steroid, dopamine hydrochloride and gabexate mesilate. At the same time, 8 g per day of ampicillin, 4 g per day of ceftriaxon and gamma globulin were administered. The culture showed *Neisseria meningitides*, proven to be group A by a serological study. His family and all of the contact medical staff took prophylactic ciprofloxacin and remained healthy. His general condition and mental status improved: He could obey simple commands on the fourth hospital day, and his consciousness level was almost clear in a week. He was discharged hospital 6 weeks later.

Neisseria meningitides is classified into serogroups, such as A, B, C, Y, and W-135, by their polysaccharide coat. Most of the isolates in Japan are groups B and Y (1), and we could find only one report on group A (2), which is regarded as highly pathogenic. The more detailed analysis by multilocus sequence typing (MLST) (3) revealed that the strain was Sequence Type 7 (ST-7), which belongs to Subgroup III. ST-7 is one of the virulent clones and has caused large epidemics in Africa (4). Subgroup III strains caused three pandemic waves originating from China; the first in the mid-1960s, the second in the early-1980s, and the last in the late-1990s (5). This one would be imported from Beijing, China, where no epidemic has been reported since 1993.

Recently, few epidemics have occurred in Asia, and Japanese tourists are not vaccinated against meningococcal disease. The development of rapid and cheap transportation has enabled short stays abroad, and increasing numbers of ordinary people may fall ill after they return home, and visit hospitals with infectious diseases that have been rare in their homeland.

Acknowledgements: Special thanks to Yoko Tagawa, who isolated the pathogen and arranged for further investigation. The laboratory research for *Neisseria meningitides* was supported by Yuko Watanabe, Kanagawa Prefectural Public Health Laboratory and by a Grant-in Aid for research from the Japanese Ministry of Health, Labour and Welfare.

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Received for publication November 6, 2004; Accepted for publication May 23, 2005

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References

1) Takahashi H, Kuroki T, Watanabe Y, et al. Characterization of Neisseria meningitidis isolates collected from 1974 to 2003 in Japan by multilocus sequence typing. J Med Microbiol **53**: 657-662, 2004 (Erratum in: J Med Microbiol **53**: 1175, 2004).
2) Nishioka K, Nakanishi Y, Matsubara K, et al. An infantile case of serogroup A meningococcal meningitis markedly improved by panipenem/betamipron. Shonika **37**: 183–186, 1996 (Japanese).
3) Maiden MC, Bygraves JA, Feil E, et al. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci USA **95**: 3140–3145, 1998.
4) Nicolas P, Decousset L, Riglet V, Castelli P, Stor R, Blanchet G. Clonal expansion of sequence type (ST-) 5 and emergence of ST-7 in serogroup A meningococci, Africa. Emerg Infect Dis **7**: 849–854, 2001.
5) Zhu P, van der Ende A, Falush D, et al. Fit genotypes and escape variants of serogroup III Neisseria meningitides during three pandemics of epidemic meningitis. Proc Natl Acad Sci USA **98**: 5234–5239, 2001.

and a micro-ELISA. Clin Diagn Lab Immunol **8**: 174–177, 2001.
6) Moreto M, Barron J. The laparoscopic diagnosis of the liver fascioliasis. Gastrointest Endosc **26**: 147–149, 1980.
7) Uribarrena R, Borda F, Munoz M, Rivero-Puente A. Laparoscopic findings in eight cases of liver fascioliasis. Endoscopy **17**: 137–138, 1985.

Identification of nitrated proteins in the normal rat brain using a proteomics approach

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Background: *The nitration of tyrosine has been suggested to play a role in the pathogenesis of neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD) and Alzheimer's disease (AD).*
Methods: *In the present study, we identified four targets of protein nitration, T-complex polypeptide 1α subunit (TCP-1), neurofilament L (NFL), glial fibrillary acidic protein (GFAP) and clathrin heavy chain (CHC), in the normal rat cortex using a proteomics approach.*
Conclusions: *There have been no reports on these proteins being identified by proteomics as nitrated forms in the brain. For further study, we have to investigate alterations in these nitrated proteins during aging and in neurodegenerative disorders.* [Neurol Res 2005; **27**: 630–633]

Keywords: Clathrin heavy chain; nitrated protein; proteomics; rat brain; T-complex polypeptide 1α subunit

INTRODUCTION

The oxidative alteration of proteins by reactive nitrogen species (RNS) has been implicated in normal aging[1] and age-related neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS)[2,3], Parkinson's disease (PD)[4] and Alzheimer's disease (AD)[5,6]. The nitration of protein may lead to several events such as the loss of specific protein function, abnormal protein clearance, depletion of the cellular redox-balance and interference with the cell cycle, ultimately resulting in neuronal cell death. The chemistry and effects of nitration on protein function have been studied extensively[7,8]. Previous reports suggest a role for protein nitration in the pathogenesis of normal aging and neurodegenerative disorders. However, studies on specific targets of protein nitration are scarce[5,9].

The identification of nitrated proteins in the normal brain provides evidence regarding the importance of RNS-related protein modification in normal as well as pathological conditions. Additionally, it is essential to identify targets of protein nitration in the normal brain before studying alterations in neurodegenerative disorders. In the present study, we determined four specific targets of protein nitration in the normal rat brain using a proteomics approach.

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MATERIALS AND METHODS

Sample preparation

After the whole brain was removed rapidly from a male Wistar rat (24 months old), the cerebral cortex was dissected, frozen immediately in liquid nitrogen and stored at −80°C until use. The tissues were homogenized with 20 volumes (v/w) of lysis buffer [7 M urea, 2 M thiourea, 40 mM dithiothreitol:DTT, 2% CHAPS, 2% Pharmalyte (pH 3–10) and one tablet of Complete mini EDTA (–) in 10 ml] using a teflon homogenizer with 20 strokes on ice. After the residual material had been removed by centrifugation at 16,000 rpm for 20 minutes at 4°C, the supernatant was subjected to first-dimensional isoelectric focusing of 2D-GE. Urea (Plusone) and Pharmalyte were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA), DTT and CHAPS were purchased from Nacalai Tesque (Kyoto, Japan), thiourea was purchased from Wako Pure Chemical Industries (Osaka, Japan), and Complete mini EDTA (–) was purchased from Roche Diagnostics (Mannheim, Germany).

Two-dimensional gel electrophoresis (2D-GE)

For first-dimensional isoelectric focusing, 125 μl of the sample was applied to rehydrated immobilized pH gradient gel (IPG gel, Amersham Pharmacia, pH 4-7 linear gradient, 180 mm) and for the second-dimensional gel electrophoresis (SDS–PAGE), IPG gels after the isoelectric focusing were equilibrated in a solution consisting of 7 M urea, 25% glycerol, 50 mM Tris–HCl (pH 6.8), 2% SDS and 33 mM DTT with 0.001% bromophenol blue for 30 minutes. Second-dimensional electrophoresis was

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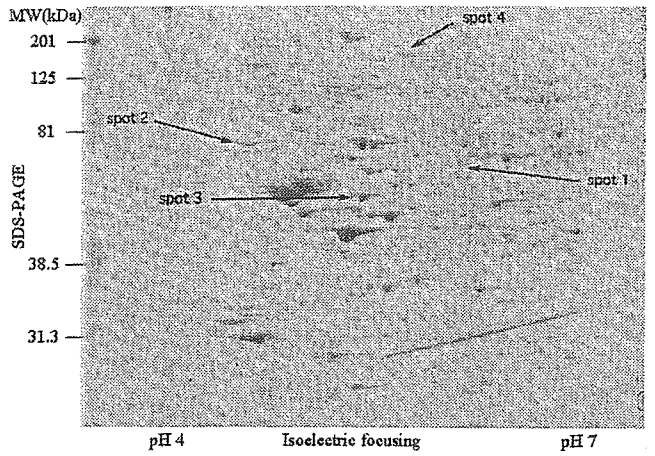

Figure 1: 2D-GE profiles of proteins from the rat cerebral cortex (20 × 20 cm, pH 4–7) After second-dimensional SDS–PAGE, the gel was stained with Coomassie Brilliant Blue

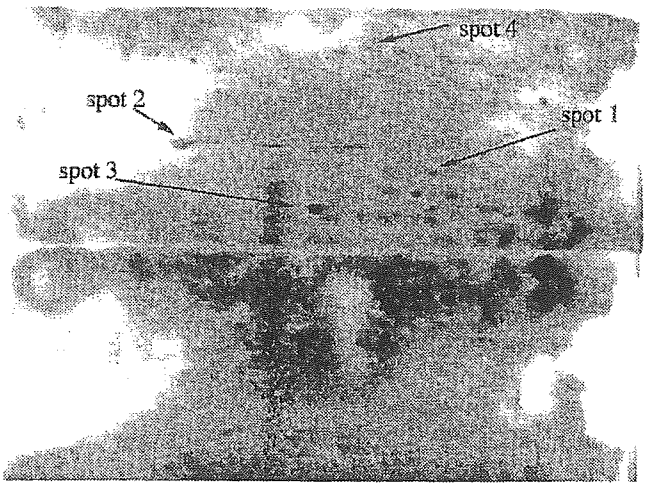

Figure 2: Western blotting of proteins from 2D-GE of the rat cortex extract. After second dimensional SDS–PAGE, the gel was blotted on a PVDF membrane and stained with anti-nitrotyrosine antibody for chemiluminescence visualization

performed with laboratory-cast SDS gel (10% T and 3% C, 20 × 20 cm with 1 mm thickness) with running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS) at 25 mA for 15 minutes followed by 35 mA for 5 h.

Western blot analysis

After 2D-GE, proteins were transferred to a polyvinylidene difluoide (PVDF) membrane. Following blocking in skimmed milk, the membrane was incubated with rabbit anti-nitrotyrosine polyclonal antibody (Upstate Cell Signaling Solutions, NY, USA) diluted to 0.5 µg/ml overnight at 4°C. Then, the membranes were washed with phosphate buffer saline and 0.1% Tween 20 (PBS-T). A secondary antibody solution (donkey anti-rabbit antibody conjugated with horseradish peroxidase, Jackson ImmunoResearch, PA, US), diluted to 1:10,000, was then added to the membranes and they were kept for 1 hour at room temperature. After washing with PBS-T, the blots were visualized using the chemiluminescence reagent ECL-PLUS (Amersham Pharmacia).

In-gel digestion of proteins for internal amino acid sequencing

Digestion of protein with peptidase was carried out as follows[10]. A piece of the gel with a spot stained with Coomassie Brilliant Blue was cut out and equilibrated with 1 ml of 50% acetonitrile for 15 minutes followed twice with 1 ml of 100 mM Tris–HCl (pH 9) for 15 minutes. Gel pieces were then placed in a 1.5 ml sampling tube containing 50 µl of 100 mM Tris–HCl (pH 9) and 0.1 µg lysylendpeptidase AP-1 (Lys-C, Wako Pure Chemical Industries, Osaka, Japan) and manually homogenized with a plastic pestle. The gel suspension was added to 50 µl of the same buffer and incubated at 37°C overnight with vigorous shaking. It was next centrifuged at 16,000 rpm for 10 minutes and the supernatant was saved. The precipitate was suspended in 100 µl of 100 mM Tris–HCl (pH 9) and the solution was shaken for 20 minutes at 37°C. Following this, the suspension was centrifuged at 16,000 rpm for 10 minutes and the supernatant was saved. The precipitate was then suspended in 100 µl of 60% acetonitrile–0.1% TFA and shaken for 20 minutes at 37°C and the suspension was centrifuged at 16,000 rpm for 10 minutes and the supernatant was saved. The precipitate was suspended in 100 µl of 60% acetonitrile–0.1% TFA containing 0.01% Tween 20, again shaken for 20 minutes at 37°C and the supernatant after centrifugation was obtained. All saved supernatant solutions were combined and centrifuged again to remove any fine pieces of gel, and the supernatant was reduced in volume by vacuum concentrator for application to HPLC.

Peptides separation by HPLC

Peptides from each spot were purified with a reversed phase HPLC column [R-300 (C8), 1 × 100 mm] using a model 172 HPLC apparatus (Applied Biosystems, Foster City, CA, USA). Elutions were carried out at equilibration with 0.1% TFA (solvent A) for 20 minutes followed by a linear grandient from solvent A to 70% acetonitrile containing 0.1% TFA (solvent B) at 50 µl/minutes in 45 minutes.

Peptide sequencing and database searching

Purified peptides were sequenced with a pulse-liquid phase protein sequencer Model 492 cLC (Applied Biosystems)[11]. The obtained amino acid sequence data was then searched in the Swiss-Prot protein database.

RESULTS

2D-GE profiles and western blotting of the normal rat cerebral cortex

To observe the protein profiles of the normal rat cerebral cortex, 2D-GE was carried out over the range of pH 4–7, to cover most proteins of the brain, with 18 cm of IPG gel for the first-dimensional isoelectric

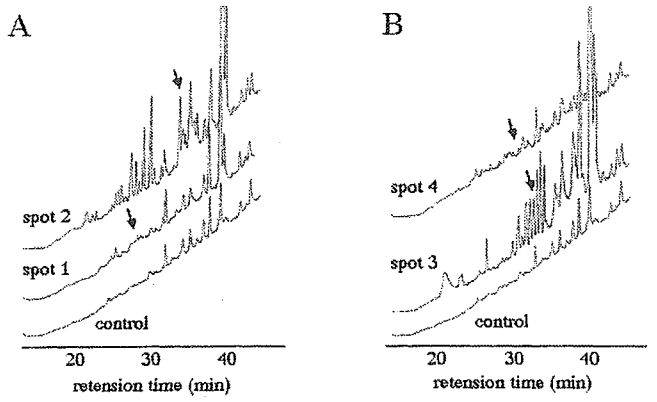

Figure 3: HPLC profiles of peptides released by the *in situ* protease (Lys-C) digestion of protein spots 1, 2, 3 and 4 (A and B). Proteins on each spot were digested with Lys-C. Extracted peptides were purified with a reverse-phase column of C8 (Aquapore RP-300, 1 × 100 mm). Gradient elution was carried out with 0–70% acetonitrile in 0.1% TFA and the elution profile was monitored at an absorbance of 215 nm. A control experiment (lowest chart) was carried out with gel pieces containing no protein. Peaks marked with an arrow were subjected to amino acid sequence analysis

focusing followed by the second-dimensional SDS–PAGE (20 × 20 cm). Protein profiles obtained by Coomassie Brilliant Blue staining are shown in *Figure 1*. Western blot analysis and subsequent immunochemical detection of nitrotyrosine was then performed (*Figure 2*). Many protein spots could be detected with anti-nitrotyrosine antibody in the smeared profiles and several spots could be matched to spots on the Coomassie Brilliant Blue stained gel by comparing both gel profiles. Among them, four proteins designated as spots 1, 2, 3 and 4 were then identified.

Protein analyses

To identify the proteins corresponding to spots 1, 2, 3 and 4, they were subjected to *in situ* proteolytic cleavage with endopeptidase Lys-C and separated by HPLC (*Figure 3A* and *B*). The peptide peaks marked with arrows on the HPLC profiles were then analysed for their amino acid sequences and the sequenced data is shown in *Table 1*. The obtained amino acid sequences were used to search in the database Swiss-Prot, with NCBI BLAST site (http://www.ncbi.nlm.nih.gov/BLAST/.) Spots 1, 2, 3 and 4 were identified as T-complex polypeptide 1α subunit (TCP-1), neurofilament L (NFL), glial fibrillary acidic protein (GFAP) and clathrin heavy chain (CHC).

Table 1: Amino acid sequences of peptides obtained by HPLC shown in *Figure 3*

Sample	AA sequence												
Spot 1	Q	A	(G)	V	F	E	P	T	I	V	K		
Spot 2	H	S	E	P	S	R	F	R	A	L	Y	E	
Spot 3	E	T	R	A	S	E	R	A	E	M	(M)	(E)	(L)
Spot 4	(A)	(H)	T	M	T	(D)	(D)	V	(T)	F			

DISCUSSION

Several studies have reported on protein nitration of the brain and the spinal cord in neurodegenerative disorders such as ALS[2,3], PD[4] and AD[5,6], although studies on specific targets of protein nitration are scarce[5,9]. In the present study, we identified four targets of protein nitration in the normal rat brain by a proteomics approach.

Previous studies showed the existence of nitrated proteins using immunohistochemistry and immunoblotting techniques. The present study applied a proteomics approach to identify nitrated proteins. There is only one other report on the identification of nitrated proteins involving a proteomics approach[5] and no studies have reported TCP-1 and CHC as nitrated forms in any organs.

The four targets of protein nitration identified in the present study were TCP-1, NFL, GFAP and CHC. Each plays a role as a cytoskeletal protein in the central nervous system (GFAP and NFL), a chaperon necessary for protein folding (TCP-1) or as a vesicular coating protein that mediates endocytosis (CHC). NFL and GFAP are neuronal- and glial-specific intermediate filaments, respectively. TCP-1 is a selective molecular chaperon for tubulin and actin biogenesis active during protein folding[12] and neurons rely on clathrin-mediated endocytosis to retrieve synaptic vesicles from presynaptic compartments after the release of neurotransmitters[13].

NFL is susceptible to nitration because of its large molar ratio of tyrosine and it accumulates in its nitrated form in the hippocampus of AD[14] and the spinal cord of ALS[15]. Although the role of intraneuronal neurofilamentous aggregates in the pathogenesis of ALS is unknown, their presence is an important neuropathological hallmark of the disease process. NFL and GFAP occur as nitrated forms in the spinal cord of an ALS model mouse[2]. No reports, however, have examined these proteins regarding their occurrence as nitrated forms in the brain. Furthermore, CHC and TCP-1 have not been reported to occur in their nitrated form in any organs thus far. Abnormal nitration of TCP-1 leads to the misfolding of β 1 tubulin molecules that cause their pathological aggregation. Nitration stress also affects intracellular vesicular trafficking via the alteration of clathrin-dependent vesicular trafficking, resulting in the disturbed release of neurotransmitters in the brain. If these proteins susceptible to nitration play roles in mechanisms responsible for normal aging and neurodegenerative disease, antioxidants such as NOS (nitric oxide synthase) inhibitors may prevent neuronal cell death under these conditions.

The search for mechanisms that induce the nitration of these proteins during aging and neurodegenerative disorders will be helpful in clarifying the pathology of these conditions. We still have to investigate alterations in these nitrated proteins during aging and in neurodegenerative disorders.

CONCLUSION

We identified four targets of protein nitration: TCP-1, NFL, GFAP and CHC via a proteomics approach in the

normal rat brain. TCP-1 and CHC have not been reported in their nitrated form in the CNS until now.

REFERENCES

1 Shin CM, Chung YH, Kim MJ, *et al.* Age-related changes in the distribution of nitrotyrosine in the cerebral cortex and hippocampus of rats. *Brain Res* 2002; **931**: 194–199

2 Bruijn LI, Beal MF, Becher MW, *et al.* Elevated free nitrotyrosine levels, but not protein-bound nitrotyrosine or hydroxyl radicals, throughout amyotrophic lateral sclerosis (ALS)-like disease implicate tyrosine nitration as an aberrant in vivo property of one familial ALS-linked superoxide dismutase 1 mutant. *Proc Natl Acad Sci USA* 1997; **94**: 7606–7611

3 Beal MF, Ferrante RJ, Browne SE, *et al.* Increased 3-nitrotyrosine in both sporadic and familial amyotrophic lateral sclerosis. *Ann Neurol* 1997; **42**: 644–654

4 Good PF, Hsu A, Werner P, *et al.* Protein nitration in Parkinson's disease. *J Neuropathol Exp Neurol* 1998; **57**: 338–342

5 Castegna A, Thongboonkerd V, Klein JB, *et al.* Proteomic identification of nitrated proteins in Alzheimer's disease brain. *J Neurochem* 2003; **856**: 1394–1401

6 Tohgi H, Abe T, Yamazaki K, *et al.* Alterations of 3-nitrotyrosine concentration in the cerebrospinal fluid during aging and in patients with Alzheimer's disease. *Neurosci Lett* 1999; **269**: 52–54

7 Beal MF. Oxidatively modified proteins in aging and disease. *Free Radic Biol Med* 2002; **32**: 797–803

8 Greenacre SA, Ischiropoulos H. Tyrosine nitration: Localisation, quantification, consequences for protein function and signal transduction. *Free Radic Res* 2001; **34**: 541–581

9 Gole MD, Souza JM, Choi I, *et al.* Plasma proteins modified by tyrosine nitration in acute respiratory distress syndrome. *Am J Physiol Lung Cell Mol Physiol* 2000; **278**: L961–967

10 Omori A, Ichinose S, Kitajima S, *et al.* Gerbils of a seizure-sensitive strain have a mitochondrial inner membrane protein with different isoelectric points from those of a seizure-resistant strain. *Electrophoresis* 2002; **23**: 4167–4174

11 Ikezono T, Omori A, Ichinose S, *et al.* Identification of the protein product of the Coch gene (hereditary deafness gene) as the major component of bovine inner ear protein. *Biochim Biophys Acta* 2001; **1535**: 258–265

12 Schuller E, Gulesserian T, Seidl R, *et al.* Brain t-complex polypeptide 1 (TCP-1) related to its natural substrate beta1 tubulin is decreased in Alzheimer's disease. *Life Sci* 2001; **69**: 263–270

13 Ihara Y, Yasuoka C, Kageyama K, *et al.* Tyrosine phosphorylation of clathrin heavy chain under oxidative stress. *Biochem Biophys Res Commun* 2002; **297**: 353–360

14 Nakamura Y, Hasimoto R, Kashiwagi Y, *et al.* Abnormal distribution of neurofilament L in neurons with Alzheimer's disease. *Neurosci Lett* 1997; **225**: 201–204

15 Strong MJ. Neurofilament metabolism in sporadic amyotrophic lateral sclerosis. *J Neurol Sci* 1999; **169**: 170–177

Pediatr Nephrol (2005) 20:1578–1582
DOI 10.1007/s00467-005-2019-1

ORIGINAL ARTICLE

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Detection of enteroviruses in renal biopsies from patients with immunoglobulin A nephropathy

Received: 31 March 2005 / Revised: 31 May 2005 / Accepted: 1 June 2005 / Published online: 25 August 2005

Abstract Viruses have been suspected to be one of the causes of IgA nephropathy (IgAN). Recent studies have detected viruses in renal tissues of patients with IgAN. Enteroviruses have been reported as pathogenic agents in some renal diseases. We previously reported that group B coxsackieviruses cause pathological changes in experimentally infected mouse kidney. The aim of the present study was to examine the participation of enteroviruses in the pathogenesis of renal diseases including IgAN. Renal biopsies of ten patients with IgAN (group 1) and of 19 patients with non-IgAN renal disease(group 2) were analyzed by polymerase chain reaction (PCR) for the presence of enteroviral RNA. Positive PCR results were obtained for three patients (30%) of group 1. We confirmed by sequencing that the positive PCR products were derived from strains of enteroviruses. One of these three patients also had a positive result for lymphocytes from peripheral blood. In contrast, enteroviral RNA was detected in none of the 19 patients of group 2. The incidence of enteroviral RNA detection in patients of group 1 was higher than that in group 2 ($P<0.05$). Our findings suggest that enteroviral infection may have the possibility of becoming one of the factors involved in the mechanism of onset or evolution of IgAN.

Keywords IgA nephropathy · Enterovirus · Polymerase chain reaction

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Introduction

IgA nephropathy (IgAN) is an idiopathic glomerulonephritis characterized by predominant IgA deposition in the mesangium and is the most common type of glomerulonephritis worldwide. However, its pathogenesis is still unclear. In IgAN, renal signs and symptoms are frequently preceded by episodes of upper respiratory tract infection and/or gastrointestinal infection, suggesting viral infection as the etiology of this disease. Recently, viral antigens [1, 2] were detected in renal tissues of patients with IgAN, and in viral DNA, by polymerase chain reaction or in situ hybridization [3, 4, 5]. Enteroviruses are established or suspected etiological agents in numerous diseases. In particular, group B coxsackieviruses (CBs), which are enteroviruses, have been implicated in several diseases, including pancreatitis, insulin-dependent diabetes mellitus, myocarditis, and myositis [6]. They have also been reported to be etiological agents in some renal diseases [7, 8, 9, 10]. For example, experimental nephritis induced by CB4 was first described by Sun et al. [11], and subsequent reports indicated relationships of CB4 with renal disease in humans [7, 8] and experimental animals [12, 13, 14]. We recently demonstrated glomerular lesions with IgA deposits induced by intraperitoneal inoculations of CB4 in mice [13].

In the present study, renal biopsies and lymphocytes from patients with IgAN or other renal diseases were investigated for the presence of enteroviral genome sequences by polymerase chain reaction (PCR) to determine whether IgAN is an enteroviral-associated disease.

Patients and methods

Patients

From March to December 2001, renal biopsy was performed in 29 patients. Whole-blood samples were collected simultaneously from these patients. The patients were divided into two groups based on histological diagnosis. Group 1 comprised 10 patients diagnosed with IgAN, while group 2 was composed of 19 patients diagnosed with non-IgAN renal disease. The non-IgAN patients consisted of 7

patients with minimal change, 10 with purpura nephritis, and 2 with membrano-proliferative glomerulonephritis. We attempted to detect enteroviral RNA in renal biopsy tissues and lymphocytes from peripheral blood in both groups.

Lymphocyte isolation

Whole blood was collected from 27 of 29 patients into tubes containing anticoagulant (EDTA). The blood was diluted by addition of an equal volume of 0.9% NaCl. The diluted blood was carefully layered over Lymphoprep (Nycomed Pharma AS, Oslo, Norway) in a centrifuge tube and was centrifuged at 800*g* for 20 min at room temperature in a swing-out rotor. After centrifugation, the mononuclear cells formed a distinct band at the sample/medium interface. The cells were removed from the interface. The harvested fraction was diluted with 0.9% NaCl, to reduce the density of the solution, and the cells were pelleted by centrifugation at 250*g* for 10 min.

RNA extraction

Total cellular RNA was extracted from the frozen renal biopsies and peripheral lymphocytes homogenized in a modified guanidine thiocyanate buffer (Isogen LS 750, Wako Pure Chemical Industries, Tokyo, Japan) by an acid phenol–chloroform extraction method.

Detection of enteroviral RNA by PCR

PCR for enteroviral RNA amplification was performed as has been previously described [15, 16]. After RNA extraction, cDNA was synthesized from the extracted RNA. Briefly, a reaction mixture, containing 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 5 mmol/l $MgCl_2$, 0.2 mmol/l each of dNTP, 1 μmol/l of primer F1 (5′-CAAGCACTTCTGTTTCCCCGG-3′) and R1 (5′-ATTGTCACCATAAGCAGCCA-3′), 20 U of ribonuclease inhibitor (Toyobo, Osaka, Japan), 50 U of reverse transcriptase RNaseH Minus (Toyobo), and RNA, isolated from the samples, was prepared. After incubation at 42°C for 60 min, the first PCR mixture, which contained 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 5 mmol/l $MgCl_2$, 0.2 mmol/l each of dNTP, 0.2 μmol/l of primers F1 and R1, and 2.5 U of Taq DNA polymerase (Perkin-Elmer, Norwalk, Conn., USA), was added and the amplification was performed in 40 cycles consisting of denaturation for 1 min at 93°C, primer annealing for 1 min at 45°C, and elongation for 2 min at 72°C.

The second PCR was performed as above, using the second primer pair [primer F2 (5′-TCCTCCGGCCCCTGAATGCG-3′) and R1] and 2 μl of the first PCR product. The nested PCR product was analyzed by electrophoresis in 2% agarose gels. Positive PCR reaction was expected to yield a 155-bp band. Zoll et al. [17] reported that the primer pairs were general primers of 60 different enterovirus serotypes (polioviruses 1 to 3; coxsackieviruses A1 to A24, except A11, A17, A24; coxsackieviruses B1 to B6; echoviruses 1 to 33 except 16, 22, 23).

Sequencing of PCR products

Each PCR product was gel-purified and sub-cloned into pGEM-T vector (Promega, Madison, Wis., USA). The sequences of the cloned genes were determined by an automated DNA sequencer (Perkin-Elmer) and screened for homology with database sequences using the BLAST search algorithm.

Pathological examination

The renal biopsy specimens were assessed by light microscopy (LM) and immunofluorescence (IF) examination. Material for histological study was fixed in 20% neutral formalin and embedded in paraffin, sliced at 2–3 μm thickness and stained with hematoxylin and eosin or periodic acid–Schiff reagent.

Tissue samples for IF were immediately fixed in ornithine carbamoyltransferase (OCT) compound and frozen at −80°C until required. IF examination was performed for IgG, IgA, IgM, C1q, C3, C4, and fibrinogen. We used fluorescein-conjugated goat antibodies (IgG) to rabbit IgG, IgA, IgM, C1q, C3, C4, and fibrinogen. The findings and classification of glomerular abnormalities are listed in Table 1. Classification was based on the second edition of the histological classification of glomerular diseases by a committee of the World Health Organization. [18]. The intensity of immunofluorescence was graded as (−) = negative, (+) = mild, (++) = moderate and (+++) = strong.

Definitions and diagnostic criteria

Hematuria was considered present if microscopic examination revealed five or more red blood cells per high-power field, and macro-hematuria if blood was visible with the naked eye [19]. Proteinuria was evaluated by 24-h quantitative measurement.

Diagnosis of IgAN was based on the presence of IgA as the sole or predominant Ig in the glomerular mesangium without systemic disease such as Henoch–Schönlein purpura (HSP) or systemic lupus erythematosus. Diagnosis of HSP was made if the major manifestations of disease were purpuric rash and abdominal pain without thrombocytopenia. Additional features, including arthritis and nephritis, were accepted as consistent with the diagnosis. Membranoproliferative glomerulonephritis (MPGN) was diagnosed by light microscopy based on findings of enlarged and lobular glomeruli, an increase in mesangial cells and matrix, and double contour of the capillary loop. In this study, MPGN was considered a primary disease itself, apart from systemic diseases such as monoclonal nephropathy, anti-phospholipid antibody syndrome, lupus nephritis, other autoimmune diseases, and hepatitis B or C viral infection.

The clinical status of each patient at the most recent observation was classified as follows: *Normal* the patient was healthy on physical examination, with normal urinalysis results and normal renal function. *Minor urinary abnormalities* the patient was healthy on physical examination, with microscopic hematuria or proteinuria less than 40 mg/m^2 per hour. *Active renal disease* the patient had proteinuria of 40 mg/m^2 per hour, or greater, or hypertension, and a 24-h creatinine clearance (Ccr) of 60 ml/min per 1.73 m^2 or greater. *Renal failure* the patient had a 24-h Ccr of less than 60 ml/min per 1.73 m^2 (including cases of dialysis/transplantation or death).

Acute exacerbation was defined as exacerbation of the clinical condition by at least one status level.

Statistics

Values are means ± SEM. Statistical analysis was performed on a Macintosh computer with a software package for statistical analysis (StatView, Abacus Concepts, Berkeley, Calif., USA). Differences among group values were assessed by the Mann–Whitney rank-sum test. Fisher's test was used to evaluate correlations. Findings of $P<0.05$ were considered significant.

Results

Comparison of baseline characteristics between groups

The baseline characteristics of the groups are shown in Tables 1 and 2. Age at onset, and mean duration of follow-up, were 11.2±3.2 years and 5.5±3.9 years, respectively, in group 1 and 8.4±3.5 years and 6.8±3.8 years, respectively, in group 2. Male-to-female ratios were 4/6 and 5/14, respectively.

Table 1 Histopathological findings in each group. Grading of pathological alternations: – to +++. (*D* diffuse, *S* segmental, *F* focal, *G* global, *M* mesangial, *HSPN* Henoch–Schoenlein purpura nephritis, *MC* Minimal change, *C* capillary, *NT* not tested)

Case no.	Age (years)/ gender	Diagnosis	Light microscopy			IF staining							PCR results		Clinical status	Acute exacerbation
			Mesangial lesion		Interstitial cell infiltration	IgG	IgA	IgM	C1	C3	C4	Fibrinogen	Renal biopsies	Lympho-cytes		
			Cell prolifer-ation	Matrix expan-sion												
Group 1																
1	13/Male	IgAN	D,S	D,S	+	M++	M+++	M+	–	M+	–	MC+	–	NT	Unknown	Unknown
2	14/Male	IgAN	D,S	-	+	M+	MC+++	M+	–	M+	–	–	–	-	Minor	-
3	8/Female	IgAN	F,S	F,S	–	–	MC++	–	–	–	–	C++	–	–	Unknown	Unknown
4	9/Female	IgAN	D,S	D,S	+	–	M+++	M++	–	–	–	MC++	–	–	Minor	–
5	16/Female	IgAN	F,S	F,S	–	M++	M+++	M+	–	M++	–	C+	–	–	Active	+
6	6/Female	IgAN	F,S	F,S	–	M++	M+++	M+	–	–	–	M+++	+	–	Minor	–
7	19/Male	IgAN	F, S	F, S	+	–	M++	M+	–	M++	–	–	+	+	Minor	–
8	14/Female	IgAN	F,S	F,S	+	–	M+++	–	–	M++	–	MC+	–	–	Minor	–
9	14/Male	IgAN	F,S	F,S	–	MC+	M+++	–	–	M+	–	M++	+	NT	Minor	–
10	9/Female	IgAN	D,S	–	+	–	MC++	MC+	–	MC+	–	C+	–	–	Normal	–
Group 2																
11	8/Male	HSPN	F,S	F,S	–	–	M+++	M+ –	–	M+	M+	M++	–	-	Unknown	Unknown
12	13/Female	MC	–	–	+	–	–	–	C++	–	–	M++	–	–	Normal	–
13	9/Male	MC	–	–	+	–	–	–	–	–	–	–	–	–	Minor	–
14	17/Male	MC	–	–	+	–	–	–	–	C++	–	–	–	–	Minor	–
15	11/Female	MC	–	–	–	–	C++	–	–	–	–	–	–	–	Minor	–
16	9/Female	HSPN	–	F,S	–	–	MC++	MC+	–	C+	–	C+	–	–	Active	+
17	11/Female	HSPN	F,G	F,G	–	–	MC++	–	–	MC+	–	MC+	–	–	Active	–
18	8/Female	HSPN	D,G	D;G	Fibrosis	–	M+++	–	–	C+	–	C++	–	–	Active	–
19	14/Female	HSPN	D,S	D,S	+	–	MC+++	MC+	–	MC++	–	MC++	–	–	Active	–
20	13/Female	MPGN	D,G	D,G	–	C++	–	–	C++	C+++	–	C+	–	–	Unknown	Unknown
21	5/Male	HSPN	D,S	–	–	–	MC++	C+	–	MC++	–	MC+	–	–	Normal	–
22	13/Female	MC	–	–	–	–	–	C+	–	–	–	C+	–	–	Normal	–
23	11/Female	MPGN	D,G	D,G	–	–	–	–	–	C+++	–	MC++	–	–	Unknown	Unknown
24	9/Female	HSPN	F,S	–	+	–	M+++	–	–	MC++	–	MC+++	–	–	Minor	–
25	11/Female	MC	–	–	–	–	–	–	–	–	–	–	–	–	Minor	–
26	8/Female	HSPN	D,G	–	+	MC+	MC+++	–	–	C+	C+	MC+++	–	–	Active	–
27	13/Female	HSPN	F,S	–	+	–	MC+++	–	–	–	–	C++	–	Minor	Minor	–
28	4/Male	MC	–	–	–	–	–	C+	–	–	–	–	–	–	Normal	–
29	7/Female	HSPN	F,S	–	+	–	MC+++	–	–	MC++	–	MC+++	–	Minor	Minor	–

Table 2 Comparison of baseline characteristics between both groups

Characteristic		Group 1	Group 2
Age at onset	(years)	11.2±3.2*	8.4±3.5*
Mean follow-up	(years)	5.5±3.9	6.8±3.8
Male:female		4:6	5:14
Mean urinary protein excretion	(g/day)	0.5±0.5	1.8±2.7
Hematuria	(case %)	10 (100%)	14 (73%)
Serum creatinine	(mg/dl)	0.6±0.2	0.7±0.5
Serum total protein	(g/dl)	6.6±0.3	6.5±1.2
24 h Ccr	(ml/min per 1.73 m^2)	145.6±30.1	103.1±51.7

*$P<0.05$

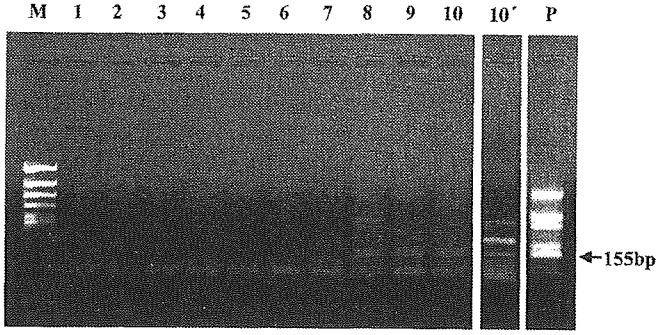

Fig. 1 Gel electrophoresis of PCR products of enteroviral amplification. *Lane M* length marker. *Lanes 1 to 10* are renal biopsies: *lane 1* case 1, *lane 2* case 2, *lane 3* case 3, *lane 4* case 4, *lane 5* case 5, *lane 6* case 8, *lane 7* case 10, *lane 8 case 9*, *lane 9* case 6, *lane 10* case 7. *Lane p* is the positive control (echovirus 7 cDNA clone). *Lane 10'* is case 7 lymphocytes

Mean urinary protein excretion ranged from 0.05 g/day to 1.5 g/day (mean 0.5±0.5 g/day) in group 1 and 0.1 g/day to 10.0 g/day (mean 1.8±2.7 g/day) in group 2. Hematuria was found in 100% of patients in group 1 and in 73% of patients in group 2. Serum creatinine, total protein, and 24-h Ccr concentrations were similar in the two groups.

Detection of enteroviral RNA

Detection of enteroviral RNA is illustrated in Figs. 1 and 2). Positive PCR results were found in three of ten (30%) frozen renal biopsies from the patients of group 1 (Figure 1). One of these three patients also had a positive result for lymphocytes obtained from peripheral blood. In contrast, enteroviral RNA was detected in none of the 19 frozen renal biopsies from patients in group 2. The incidence of enteroviral detection was higher in group 1 than in group 2 ($P<0.05$). To confirm that the PCR products represented enteroviral genome sequences, the sequences of the cloned genes from renal biopsy specimens of cases 6 and 7 were screened for homology with a database using the BLAST search algorithm (Fig. 2). The sequences of the PCR products from cases 6 and 7 were most strongly homologous with a part of human coxsackievirus B1. However, the sequence deviated from that of human coxsackie B1 virus at one base in case 7, and at three bases in case 6. In group 1, none of the clinical manifestations, the degree of exacerbation after onset, pathological findings, and prognosis differed between patients with and without detection of enteroviral RNA (Table 1).

Discussion

There have been many reports on the relationship between viral infection and renal injury [1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14]. Clinically, it has been noted that viral infection often provokes transient proteinuria, hematuria, and renal dysfunction in patients who develop IgAN. Although IgAN is a very common form of glomerular disease, its pathogenesis has yet to be elucidated. Previous studies have revealed that human cytomegalovirus (HCMV) participated in the pathogenesis of IgAN [2, 3]. Recently, Iwama et al. [5] reported that Epstein–Barr

```
Case 6   *---------- ---------- ---------- ---------- ---------- ----------
Case 7   ---------- ---------- ---------- ---------- ---------- ----------
                446bp                                                505bp
Human coxsackievirus B1 ggctaatcct aactgcggag caggcaatca caatccagtg ggtggcctgt cgtaacgggc
     Human echovirus 26 ***------- ---------- ------cc-- ---g------ --ca------ ----------
   Human enterovirus 71 ---------- ---------- ----tgcc-- ---c------ ---------- ----------
          Echovirus 6   --------t- ---------- ----tgct-- ---------- ---------- ----------

Case 6   --a------- ---------- ---------* -----*---- ---------- ------
Case 7   ---------- ---------- ---------- ------*--- ---------- ------
              506bp                                                561bp
Human coxsackievirus B1 aactccgcag cggaaccgac tactttgggt gtccgtgttt ctttttattc ttacat
     Human echovirus 26 -----t---- ---------- ---------- ------g--- ---------- -----
   Human enterovirus 71 -----t---- ---------- ---------- ------g--- -c-------- --
          Echovirus 6   -----t---- ---------- ---------- ------
```

Fig. 2 Comparison of sequences of PCR products and RNA sequences of enteroviruses

virus (EBV)-specific DNA in renal biopsies was detected by PCR in seven (58%) of 12 patients with IgAN, three (50%) of six patients with membranous nephropathy, none (0%) of ten patients with minor glomerular abnormalities, and two (100%) of two patients with focal segmental lesions. EBV genome detection did not yield findings specific for IgAN.

Enteroviruses have been implicated in a wide variety of clinical disorders [6] and have been suggested to be pathogenic agents in renal diseases such as hemolytic uremic syndrome (HUS) and glomerulonephritis [9, 10]. By using pure cultures of human glomerular and tubular cells, Conaldi et al. demonstrated that the infection of six CBs led to cytolysis in proximal tubular epithelial cells and glomerular podocytes [20]. Previously, we reported that CB4 caused pathological changes in experimentally infected mouse kidney [12, 13, 14]. We recently observed lesions similar to those of human IgA nephropathy in mice intravenously inoculated with CB4 once a month from 1 to 5 months of age, and we detected CB4 viral RNA in the mesangial lesions, using in situ hybridization [13]. We therefore attempted to detect enteroviral RNA in renal biopsies from patients with IgAN, using PCR. The PCR method we used could detect almost all prevalent enteroviruses in Japan, with a sensitivity a thousand times that of the virus isolation method [15].

In the present study, we detected enteroviral RNA in frozen renal biopsies from three (30%) of ten patients with IgAN, one of whom also had an enteroviral genome in peripheral lymphocytes. In contrast, enteroviral RNA was detected in none of the frozen renal biopsies from patients with non-IgAN renal disease. Our findings suggest that the detection of enteroviral RNA in renal tissue may be specific for IgAN. No specific findings are available regarding the ability of enterovirus persistently to infect human renal tissue in vivo. However, it has become apparent that CBs are associated with persistent infections in vitro [21, 22, 23]. It thus appears possible that persistent enteroviral infection might be involved in the pathogenesis of IgAN.

In conclusion, our findings suggest that enteroviral infection may have the possibility of becoming one of the factors involved in the mechanism of onset or evolution of IgAN. However, further studies will be needed to determine whether enteroviral RNA can be detected in renal biopsies in other patient populations and whether enteroviral infection plays an important role in the pathogenesis of IgAN.

References

1. Tomino Y, Yagame M, Omata F, Nomoto Y, Sakai H (1987) A case of IgA nephropathy associated with adeno- and herpes simplex viruses. Nephron 47:258–261
2. Gregory M, Hammond ME, Brewer ED (1988) Renal deposition of cytomegalovirus antigen in immunoglobulin A nephropathy. Lancet 2:11–14
3. Müller GA, Müller CA, Blum GE, Kühn W, Risler T, Bohle A, Lipkovski JM (1992) Human cytomegalovirus in immunoglobulin A nephropathy: detection by polymerase chain reaction. Nephron 62:389–393
4. Sinniah R, Khan TN, Dodd S (1993) An in situ hybridization study of herpes simplex and Epstein Barr viruses in IgA nephropathy and non-immune glomerulonephritis. Clin Nephrol 40:137–141
5. Iwama H, Horikoshi S, Shirato I, Tomino Y (1998) Epstein–Barr virus detection in kidney biopsy specimens correlates with glomerular mesangial injury. Am J Kidney Dis 32:785–793
6. Grist NR, Bell EJ, Assaad F (1978) Enteroviruses in human disease. Prog Med Virol 24:114–157
7. Burch GE, Colcolough HL (1969) Progressive Coxsackie viral pancarditis and nephritis. Ann Intern Med 71:963–970
8. Burch GE, Chu KC, Colocolough HL, Sohal RS (1969) Immunofluorescent localization of Coxsackie B antigen in the kidney observed at routine autopsy. Am J Med 47:36–42
9. Austin TW, Ray CG (1973) Coxsackie virus group B infections and the hemolytic uremic syndrome. J Infect Dis 127:698–701
10. Aronson MD, Phillips CA (1975) Coxsackievirus B5 infections in acute oliguric renal failure. J Infect Dis 132:303–306
11. Sun SH, Burch GE, Sohal RS, Chu KC (1967) Coxsackie B4 viral nephritis in mice and its autoimmune-like phenomena. Proc Soc Exp Biol Med 126:882–885
12. Mutoh S, Kumada K, Kume K, Suzuki J, Yokota T, Suzuki H (1994) Detection of coxsackie B4 virus RNA in infected mouse kidneys by in situ hybridization. Nephron 67:340–345
13. Yoshida K, Suzuki S, Kume K, Mutoh S, Suzuki J, Kato K, Suzuki H (1997) Experimental IgA nephropathy induced by Coxsackie B4 virus in mice. Am J Nephrol 17:81–88
14. Isome M, Yoshida K, Suzuki S, Kume K, Suzuki J, Kato K, Suzuki H (1997) Experimental glomerulonephritis following successive inoculation of five different serotypes of group B coxsackieviruses in mice. Nephron 77:93–99
15. Hosoya M, Honzumi K, Suzuki H (1997) Detection of enterovirus by polymerase chain reaction and culture in cerebrospinal fluid of children with transient neurologic complications associated with acute febrile illness. J Infect Dis 175:700–703
16. Hosoya M, Honzumi K, Sato M, Katayose M, Kato K, Suzuki H (1998) Application of PCR for various neurotropic viruses on the diagnosis of viral meningitis. J Clin Virol 11:117–124
17. Zoll GJ, Melchers WJG, Kopecka H, Jambroes G, Poel HJA, Galama JMD (1992) General primer-mediated polymerase chain reaction for detection of enteroviruses: application for diagnostic routine and persistent infections. J Clin Microbiol 30:160–165
18. Churg J, Bernstein J, Glassock RJ (1995) Renal disease. Classification and atlas of glomerular diseases (edn 2). Igaku-shoin, Tokyo, Japan
19. Southwest Pediatric Nephrology Study Group (1985) A clinicopathologic study of crescentic glomerulonephritis in 50 children: a report of the Southwest Pediatric Nephrology Study Group. Kidney Int 27:450–458
20. Conaldi PG, Biancone L, Bottelli A, De Martino A, Camussi G, Toniolo A (1997) Distinct pathogenic effects of group B coxsackieviruses on human glomerular and tubular kidney cells. J Virol 71:9180–9187
21. Matteucci D, Paglianti M, Giangregorio AM, Capobianchi MR, Dianzani F, Bendinelli M (1985) Group B coxsackieviruses readily establish persistent infections in human lymphoid cell lines. J Virol 56:651–654
22. Schnurr DP, Schmidt NJ (1984) Persistent infection of mouse fibroblasts with coxsackievirus. Arch Virol 81:91–101
23. Frank JA, Schmidt EV, Smith RE, Wilfert CM (1986) Persistent infection of rat insulinoma cells with coxsackie B4 virus. Acta Virol 87:143–150

Epilepsia, **46**(Suppl. 5):152–158, 2005
Blackwell Publishing, Inc.

Autoantibodies and Cell-mediated Autoimmunity to NMDA-type GluRε2 in Patients with Rasmussen's Encephalitis and Chronic Progressive Epilepsia Partialis Continua

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Summary: *Purpose:* To evaluate antibody-mediated and cytotoxic T cell–mediated pathogenicity that has been implicated as the autoimmune pathophysiological mechanism in Rasmussen's encephalitis.

Methods: We examined autoantibodies against the *N*-methyl-D-aspartate glutamate receptor (NMDA-type GluR) ε2 subunit and its epitopes in serum and CSF samples from 20 patients [five histologically proven (definitive) Rasmussen's encephalitis with epilepsia partialis continua (EPC), four definitive Rasmussen's encephalitis without EPC, and 11 clinical Rasmussen's encephalitis with EPC]. We examined ^{3}H-thymidine uptake into lymphocytes after stimulation by GluRs.

Results: All nine definitive patients (five patients with EPC and four without EPC), and 10 of 11 clinical Rasmussen's encephalitis patients had the autoantibodies. In four patients, the autoantibodies were absent in early stage when epileptic seizures had already become frequent, and appeared subsequently. In two patients, the autoantibodies persisted in the serum after frontal lobe resection or functional hemispherectomy, although epileptic seizures were completely controlled. Autoantibodies to the C2 epitope predominated, while autoantibodies to the extracellular N epitope were rare. The mean ^{3}H-thymidine uptake ratios (stimulation by GluRε2-containing homogenates/stimulation by PHA) were significantly higher in definitive and clinical Rasmussen encephalitis patients than in controls. The mean ^{3}H-thymidine uptake ratios (relative to PHA) were significantly higher for GluRε2-containing homogenate than for control homogenate or GluRδ2-containing homogenate.

Conclusions: Autoantibodies against GluRε2 may be one of the diagnostic markers for Rasmussen's encephalitis with and without EPC. Patients have activated T cells stimulated by GluRε2 in peripheral blood circulation. We speculate that cellular autoimmunity and the subsequent humoral autoimmunity against GluRε2 may contribute to the pathophysiological processes in Rasmussen's encephalitis. **Key Words:** Rasmussen syndrome—EPC—GluRε2—autoantibodies—Cell-mediated autoimmunity.

Epilepsia partialis continua (EPC) is characterized by continuous myoclonic jerks of the extremities and/or the face, usually without impairment of consciousness. In patients with chronic EPC, myoclonic jerks continue for periods of days, weeks, or months, and are usually localized in one part of the body, except in serious cases (1). The clinical evolution of EPC is variable, depending on the patient's age and underlying brain diseases (1,2). Bancaud (3) classified EPC into two groups: a group with stable neurological deficit, and another group with slowly progressive neurological deficit. 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 syndrome (5) after histological confirmation. However, biopsy of brain lesion is not generally accepted in Japan, and surgery for patients with a tentative diagnosis of Rasmussen's encephalitis is rare. Therefore, Japanese patients are generally diagnosed as

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chronic progressive EPC, even though they may have the pathology of Rasmussen's encephalitis.

Rogers et al. (6) reported glutamate receptor 3 (GluR3) as an autoantigen in Rasmussen's encephalitis, speculated to be one cause of Rasmussen's encephalitis. After their report, epilepsies causally related to autoantibodies against GluRs were highlighted. GluRs in the central nervous system (CNS) play important roles in brain function, and they are divided into ionotropic and metabotropic receptors. The ionotropic GluRs are subdivided into three major subtypes: the *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), and kainate receptors. The NMDA-GluRs are heterodimers composed of GluRε (ε1-ε4) (NMDAR2A-2D), GluRζ(NMDAR1) subunits, and NR3 subunits (7,8). The molecular diversity of NMDA-GluRs contributes not only to their important physiological roles in development, synaptic plasticity, learning, and memory, but also to the pathological processes in ischemic brain injury, neurodegenerative diseases, and epilepsy (7–11).

GluRε2 is the essential molecule for synaptic plasticity and development (12). In Rasmussen's encephalitis, not only epileptic seizures, but also impairment of higher brain function, such as mental retardation and visual disturbance, occur frequently. Therefore, we examined autoantibodies against NMDA-GluRε2 and their epitopes in the serum and cerebrospinal fluid (CSF) in patients with chronic EPC (13). We detected NMDA-type GluRε2 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). The presence of autoantibodies against NMDA-GluRε2 might suggest autoimmune pathological mechanisms, but is not a hallmark of Rasmussen's encephalitis. Epitope analyses showed that the autoantibodies were predominantly against C-terminal epitopes and rarely against N-terminal epitope, with inconsistency in the profile during the course of the disease. The 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 the disease progressed in some patients. Therefore, we speculated that the autoantibodies are produced in the CNS after cytotoxic T cell–mediated neuronal damages (13).

In this study, as an extension to our previous study in Rasmussen's encephalitis patients with EPC (13), we examined autoantibodies against NMDA-GluRε2 in 10 new Rasmussen's encephalitis patients. These new patients included four histologically proven Rasmussen's encephalitis patients without EPC (this category of Rasmussen's encephalitis was not reported in our previous paper), two histologically proven Rasmussen's encephalitis patients with EPC, and four clinical Rasmussen's encephalitis patients with EPC. Consequently, we could evaluate the autoantibodies in 20 patients with Rasmussen's encephalitis. Furthermore, as an original study, we examined lymphocyte stimulation by GluRs to estimate the cellular autoimmune pathophysiology of Rasmussen's encephalitis.

PATIENTS AND METHODS

Patients

We examined the samples from 11 clinical Rasmussen's encephalitis patients with chronic progressive EPC (Patients 1–4, 11–16, and 18), and nine histologically proven Rasmussen's encephalitis patients with EPC (Patients 5, and 7–10) or without EPC (6,17,19, and 20) (Table 1). Eleven clinical patients with chronic progressive EPC manifested intractable EPC for >1 month, and all showed neuropsychological impairment. They had progressive EPC, neuroimage findings characteristic of Rasmussen's encephalitis (14), and neuropsychological impairment (clinical Rasmussen type). Detailed clinical manifestations of Patients 1–3, 13, 14, 16, and 18 were reported in our previous article (13), and those of Patient 10 were also reported (15). Patient 14 had negative tests for anti-GluR3 autoantibodies in another hospital. Nine patients were histologically diagnosed as Rasmussen's encephalitis (definitive Rasmussen type) after functional hemispherectomy (Patient 7–10) and focal resection surgeries (Patients 5, 6, 17, 19, and 20).

Informed consent was obtained from each patient or guardian after oral or written explanation, according to the ethical principle of Declaration of Helsinki.

Establishment of stable transformant cells expressing GluRε2

Using the tetracycline-induction system (16), cDNA of GluRε2 (17), pSTneoB (18), and G418 selection (19), we established stable NIH3T3 transformant cell lines expressing full-length GluRε2 (B18) and GluRδ2 subunits (D33) (13). A1 is a control cell line without GluR expression, B18 is a cell line expressing recombinant GluRε2, and D33 is a cell line expressing recombinant GluRδ2.

Detection of autoantibodies against GluRε2 in sera and CSF

Supernatants of cell extracts prepared from B18 and A1 cultured for 48 h with doxycycline (1 μg/ml) were subjected to 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 patients' sera (diluted 20-fold with blocking buffer) or CSF (diluted 15-fold with blocking buffer) for 48 h at 4 °C, and were stained by alkaline phosphatase-labeled second antibodies (IgG, IgA, or IgM) (Jackson ImmunoResearch, West Grove, Philadelphia, PA, U.S.A.). The presence of autoantibodies against GluRε2 was judged by a positively

TABLE 1. *Clinical characteristics of patients*

Patient	Onset age		Sex	Causative or underlying factors	Seizure type	Ope	Outcome			
	Epilepsy	EPC				Age	Neurological	Mental	Seizure	At age
1	2 days	1.1 yr	M	DPT vaccination, status	CPS, EPC		QP	MR+++	Daily	4.5 yr
2	2 mo	7 mo	M	Viral meningitis	CPS, EPC		QP	MR+++	Daily	4 yr
3	2 mo	4 mo	M	Polio vaccination, status	CPS, EPC		QP	MR+++	Disappeared	8 yr
4	8 mo	8 mo	M	Respiratory infection	CPS, EPC		HP	MR+++	Daily	4 yr
5	2 yr	9 yr	M		CPS, EPC	8 yr	-	MR++	Daily	9 yr
6	2.6 yr	-	M		CPS	14 yr	-	Normal	Disappeared	15 yr
7	3.6 yr	3.7 yr	M	Infection	CPS, EPC	4 yr	HP, HA	Normal	Disappeared	5yr
8	3.8 yr	3.8 yr	M	Head trauma	EPC, CPS	9 yr	HP, HA	MR+	Disappeared	11 yr
9	3.9 yr	5.3 yr	M	Influenza	CPS, EPC	5.8 yr	HP	Normal	Disappeared	5.9 yr
10	5.2 yr	6.1 yr	F	Status	CPS, EPC	6 yr	HP, HA	Normal	Daily	6 yr
11	5.8 yr	6 yr	F		CPS, EPC		MP	Normal	Daily	20 yr
12	5.9 yr	6 yr	F		CPS, EPC		HP	MR++	Daily	6.2 yr
13	6.1 yr	6.9 yr	M		EPC, CPS		HP	MR+	Daily	10 yr
14	8.9 yr	9.5 yr	F	Infection, status	CPS, EPC		HP	MR++	Daily	15 yr
15	12 yr	15 yr	M		CPS, EPC		QP	MR+++	Daily	16 yr
16	12.5 yr	12.9 yr	M		CPS, EPC		QP	MR++	Weekly	20 yr
17	16 yr	–	M	Head trauma	CPS	34 yr	MP	MR++	Weekly	34 yr
18	23 yr	31yr	F		EPC, CPS		Normal	MR+	Daily	38 yr
19	25 yr	–	F	–	CPS	29 yr	Normal	Normal	Weekly	31 yr
20	28 yr	–	F	–	CPS	34 yr	HP	MR++	Weekly	38 yr

EPC, epilepsia partialis continua; M, male; F, female; NMD, neuronal migration disorder; DPT, diphtheria-pertussis-tetanus; Status, status epilepticus; SPS, simple partial seizure; CPS, complex partial seizure; QP, quadriplegia; HP, hemiplegia; MP, monoplegia; HA, hemianopsia; MR, mental retardation.

stained band with molecular size ~180 kDa, which was found only on the B18 strip and not on the A1 strip (13).

Preparation of bacterial fusion proteins containing peptides from the GluRε2 subunit

DNA fragments encoding amino acid residues 1–48 (an amino-terminal peptide; N), 998–1074 (a carboxyl-terminal peptide; C1), 1053–1153 (a carboxyl-terminal peptide; C2), and 1353–1432 (a carboxyl-terminal peptide; C3) (17) 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, MA, U.S.A.) for the production of fusion protein with glutathione S-transferase (GST) or maltose binding protein (MBP) (13,20). The expression plasmids were transformed into *Escherichia coli* (BL21 or TB1), and subsequent induction with isopropyl-beta-D-thiogalactopyranoside (IPTG) was performed by routine methods (21). The transformed *Escherichia coli* were sonicated to obtain supernatants of the bacterial homogenates.

Epitope analyses of autoantibodies against GluRε2 in sera and CSF

Supernatants of bacterial homogenates containing induced GluRε2 polypeptide fusion proteins synthesized by the methods described previously 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 h at 4°C, and stained by alkaline phosphatase-labeled second antibodies (IgG or IgM). A positive reaction with an epitope was judged by obtaining a stained band at the appropriate molecular weight (13).

Lymphocyte stimulation test by GluRs

Peripheral venous blood was collected in heparinized sterile vacutainer tubes (Becton Dickinson, Oxford, U.K.). Blood cells and serum were separated by centrifugation at 1,800 rpm for 5 min. Blood cells were diluted with PBS (-) and applied on Ficoll-Conray (d = 1.077), and centrifuged at 1800 rpm for 20 min to separate the lymphocytes. The lymphocytes were washed twice in PBS by centrifugation at 2,000 rpm for 5 min. Then lymphocytes were diluted to 1×10^6 cells/ml in complete RPMI-1640 supplemented with 20% autologous serum. These cells were cultured in triplicate in 96-well microplates for 3 days with the corresponding antigens. Then ^{3}H-thymidine (0.25μCi/well) was added to each well. After incubation for 16 h, the cells were harvested using an automatic cell harvester and the incorporated radioactivity was measured by liquid scintillation counter. The supernatants of homogenates from A1, D33, and B18, and phytohemagglutinin (PHA) were used as antigens.

Statistical analysis

For the comparison of LST data, we used Mann–Whitney's U test or Wilcoxon signed-ranks test, using statcel software. The numbers indicate mean ± SD (standard deviation).

TABLE 2. *Autoantibodies against glutamate receptors*

			EPC				Whole GluRε2			Epitope analysis			
Pt	Age at exam	Latency	Ext	Face	CPS	Sample	IgG	IgM	IgA	N	C1	C2	C3
1	0.8 yr	0.8 yr	–	–	D	Serum	–	–					
	0.9 yr	0.9 yr	–	–	D	Serum	+	+	–	–	–	+	–
2	1.1 yr	0.9 yr	+	–	D	Serum	+	+	–	–	–	–	–
	1.1 yr	0.9 yr	+	–	D	CSF	+	+	–	+	+	+	+
	4.0 yr	3.8 yr	+	–	W	Serum	+	–	–	–	–	+	–
3	3.8 yr	3.7 yr	–	–	C	Serum	–	–	–	–	–	–	–
4	3.5 yr	2.8 yr	+	+	–	CSF	–	–					
	4.0 yr	3.3 yr	+	+	–	CSF	+	–					
5	9 yr	7 yr	–	+	D	CSF	+	–					
6	11 yr	7 yr	–	–	D	Serum	+	+		–	–	+	–
	15 yr	12 Y	–	–	C	Serum	+	+					
7	3.9 yr	0.2 yr	+	–	M	Serum	+	–		–	+	+	+
	3.9 yr	0.2 yr	+	–	M	CSF	+	–		–	+	+	+
8	3.9 yr	0.1 yr	+	–	M	Serum	–	–	–				
	4.7 yr	0.9 yr	+	–	M	Serum	+	+		–	–	–	–
	5.2 yr	1.4yr	–	–	Y	Serum	–	–					
	8.1 yr	4.3 yr	+	+	M	Serum	+	–		–	–	–	–
	10 yr	6.2 yr	–	–	C	Serum	+	+					
9	5.4 yr	2.5 yr	+	–	W	Serum	+	–					
	5.4 yr	2.5 yr	+	–	W	CSF	+	–					
10	6.1 yr	0.9 yr	+	–	W	Serum	+	–		–	+	–	+
	6.7 yr	1.5 yr	+	–	D	Serum	+	–		–	+	–	+
11	20 yr	14 yr	+	–	C	Serum	+	+					
12	6.1 yr	0.2 yr	–	+	D	Serum	+	–					
	6.1 yr	0.2 yr	–	+	D	CSF	+	–					
13	7.1 yr	1 yr	+	+	C	Serum	–	–		–	–	+	–
	7.1 yr	1 yr	+	+	C	CSF	–	+		–	–	+	–
	11.3 yr	5.2 yr	–	+	Y	Serum	+	+					
14	12 yr	3 yr	+	–	D	Serum	+	–		–	–	+	–
15	15 yr	3 yr	+	–	D	Serum	+	+					
	16 yr	4 yr	+	–	D	Serum	–	–					
16	18.5 yr	6 yr	–	–	W	Serum	+	–		+	–	+	+
	19.5 yr	7 yr	–	–	W	CSF	+						
17	34 yr	18 yr	–	–	W	Serum	+	+					
18	38 yr		–	–	D	Serum	+	+		–	+	–	+
19	29 yr	4 yr	–	–	D	Serum	+	+		+	+	–	+
20	36 yr	8 yr	–	–	W	Serum	–	–					
	38yr	10 yr	–	–	W	Serum	+	+					

Latency, latency from onset to examination; EPC, epilepsia partialis continua; Age at exam, age at examination of autoantibodies; Ext, EPC involving extremities; Face, EPC involving facial muscles, tongue, or throat; CSF, cerebrospinal fluid; GluR, glutamate receptor; NT, not tested. In the column of CPS, D indicates daily frequency of CPS, W indicates weekly, M indicates monthly, Y indicates yearly, C indicates controlled. In the column of whole GluRε2 and epitope analysis, + indicates presence of autoantibodies, and – indicates absence of autoantibodies.

RESULTS

Autoantibodies against GluRε2 molecules in sera and CSF

Autoantibodies against full-length GluRε2 molecule were studied in the sera and or CSF of 20 patients with definitive or clinical Rasmussen encephalitis (Table 2). All nine patients with definitive Rasmussen's encephalitis (five with EPC and four without EPC), and 10 of 11 patients (except Patient 3) with clinical Rasmussen's encephalitis had the autoantibodies in serum or CSF. Although Patient 3 had no autoantibodies against GluRε2, the test was done at the residual stage (22) when EPC and CPS had both remitted. Nineteen patients had IgG autoantibodies against the GluRε2 molecule during the course of disease, and 11 patients had IgM autoantibodies. None of the patients we examined had IgA autoantibodies against GluRε2. In Patients 1, 4, 8, and 13, the IgG and IgM autoantibodies against GluRε2 were absent in the early stage when epileptic seizures already became frequent, and appeared subsequently. In Patient 8, after intravenous high-dose gamma-globulin infusion therapy (200 mg/kg weight, 5 days), EPC remitted and CPSs decreased to a 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 8 showed fluctuation of autoantibodies in relation to the severity of clinical symptoms modulated by immunotherapy. In Patient 15, the autoantibodies became negative after steroid pulse therapy, but epileptic seizures persisted. In Patients 6 and 8, the autoantibodies persisted in the serum after frontal lobe resection or functional hemispherectomy, although epileptic seizures were controlled.

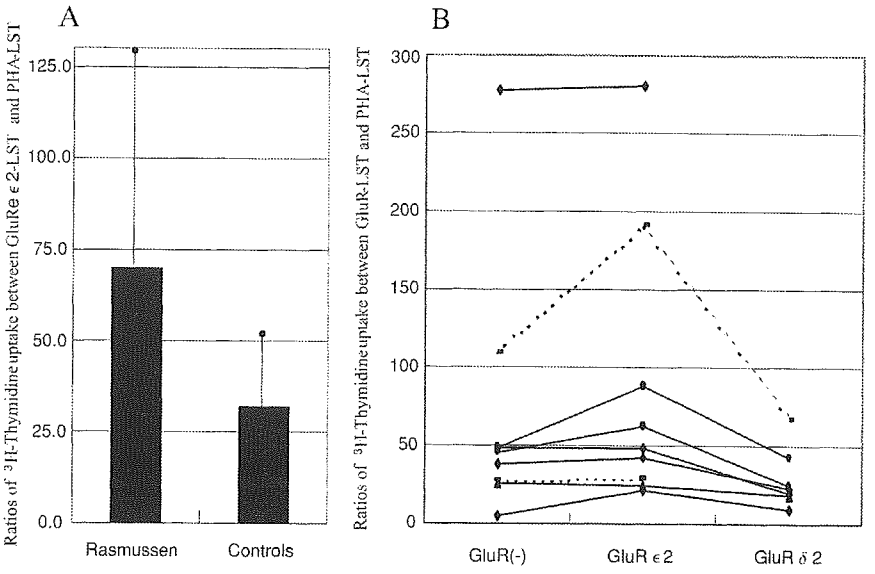

FIG. 1. A: Mean ^{3}H-thymidine ratios of uptake stimulated by GluRε2 relative to PHA in lymphocyte stimulation test (LST) using lymphocytes from patients with Rasmussen syndrome and healthy controls. GluRε2-LST and PHA-LST were conducted using supernatants of cell culture homogenates containing recombinant GluRε2 (B18) and PHA. Vertical axis indicates ratios of uptake stimulated by supernatant of B18 homogenate containing recombinant GluRε2 to that stimulated by PHA (GluRε2/PHA ×1000). **B:** Ratios of uptake stimulated by GluRε2 relative to PHA, those stimulated by GluRδ2 relative to PHA, and those stimulated by control homogenate [GluR (-)] relative to PHA for each patient. GluR (-): Uptake stimulated by control homogenates (A1)/uptake stimulated by PHA ×1000. GluRε2: Uptake stimulated by B18 homogenate containing recombinant GluRε2/uptake stimulated by PHA ×1000. GluRδ2: Uptake stimulated by D33 homogenate containing recombinant GluRδ2/uptake stimulated by ×1000 PHA.

Epitope analyses of autoantibodies against GluRε2 in sera and CSF

IgG autoantibodies against the putative epitopes (N, C1, C2, and C3) of the GluRε2 molecule were examined using bacterial fusion proteins containing the corresponding GluRε2 polypeptides (13). Of 12 patients with IgG autoantibodies against the GluRε2 molecule, three patients had serum autoantibodies to N epitope, four had autoantibodies to C1, seven had autoantibodies to C2, and six had autoantibodies to C3 (Table 2). Autoantibodies to C2 epitope predominated, while autoantibodies to the extracellular N epitope were rare. The epitope pattern in patients without EPC was not different from that in patients with EPC. 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.

Lymphocyte stimulation by GluRs

In a preliminary study, we examined ^{3}H-thymidine uptake after stimulation with B18 homogenates at different protein concentrations in six definitive patients, and the uptake was highest at the concentration of 40 μg/ml. Subsequently, we conducted lymphocyte stimulation tests (LSTs) using homogenates of D33 (cell line expressing GluRδ2 subunits), B18 (cell line expressing GluRε2), and A1 (control cell line without GluR expression) at protein concentrations of 40 and 400 μg/ml. The mean ^{3}H-thymidine uptake ratios for B18 stimulation (uptake stimulated by B18 divided by uptake stimulated by PHA × 1000) were 69.3 ± 60.7 (n = 23) in patients with definitive and clinical Rasmussen encephalitis and 23.1 ± 19.7 (n = 7) in controls ($p < 0.05$, Mann–Whitney's U test) (Fig. 1A). In patients with definitive Rasmussen encephalitis, the mean ^{3}H-thymidine uptake ratios were 69.3 ± 82.8 (n = 9) for A1 stimulation (uptake stimulated by A1 divided by uptake stimulated by PHA ×1000), 87.6 ± 89.4 (n = 9) for B18 stimulation (uptake stimulated by B18 stimulation divided by uptake stimulated by PHA ×1000), and 29.0 ± 19.7 (n = 7) for D33 stimulation (uptake stimulated by D33 divided by uptake stimulated by PHA ×1000). The uptake ratios for B18 stimulation were significantly higher than those for A1 ($p < 0.05$, Wilcoxon signed-ranks test) and D33 stimulation ($p < 0.05$, Wilcoxon signed-ranks test). The uptake ratios of GluRε2 to PHA were relatively low in Patients 6 and 8, after seizures were controlled after epilepsy surgeries, comparing with those in patients with seizures.

DISCUSSION

Rasmussen's encephalitis is divided into two clinical subtypes by the existence of EPC. In our previous article (13), we reported that autoantibodies against NMDA-type GluRε2 were detected in Rasmussen's encephalitis patients with EPC (definitive Rasmussen's encephalitis patients, 3/3 patients, and clinical Rasmussen's encephalitis patients, 6/7 patients). Subsequently, the present study demonstrated the autoantibodies also in patients with definitive Rasmussen's encephalitis without EPC

(4/4 patients). This suggests that autoantibodies against GluRε2 are important for the diagnosis of both subtypes of Rasmussen's encephalitis, independent of EPC. Because EPC is a typical clinical diagnostic marker of Rasmussen's encephalitis, presurgical diagnoses of Rasmussen's encephalitis are usually difficult in patients without EPC, compared with those with EPC. Detection of autoantibodies against GluRε2 can contribute to the presurgical diagnosis and may facilitate immunological treatment in patients with dominant side involvement.

In these 19 patients with Rasmussen's encephalitis and autoantibodies against the NMDA GluRε2 subunit, the epitope pattern in patients with EPC was not different from that in patients without EPC. Clinical subtypes might be determined by some factors other than difference of epitopes of the autoantibodies. We need to investige further into the distribution of immunological lesions to decide the factors.

In this study, we could reveal the existence of peripheral lymphocytes selectively stimulated by GluRε2 in patients with Rasmussen's encephalitis using LST. SD in patients' data of the mean ^{3}H-thymidine uptake ratios is slightly large. This might be attributed to the effect of treatments and the stage of Rasmussen's encephalitis. The ^{3}H-thymidine uptake ratios were lower mainly in patients with steroid therapy, and the ratios tended to become lower in the progressed stage (data not shown). Lymphocytes stimulated by LST are usually T cells. Although we could not confirm a subset of stimulated T cells ($CD4^+$ or $CD8^+$) by GluRε2, activated effector T cells that could invade the CNS beyond the blood brain barrier definitively exist in peripheral blood circulation. We estimate that these activated T cells are produced by cross-reaction using molecular mimicry after an infection and play an important role in the subsequent onset of Rasmussen's encephalitis.

In previous reports, lymphocytic infiltration containing predominantly T cells and sparsely B cells were reported in resected tissues from Rasmussen's encephalitis patients (23), and local CNS immune responses in Rasmussen's encephalitis included local clonal expansion of T cells responding to discrete antigen epitopes (24). Our proof about the existence of peripheral lymphocytes stimulated by GluRε2 can explain the infiltration of T cells in the CNS. $CD8^+$ 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 speculated to generate secondary antibody-mediated damage (25,26). The probable heterogeneity of autoantibodies in Rasmussen's encephalitis, epitope spreading and autoantibodies directed predominantly against intracellular epitopes of NMDA GluRε2, also suggests an important role of T cell-mediated autoimmunity (13). Mice immunized with the GluR3B peptide produced autoantibodies but exhibited no epilepsy, even by facilitated entry of the autoantibodies into the brain (27). These data have suggested that the primary cause of Rasmussen's encephalitis is not autoantibodies, but T cell–mediated autoimmunity. In four patients, the autoantibodies against GluRε2 were absent in the early stage, even when epileptic seizures had already become frequent, and appeared later. This delayed appearance of autoantibodies against GluRε2 also supports the hypothesis about primary cause of Rasmussen's encephalitis.

After epilepsy surgeries, Patients 6 and 8 became seizure free, but the autoantibodies persisted in the serum. However, the uptake ratios for GluRε2 to PHA in LST were relatively low in Patients 6 and 8, when seizures were controlled after epilepsy surgeries. These data suggest that autoantibodies against the GluRε2 in sera are not causally related to active pathophysiological processes, and is not a marker of activity of Rasmussen's encephalitis, while the uptake ratio of GluRε2 to PHA in LST might be a marker of disease activity.

International collaborating studies on the contribution of multiple autoantibodies and cellular immunity to the pathophysiology of Rasmussen's encephalitis are expected to elucidate the pathophysiological mechanisms mediated by autoimmunity in Rasmussen's encephalitis and other neurological diseases.

Acknowledgment: This project was supported by grants-in-aid for Science Research (C) 16590859 and 17591133, Research Grant 16A-3, and a grant from the Japan Epilepsy Foundation.

REFERENCES

1. Takahashi Y, Kubota H, Fujiwara T, et al. Epilepsia partialis continua of childhood involving bilateral brain hemispheres. *Acta Neurol Scand* 1997;96:345–52.
2. Thomas JE, Reagan TJ, Klass DW. Epilepsia partialis continua. A review of 32 cases. *Arch Neurol* 1977;34:266–75.
3. Bancaud J. Kojewnikow's syndrome (epilepsia partialis continua) in children. In: Roger J, ed. *Epileptic syndrome in infancy, childhood and adolescence*. 2nd edition, London: John Libbey, 1992:363–79.
4. Commission on classification and terminology of the International League Against Epilepsy. Proposal for revised classification of epilepsies and epileptic syndromes. *Epilepsia* 1989;30:389–99.
5. Rasmussen T, Olszewski J, Lloyd-Smith D. Focal seizures due to chronic localized encephalitis. *Neurology* 1958;8:435–45.
6. Rogers SW, Andrews PI, Gahring LC, et al. Autoantibodies to glutamate receptor GluR3 in Rasmussen's encephalitis. *Science* 1994;265:648–51.
7. Mori H, Mishina M. Structure and function of the NMDA receptor channel. *Neuropharmacology* 1995;34:1219–37.
8. Mishina M. Molecular diversity, structure, and function of glutamate receptor channels, In: Endo M, Kurachi Y, Mishina M, eds. *Handbook of experimental pharmacology 147: pharmacology of ionic channel function*. Berlin: Springer-Verlarg, 2000:393–414.
9. Morikawa E, Mori H, Kiyama Y, et al. Attenuation of focal ischemic brain injury in mice deficient in the epsilon1 (NR2A) subunit of NMDA receptor. *J Neurosci* 1998;18:9727–32.
10. Nakanishi S, Masu M. Molecular diversity and functions of glutamate receptors. *Annu Rev Biophys Biomol Struct* 1994;23:319–48.
11. Köhr G, De Koninck Y, Mody I. Properties of NMDA receptor channels in neurons acutely isolated from epileptic (kindled) rats. *J Neurosci* 1993;13:3612–27.

12. Mori H, Manabe T, Watanabe M, et al. Role of the carboxy-terminal region of the GluR epsilon2 subunit in synaptic localization of the NMDA receptor channel. *Neuron* 1998;21:571–80.
13. Takahashi Y, Mori H, Mishina M, et al. Autoantibodies to NMDA receptor in patients with chronic forms of epilepsia partialis continua. *Neurology* 2003;61:891–6.
14. Bien CG, Urbach H, Deckert M, et al. Diagnosis and staging of Rasmussen's encephalitis by serial MRI and histopathology. *Neurology* 2002;58:250–7.
15. Maeda Y, Oguni H, Saitou Y, et al. Rasmussen syndrome: multifocal spread of inflammation suggested from MRI and PET findings. *Epilepsia* 2003;44:1118–21.
16. Gossen M, Freundlieb S, Bender G, et al. Transcriptional activation by tetracyclines in mammalian cells. *Science* 1995;268:1766–9.
17. Kutsuwada T, Kashiwabuchi N, Mori H, et al. Molecular diversity of the NMDA receptor channel. *Nature* 1992;358:36–41.
18. Katoh K, Takahashi Y, Hayashi S, Kondoh H. Improved mammalian vectors for high expression of G418 resistance. *Cell Structure and Function* 1987;12:575–80.
19. Takahashi Y, Sakaguchi N, Kondo N, Mori H, Mishina M. Early diagnosis and treatment in intractable epileptic patients with autoantibodies against glutamate receptor. *Ann Rep Jpn Epi Res Found* 1999;11:45–52.
20. Watanabe M, Fukaya M, Sakimura K, et al. Selective scarcity of NMDA receptor channel subunits in the stratum lucidum (mossy fiber-recipient layer) of the mouse hippocampal CA3 subfield. *Eur J Neurosci* 1998;10:478–87.
21. Smith DB, Johnson KS. Single-step purification of polypeptides expressed in *Echerichia coli* as fusions with glutathione S-transferase. *Gene* 1988;67:31–40.
22. Bien CG, Widman G, Urbach H, et al. The natural history of Rasmussen's encephalitis. *Brain* 2002;125:1751–9.
23. Farrell MA, Droogan O, Secor DL, et al. Chronic encephalitis associated with epilepsy: immunohistochemical and ultrastructural studies. *Acta Neuropathol* 1995;89:313–21.
24. Li Y, Uccelli A, Laxer KD, et al. Local-clonal expansion of infiltrating T lymphocytes in chronic encephalitis of Rasmussen. *J Immunol* 1997;158:1428–37.
25. Bien CG, Bauer J, Deckwerth TL, et al. Destruction of neurons by cytotoxic T cells: a new pathogenic mechanism in Rasmussen's encephalitis. *Ann Neurol* 2002;51:311–8.
26. Gahring LC, Carlson NG, Meyer EL, Rogers SW. Cutting edge: granzyme B proteolysis of a neuronal glutamate receptor generates an autoantigen and is modulated by glycosylation. *J Immunol* 2001;166:1433–8.
27. Levite M, Hermelin A. Autoimmunity to the glutamate receptor in mice –A model for Rasmussen's encephalitis? *J Autoimmunity* 1999;13:73–82.

GDEV 158935—6/2/2006—MADHAVANS—200444

Clinical & Developmental Immunology, 2006; 00(0): 1–7

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Vaccination and infection as causative factors in Japanese patients with Rasmussen syndrome: Molecular mimicry and HLA class I

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Abstract

Rasmussen syndrome is an intractable epilepsy with a putative causal relation with cellular and humoral autoimmunity. Almost half of the patients have some preceding causative factors, with infections found in 38.2%, vaccinations in 5.9% and head trauma in 8.9% of Japanese patients. In a patient with seizure onset after influenza A infections, cross-reaction of the patient's lymphocytes with GluRε2 and influenza vaccine components was demonstrated by lymphocyte stimulation test. Database analyses revealed that influenza A virus hemagglutinin and GluRε2 molecules contain peptides with the patient's HLA class I binding motif (HLA − A*0201). The relative risks of HLA class I genotypes for Rasmussen syndrome are 6.1 (A*2402), 6.4 (A*0201), 6.3 (A*2601) and 11.4 (B*4601). The relative risks of HLA class I-A and B haplotypes are infinity (A*2601 + B*5401), 21.1 (A*2402 + B*1501), 13.3 (A*2402 + B*4801) and 5.1 (A*2402 + B*5201). Some alleles and haplotypes of HLA class I may be the risk factors in Japanese patients. Cross-reactivity of cytotoxic T lymphocytes may contribute to the processes leading from infection to the involvement of CNS.

Keywords: *Rasmussen syndrome, HLA, cytotoxic T cells, influenza, vaccination, epilepsy*

Introduction

Rasmussen's encephalitis is a slowly progressive, autoimmune-mediated chronic inflammatory disease of the CNS. The mean age of onset is 7.4 years. The disease may be preceded by some causative factors including infection, and the initial seizure episode manifests various forms such as partial onset generalized tonic-clinic (pGTC) seizures (30%), focal motor seizures (26%) and complex partial seizures (CPS) (26%) (Andermann 1991). One third of the patients have preceding infections within 1 month before onset. Patients with typical Rasmussen's encephalitis manifest frequent intractable partial motor seizures in the acute phase, characteristically epilepsia partialis continua (EPC) (56%). Patients begin to manifest EPC 1.8 years after the onset of epilepsy, but the seizure frequency decreases markedly in the residual stage (Andermann 1991, Bien et al. 2002b) (Figure 1). Patients in the residual stage are affected by hemiplegia (96%), mental deterioration (85%), visual field defect (49%), and cortical sensory defect (29%) (Andermann 1991). Histological examination reveals infiltration of T lymphocytes and microglia cells, astrocytosis, and neuronal loss in the lesion (Aguilar and Rasmussen 1960, Andermann 1991, Farrell et al. 1995). Functional hemispherectomy is the only reliable therapy when the non-dominant side is involved, but hemiparesis and hemianopsia are

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ISSN 1740-2522 print/ISSN 1740-2530 online © 2006 Taylor & Francis
DOI: 10.1080/17402520600589522