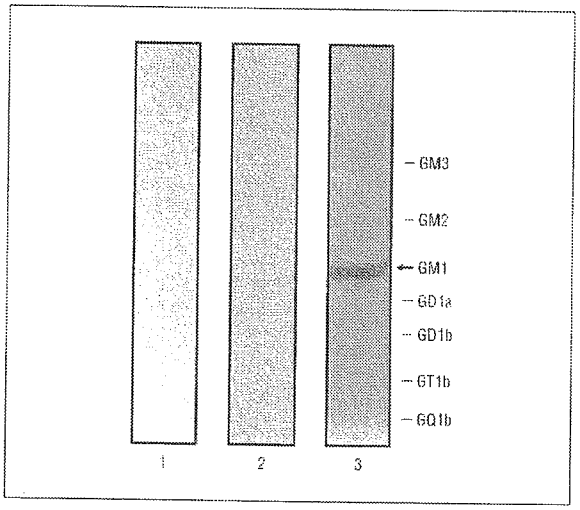

Figure. Electrothermal blotting of gangliosides following immunoblot analysis with serum. Various ganglioside subspecies (GM3, GM2, GM1, GD1a, GD1b, GT1b, GQ1b, and asialo GM1) were electrothermally blotted onto a polyvinylidene difluoride membrane. The membrane was probed with serum from the present patient obtained before (at day 1; lane 1) and after (at day 20; lane 2) intravenous immunoglobulin treatment, and with serum samples from positive controls who have anti-GM1 antibody in the serum (lane 3). The positions of each ganglioside were determined on a thin-layer chromatography plate developed simultaneously without electrothermal blotting. Gangliosides were visualized with the resorcinol reagent.[16] These experiments were performed at least 3 times using different serum samples, with essentially identical results. The arrow indicates the position of GM1 ganglioside.

rus DNA in the CSF, however, is considered a reliable diagnostic tool for infections of the central nervous system,[12] although a DNA polymerase chain reaction can occasionally yield false-positive results.[13] In our case, HHV-7 DNA was the only viral DNA detected in the CSF, and, more importantly, evidence of HHV-7 DNA disappeared following therapy. These changes were accompanied by concomitant changes in anti–HHV-7 serum antibody titers. Judging from this evidence, we speculate that the development of the clinical features of the patient is closely related to the reactivation of HHV-7 in the nervous system. At present, however, we do not know why our patient developed a reactivation of HHV-7 in the nervous system, even though an immunologically competent state was absent as described in other cases of HHV-6.[14] Based on the time sequence of the onset of the symptoms, we also speculate that HHV-7 might result in the production of neuronal autoantibodies, as has been described with antiglycosphingolipid antibody following CMV infection,[5] although there was no antiganglioside antibody in the serum of this patient. Primary HHV-7 infection of the spinal cord also remains a distinct possibility.

The clinical presentation of this case exhibited several characteristics of GBS. This was especially true for the nerve conduction study data. However, there were also signs of modest involvement of the spinal cord as evidenced by the transient presence of positive pathological reflexes. Previous analyses on 229 patients with GBS have suggested that all of the patients with CMV or Epstein-Barr virus infection showed demyelinating neuropathy[15] whereas the present patient showed an axonal neuropathy.

This case supports the contention that HHV-7 may be a pathological factor in the development of acute myeloradiculoneuropathy.

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CD16+CD57– Natural Killer Cells in Multifocal Motor Neuropathy

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Key Words
Natural killer cells · Multifocal motor neuropathy · Fc receptor · Intravenous immunoglobulin · Cyclophosphamide

Abstract
We analyzed the CD16+CD57– lymphocyte subset, which is considered to have strong natural killer (NK) cell activity, in peripheral blood from patients with chronic immune-mediated neuropathies and patients with other neurological diseases. We found that the ratio of CD16+CD57– NK cells to total lymphocytes was increased in 4 of 6 patients with multifocal motor neuropathy (MMN) with persistent conduction block. Since the CD16 molecule is an Fc receptor for immunoglobulin G (IgG), high-dose intravenous immunoglobulin (IVIg) may interfere with CD16+CD57– NK cells via Fc receptor blockade. In addition, cyclophosphamide (Cy) is often used to suppress NK cells. Therefore, our findings may partly account for the effectiveness of IVIg or Cy, which is the current treatment of choice for MMN.

Introduction

Natural killer (NK) cells have the ability to lyse a variety of cells without major histocompatibility complex restriction, and NK cell lymphoproliferative disease is occasionally complicated by peripheral neuropathies [1–4]. However, the roles of NK cells in peripheral neuropathies have not been fully clarified. The CD16 (Leu11) molecule, known as one of the NK cell markers, is an Fc receptor for immunoglobulin G (IgG) [5]. Lanier et al. [6] demonstrated that CD16+CD57– cells in human peripheral blood (PB) have strong NK activity. In this study, we performed flow cytometric analysis of the CD16+CD57– NK cell subset in PB obtained from patients with chronic immune-mediated neuropathies and patients with other neurological diseases. As a result, some patients with multifocal motor neuropathy (MMN) had an increase in the NK cell subpopulation.

Patients and Methods

We studied 12 patients with chronic inflammatory demyelinating polyneuropathy (CIDP; 4 women, 8 men, age range 25–83 years, mean age 53.0 years, disease duration range 3 months to 12 years) and 6 patients with MMN (3 women, 3 men, age range 17–

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Table 1. Clinical profile of 8 patients who showed an increase in the ratio of CD16+CD57– natural killer cells

Patient No.	Age years/sex	CD16+CD57– cells, %[a]	Diagnosis
1	40/M	32.6	MMN
2	71/M	20.9	Viral encephalitis
3	59/F	15.1	Viral encephalitis
4	17/F	13.5	MMN
5	46/F	12.9	MMN
6	30/F	12.4	CIDP
7	67/M	12.2	MMN
8	28/M	12.1	Viral encephalitis

MMN = Multifocal motor neuropathy; CIDP = chronic inflammatory demyelinating polyneuropathy.

[a] Cutoff value = 10.4%.

Table 2. Ratio of CD16+CD57– natural killer cells in each patient group

Patient group	Ratio, %
MMN (n = 6)	13.5 ± 4.2
CIDP (n = 12)	4.6 ± 1.0[a]
HMSN (n = 6)	4.7 ± 1.0[a]
Motor neuron disease (n = 22)	4.1 ± 0.4[b]
Parkinson's disease (n = 7)	3.8 ± 0.6[a]
HTLV-I-associated myelopathy (n = 12)	2.5 ± 0.4[b]
CNS infections (n = 12)	7.2 ± 1.8
Cerebrovascular disease (n = 8)	3.6 ± 0.4[a]
Epilepsy (n = 8)	4.0 ± 0.5[a]

MMN = Multifocal motor neuropathy; CIDP = chronic inflammatory demyelinating polyneuropathy; HMSN = hereditary motor and sensory neuropathy; CNS = central nervous system.

Values represent mean ± SEM. Statistical analysis was performed using the Mann-Whitney U test.

[a] $p < 0.05$; [b] $p < 0.01$ compared to the MMN patient group.

67 years, mean age 45.5 years, disease duration range 16 months to 15 years). Diagnosis of CIDP was based on the criteria of the American Academy of Neurology, and MMN according to the criteria of the American Association of Electrodiagnostic Medicine as described previously [7]. Serum IgM anti-GM1 antibodies were positive in 3 of 6 patients with MMN. Disease controls were as follows: hereditary motor and sensory neuropathy (n = 6); motor neuron disease (n = 22); Parkinson disease (n = 7); HTLV-I-associated myelopathy (n = 12), and infections of the central nervous system (CNS) such as encephalitis (n = 12), cerebrovascular disease (n = 8) and epilepsy (n = 8).

Flow Cytometric Analysis

PB mononuclear cells (PBMCs) were separated from 5 ml of CPD (citrate, phosphate, dextrose)-blood of each patient. Then 1×10^5 PBMCs in RPMI-1640 were incubated with PE-conjugated monoclonal antibody (MoAb) against CD16 (Becton Dickinson Immunocytometry Systems, Oxnard, Calif., USA) and FITC-conjugated MoAb against CD57 (Becton Dickinson Immunocytometry Systems) at 4°C for 30 min. Two-color flow cytometric analysis was performed using CYTRON (Ortho-Clinical Diagnostics K.K, Tokyo, Japan). Gates were set on lymphocytes. Results were expressed as the ratio of CD16+CD57– cells to total lymphocytes in percentage. The cutoff value was determined as mean + 2 SD (10.4%) based on the results of normal subjects (n = 72) in Falco Bio Systems Ltd. (Kyoto, Japan). The Mann-Whitney U test was used to assess intergroup comparisons.

Results

The ratio of CD16+CD57– NK cells to total lymphocytes was increased in 8 patients including 3 with viral encephalitis, 1 with CIDP, and 4 with MMN (patients 1, 4, 5, and 7), as shown in table 1. In the 4 MMN patients, there was no clinical sign or evidence of infection. These 4 patients were retested and similar results were obtained (data not shown). Especially, a marked increase in CD16+CD57– NK cells and a decrease in CD3+ cells and in the CD4/CD8 ratio were demonstrated in patient 1, who has previously been reported elsewhere as a typical case of MMN [8, 9].

The mean ratio of CD16+CD57– NK cells was significantly higher in the MMN group than in the other patient groups except for the group with CNS infections (table 2).

Discussion

In disease controls, 3 patients with viral encephalitis had an increase in the proportion of CD16+CD57– NK cells in PB. This finding is in conformity with the previous concept that NK cell number and activity may change in relation to acute infections. In addition, we found that 4 of 6 patients with MMN had an increase in CD16+CD57– NK cells. MMN is a slowly progressive neurological disorder characterized by asymmetrical limb weakness with persistent conduction block. Although the disease etiology remains unknown, immunological processes are thought to be involved.

In the MMN patient with the highest percentage of CD16+CD57– NK cells (patient 1), focal gadolinium enhancement of the enlarged median nerve was observed

by magnetic resonance imaging, suggesting disruption of the blood-nerve barrier (BNB) at the conduction block site [9]. This BNB impairment may partly contribute to the persistence of motor conduction block in MMN through interference with remyelination of the affected nerve [9]. Since activated NK cells were shown to bind to vascular endothelial cells [10] and cause their lysis [11], the increase in CD16+CD57- NK cells in PB might be associated with the breakdown of BNB. Interestingly, an increase in NK cells in PB was observed in amyotrophic lateral sclerosis with conduction block, but not in amyotrophic lateral sclerosis without such block [12]. Therefore, abnormalities in NK cell subpopulations might be related to the conduction block.

Steroid therapy is often effective in CIDP, whereas MMN patients do not improve and occasionally even deteriorate with steroid treatment [13–18] despite the fact that MMN is pathologically a demyelinating disorder [8]. In addition, plasma exchange is likewise ineffective in MMN [13, 14, 16, 19], although levels of IgM anti-GM1 antibodies are elevated in some patients with MMN [13–17, 20]. However, treatment with intravenous immunoglobulin (IVIg) [14–17] or cyclophosphamide (Cy) [8, 13, 16, 20] often improves clinical symptoms in those patients. In the present series, both IVIg and Cy were effective in patient 1, and IVIg was effective in patients 4, 5, and 7. Since the CD16 molecule is one of the Fc receptors for IgG (FcγR III) [5], high-dose IVIg may interfere with CD16+CD57- NK cells through Fc receptor blockade. Engelhard et al. [21] reported that NK activity was decreased in patients with autoimmune neutropenia or idiopathic thrombocytopenic purpura after IVIg. Interestingly, IVIg has recently been reported to modulate monocyte FcγR II expression in patients with chronic inflammatory neuropathies [22]. Moreover, Cy is often used in the field of hematology to suppress NK cells [23–25]. Thus, the beneficial effects of IVIg or Cy in the treatment of MMN may be partly explained if NK cells play a role in its pathogenesis.

We cannot explain clearly why discordant results were observed in 1 CIDP case (patient 6) and in 2 MMN cases. However, heterogeneity in the immunopathogenesis of each disease might be related. Accumulation of further data from a large number of patients is necessary to verify our hypothesis.

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Acute limbic encephalitis: A new entity?

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Abstract

Clinical cases similar to herpes simplex virus (HSV) encephalitis have accumulated in Japan. Detailed examinations have failed to demonstrate HSV infection. Recently, these cases have been named "non-herpetic acute limbic encephalitis". Only a single autopsy case was so far reported in an abstract form, because many cases showed a good prognosis. The case presented here was that following fever, a 59-year-old woman developed disturbance of consciousness and uncontrollable generalized seizures. Brain MRI revealed abnormal signals in the bilateral medial temporal lobe and along the lateral part of the putamen. Autoantibody against the NMDA glutamate receptor (GluR) IgM-ε2 was detected in the serum, and the GluR IgG-δ2 antibody was positive in cerebrospinal fluid. She died 12 days after onset. An autopsy examination revealed scattered foci consisting of neuronal loss, neuronophagia and some perivascular lymphocytic infiltration in the hippocampus and amygdala, but no haemorrhagic necrosis in the brain. HSV-1, -2 and human herpes virus-6 were negative immunohistochemically. We believe that our autopsy case may contribute to understanding the neuropathological background of non-herpetic acute limbic encephalitis.

Keywords: Acute encephalitis; Status epilepticus; Autopsy; Non-herpetic acute limbic encephalitis; Herpes simplex encephalitis

Limbic encephalitis is usually considered to be paraneoplastic, occurring subacutely in association with specific neuronal antibodies [2]. Among the cases with reversible acute or subacute non-paraneoplastic limbic encephalitis, voltage-gated potassium channel (VGKC) antibodies have been reported [12]. Autoantibodies against the NMDA glutamate receptor (GluR), which is considered to be related causally to partial seizures [11], were detected in the acute non-herpetic encephalitis [3].

In Japan, acute encephalitis, in which the clinical picture was comparable with that of herpes simplex virus (HSV) encephalitis but where evidence of HSV infection was not demonstrated, has been reported [5]. Recently, these cases have been named "non-herpetic acute limbic encephalitis" as a possible new subgroup of limbic encephalitis [5,9]. It has been proposed that mild infections and immunological process are the cause of this disease from clinical findings and cerebrospinal fluid (CSF) cytokine levels, elevated level of interleukin-6 [5,9] and unelevated level of interferon-γ [1]. Moreover, it has been indicated that acute limbic encephalitis, HSV encephalitis and other acute limbic encephalitis were etiologically interrelated, because cases of limbic encephalitis similar to non-herpetic acute limbic encephalitis were reported [1,9].

Many previously reported cases of non-herpetic acute limbic encephalitis have shown a rather favorable prognosis [1,4,5,7,8,10]. For this reason, only a single autopsy case was so far reported in an abstract form [7]. We believe that this report contributes to understanding the neuropathological background of the acute limbic encephalitis of unknown etiology.

One week after a fever, a 59-year-old woman developed progressive disturbance of consciousness following generalized tonic seizures. The brain computed tomography showed no abnormalities. CSF examinations showed mononuclear cells 10 μl/l, protein 50 mg/dl and glucose 143 mg/dl. The seizures continued, even though multiple anticonvulsants were administered and mechanical ventilation was performed. Eight days after the onset of unconsciousness and seizures, brain magnetic resonance imaging (MRI) with T2-weighted and FLAIR images revealed high signal intensities in the bilateral medial temporal lobes and along the lateral part of the putamen (Fig. 1). She was admitted to our hospital 10 days after the onset of the seizures. She showed marked emaciation and pneumonia complications. Recurrence of generalized tonic seizures

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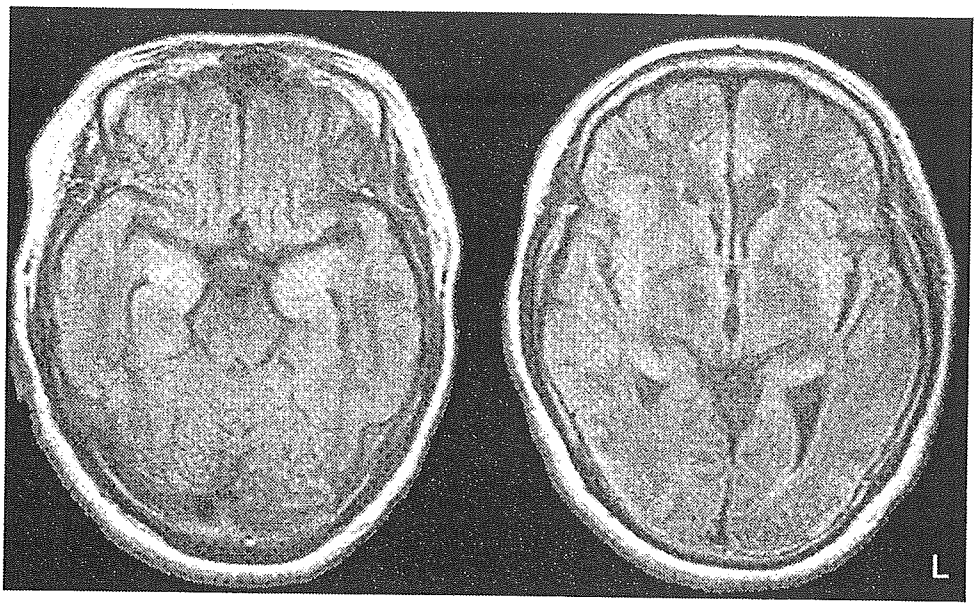

Fig. 1. FLAIR MRI images. High signal intensity is seen in the bilateral medial temporal lobe and the lateral part of the putamen.

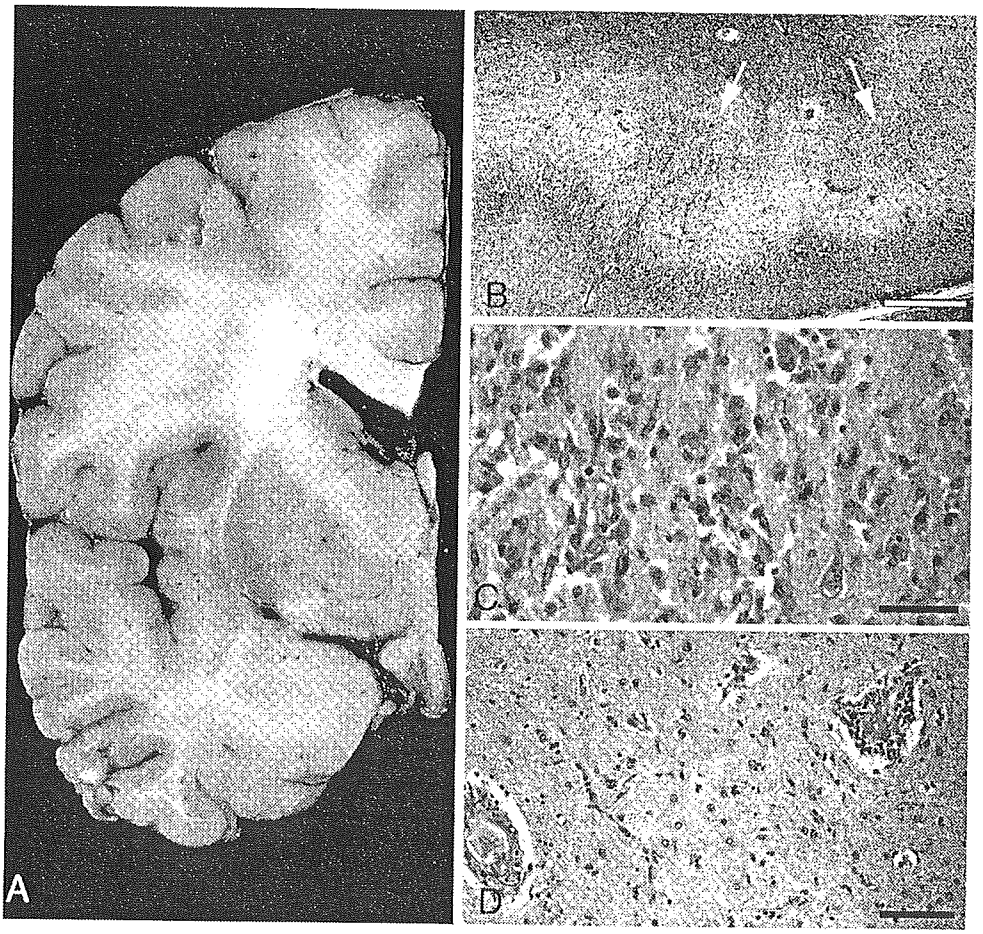

Fig. 2. Neuropathological findings: (A) coronal slice through the left cerebrum. No lesions visible on macroscopic examination; (B) foci of neuronal loss (arrows) surrounded by spongy state in the rostral CA1 of hippocampus. Klüver–Barrera staining (Bar 500 μm); (C) foci of neuronal loss and neuronophagia in the rostral CA1 of hippocampus. Hematoxylin and eosin (HE) staining (Bar 50 μm) and (D) neuronal loss, fibrillary astrocytosis and lymphocytic perivascular cuffing were seen in the rostral part of amygdala. HE staining (bar 50 μm).

failed to be controlled with propofol and acyclovir in addition to the anticonvulsants. An electroencephalogram revealed multifocal spikes without periodic synchronous discharges and periodic lateralized epileptiform discharges. Autoantibodies, including antinuclear antibody, anti-SS-A/B antibodies, and anti-Hu antibodies were all negative. Autoantibody against the GluR IgM-ε2 [11] in the serum was positive, autoantibody against GluR IgG-δ2 in the CSF was positive, and VGKC antibody and P/Q-type voltage-gated calcium channel antibodies were negative in the serum and CSF. Antibodies for several viruses including HSV in the serum and CSF were negative 10 days after the onset of seizures. She had acute renal failure complications and died 12 days after the onset of the seizures.

The direct cause of death was acute renal tubular necrosis and purulent pneumonia. Both laboratory data and pathological examination revealed that the patient did not have any malignant tumors or collagen disease. The brain, weighing 1183 g, was macroscopically unremarkable except for mild swelling (Fig. 2A). Microscopically, there were no leptomeningitis. The amygdala and hippocampus showed small foci of neuronal loss with neuronophagias, proliferation of microglias and hypertrophic astrocytes (Fig. 2B and C). These foci were surrounded by a spongy state. Only a few lymphocytic perivascular cuffings occurred in the amygdala (Fig. 2D). No intranuclear inclusion bodies were found anywhere. Immunohistochemistry for HSV-1, -2 and human herpesvirus-6 was negative. No tissue necrosis or haemorrhage were found in the cerebral cortex including the cingulate, insular, and parahippocampal cortex.

Besides demonstrating evidence of HSV infection, HSV encephalitis shows extensive necrosis with haemorrhage in the medial temporal lobe, insular and cingulate gyri bilaterally [6], where the brain MRI shows high signal intensities. Furthermore, the lesions are bilateral, but not always symmetrical in distribution. In our case, however, abnormal signal intensities were limited in the hippocampus and amygdala bilaterally and symmetrically. No haemorrhagic necrosis was found anywhere, even though there would not have been sufficient time for our patient to develop it. Finally, there were no intranuclear inclusions or immunohistological evidence of HSV infection.

Recently, it is suggested that the presence of autoantibodies against the GluR-ε2 in the CSF of non-herpetic acute encephalitis involves in autoimmune pathogenic mechanism [3,9]. In the CSF of this patient, autoantibody against the GluR-ε2 was negative, while the autoantibody against the GluR-δ2, which is against cerebellar Purkinje cell-specific antibody [11] was positive. The other similar cases as shown in Table 1 [1,4,5,7,8,10] were not examined for the presence of these antibodies. Unfortunately, it remains obscure that this antibody played a role in development of the disease in our case.

There has been only one pathological report of a patient similar to our case: a 53-year-old woman who died 36 days after the onset of illness, and showed neuronal loss in the hippocampus, and neuronophagia and gliosis in the amygdala [7]. As seen in the present patient, this patient showed no evidence of HSV infection, no apparent necrosis in the brain, and the

Table 1
Clinical characteristics and MRI abnormalities of patients with non-herpetic acute limbic encephalitis

Patients	Kohira et al. [4]	Kusuhara et al. [5]				Asaoka et al. [1]						Nonaka et al. [8]	Takahashi et al. [10]		Maki et al. [7]	Present case
		Case 1	Case 2	Case 3	Case 4	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6		Case 1	Case 2		
Age (year)	25	40	38	38	53	34	60	73	35	23	18	42	25	58	53	59
Sex	M	M	F	M	F	M	F	F	M	M	F	F	F	M	F	F
Clinical symptoms																
Impaired consciousness	2+	3+	3+	+	3+	2+	+	2+	2+	2+	2+	3+	+	+	3+	3+
Seizures	2+	2+	3+	+	–	+	+	+	2+	+	3+	3+	2+	–	3+	3+
Cerebrospinal fluid																
Cells (mm^3)	52	17	47	9	10	10	5	32	8	5	320	10	1	76	normal	10
Protein(mg/dl)	25	325	55	27	50	72	32	29	41	28	86	40	15	45	normal	50
MRI abnormalities																
Hippocampi	B	B	B	B	L	B	R>L	B	B	B	L>R	B	B	B	B	B
Amygdalae	B	B	B	B	L	B	R>L	B	B	B	L>R	B	B	B	B	B
Cingulate gyri	–	–	L	–	–	–	–	B	–	–	–	–	–	–	–	–
Sequelae	+	+	+	+	+	+	2+	2+	+	+	+	+	+	+	died	died

M: male; F: female; B: bilateral; L: left; R: right, (–): negative; (+): mild; (2+): moderate and (3+): severe.

lesions were exclusively limited to the hippocampus and amygdala. In this regard, similar clinical cases with acute encephalitis have accumulated in Japan, as shown in Table 1 [1,4,5,7,8,10]. Many cases with this type of encephalitis showed good prognosis, although patients died because of uncontrollable generalized seizures during the clinical course. It is likely that our case showed the neuropathological changes of non-herpetic acute limbic encephalitis as a possible clinicopathological new entity.

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Paraneoplastic Neurologic Syndrome and Autoimmune Addison Disease in a Patient with Thymoma

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ABSTRACT: A 48-year-old man with autoimmune Addison disease developed the following paraneoplastic neurologic syndromes (PNNS): limbic encephalitis, opsoclonus/myoclonus, and sensorimotor and autonomic neuropathies. An anterior mediastinal mass detected on a chest computed tomographic scan was found on resection to be a noninvasive lymphocytic thymoma. The PNNS went into remission 1 year after the thymectomy. This is the first case of thymoma associated with autoimmune Addison disease and PNNS to be described in the literature. **KEY INDEXING TERMS:** Addison disease; Hyponatremia; Paraneoplastic neurologic syndrome; Thymoma. **[Am J Med Sci 2005;329(1):48–51.]**

Thymomas are epithelial tumors of the thymus and are associated with the highest frequency of paraneoplastic autoimmune diseases among human neoplasms.[1] Autoantibodies often appear in patients with paraneoplastic neurologic syndrome (PNNS) and with thymoma are directed mainly against skeletal muscles or the nervous system.[2] PNNS is immune-mediated and associated with a neoplasm but lies anatomically remote from it and is not due to any direct effects of the tumor itself, metastases, opportunistic infections, complications of drug or radiation therapy, or malnutrition.[3,4] Myasthenia gravis is the most common of the various types of thymoma-related PNNS reported.[5–8]

Autoimmune Addison disease is a chronic disease with a long dormant period and results from progressive autoimmune destruction of more than 90% of the adrenals.[9] Autoantibodies against 21-hydroxylase are found in more than 90% of newly symptomatic patients and help diagnose this disorder.[10] The destruction is probably secondary to cytotoxic T-cells, and the significance of these antibodies in the pathogenesis of adrenal insufficiency is unknown.[9]

We report here a unique patient with thymoma who developed autoimmune Addison disease and PNNS 1 year apart and discuss the role of thymomas in the pathogenesis of these disorders.

Case Report

A 47-year-old man, suffering from malaise and with a 30% weight loss over the past year, was admitted to the Gifu Red Cross Hospital in April 1996. He had no family history of autoimmune or endocrine disease. Generalized pigmentation and hyponatremia (132 mmol/L) were observed. He was diagnosed with autoimmune Addison disease based on high plasma ACTH level (174 pmol/L), undetectable plasma cortisol level (<28 nmol/L) and aldosterone level (<69 pmol/L), and low urinary free cortisol level (<28 nmol/d) and 17-OHCS (4.1 μmol/d) together with bilaterally atrophic adrenals revealed by computed tomography (CT) scan. With a replacement dosage of cortisone acetate (37.5 mg/d) in two divided doses, malaise, body weight, and hyponatremia improved.

In September 1997, the patient experienced proximal weakness of the lower extremities and numbness in the hands and legs with a 15% weight loss. Hyponatremia (131 mmol/L) was again noted despite cortisone acetate therapy. Physical examination revealed sinus tachycardia (110/min), sweating, finger tremor without goiter, muscle atrophy in the thigh, and distal sensory loss with reduced deep tendon reflexes in the lower extremities. His disease progressed and clinical state deteriorated. Myoclonus, muscle cramps, saccadic eye movement, gait disturbance, and dysar-

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thria due to cerebellar ataxia, delirium, auditory hallucinations, and a neurogenic bladder developed in November 1997. Axonal loss, with low-amplitude sensory and motor amplitudes and normal conduction velocities, was found on electrophysiologic studies. Cerebrospinal fluid (CSF) analysis revealed normal cell number and protein concentration with no oligoclonal band. Serum anti-acetylcholine receptor, anti-Hu, anti-Yo, and anti-Ri autoantibodies were absent. Anti-voltage-gated potassium channel antibody, which specifically appears in patients with Isaacs syndrome and is measured by the patch clamp method using human neuroblastoma cell line (NB-1), was also negative.[11] Neither cranial CT scan nor magnetic resonance imaging scan was remarkable. Chest CT scan revealed a mass with homogeneous density enhanced by contrast medium in the anterior mediastinum without metastases or invasion of other organs.

Hyponatremia (123 mmol/L) and low plasma osmolarity (263 mmol/kg) were observed despite high urinary osmolarity (774 mmol/kg). Other electrolyte and serum thyroid hormone levels and renal function were normal. Plasma antidiuretic hormone (ADH) concentration was inappropriately elevated (17.2 pmol/L), but urinary excretion of ADH was within the normal range (127 ng/d) after ingestion of 75 mg cortisone acetate at 0830 hours and 37.5 mg at 2030 hours. Plasma ACTH at 0800 hours was reduced to 11 pmol/L after taking 1 mg of dexamethasone at 2300 hours on the previous day. Plasma renin activity (PRA) was high (3.0 ng/L/s), and plasma atrial natriuretic peptide (ANP) was undetectable (<10 ng/L). With isotonic saline infusion (500 mL/d) and fludrocortisone administration (0.05 mg/d) in addition to the cortisone acetate for 3 weeks, the serum sodium concentration returned to normal.

An encapsulated mass (88 × 64 × 25 mm) without macroscopic invasion was surgically removed in January 1998. The pathologic finding was a noninvasive lymphocytic thymoma with negative immunohistochemical staining for ADH. Electron microscopy revealed no secretory granules corresponding to ADH in the cytoplasm. ADH content in the thymoma was 0.1 pg/mg wet tissue, which was too low for an ADH-producing thymoma.[12] Immunoblotting with the patient's serum demonstrated specific bands at 72, 54, and 48 kD with human adrenal (Figure 1A), at 48 kD with his thymoma (Figure 1B), and at 85 kD with NB-1 cells (Figure 2). No obvious change in the serum autoantibodies was observed 10 months after the surgery.

The patient completely recovered from PNNS 1 year after the surgery while taking a replacement dosage of prednisolone (7.5 mg/d) for Addison disease. Serum sodium concentrations have remained in the normal range, although fludrocortisone administration was stopped in April 1998. Twenty-four-hour urinary ADH excretion was within the normal range but plasma ADH concentrations were still high (16.7 pmol/L on the 16th day and 3.0 pmol/L 1 year after the thymectomy). PRA and plasma ANP normalized in August 1998. No recurrence of the thymoma or PNNS has been observed so far.

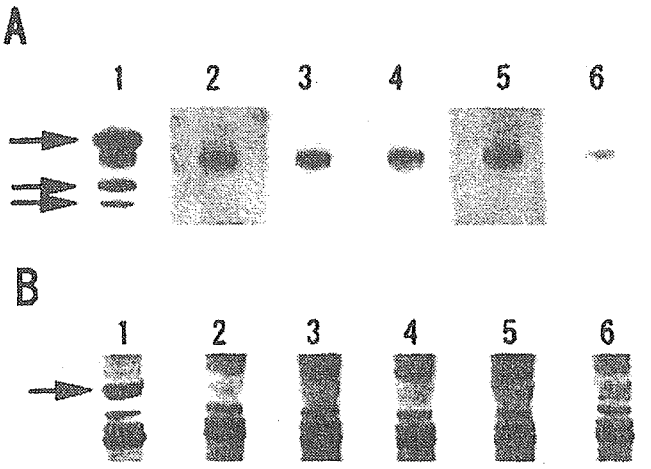

Figure 1. Antiadrenal autoantibodies (A) and antithymoma autoantibody (B) detected by Western blots. Thymoma of the patient and a human adrenal (obtained with informed consent at surgery from a patient with renal carcinoma) were homogenized in lysis buffer. The homogenates were centrifuged at 3000 × g for 10 minute followed by the supernatant being incubated with protein A Sepharose to precipitate nonspecific immunoglobulin.[6] Proteins (100 μg per lane) were subjected to sodium dodecyl sulfate-7.5% polyacrylamide gel electrophoresis, and transferred by electroblotting to a nitrocellulose membrane. The membrane was separated into six pieces and each was incubated overnight at 4°C with serum diluted to 1:200 of the patient (lane 1) and five healthy control subjects (lane 2 to 6). After washing, the membranes were incubated for 15 minutes with peroxidase-conjugated rabbit antihuman IgG diluted to 1:20,000. The immunoreactive proteins were developed with the enhanced chemiluminescence system. Arrows indicate the specific bands. They were estimated to be 72, 54, and 48 kD for the adrenal and 48 kD for the thymoma by multiple regression analysis with reference to molecular weight standards.

Discussion

A syndrome of inappropriate secretion of ADH related to thymoma has been reported.[13] However, the hyponatremia with hyperosmolar urine and elevated plasma ADH concentration observed in the present case could not have been due to this syndrome of inappropriate secretion of ADH but rather to insufficient glucocorticoid replacement, because of (1) high PRA and suppressed ANP, and (2) no evidence of ADH production from the thymoma given the immunohistochemistry and electromicroscopy results, normal 24-hour urinary excretion, and little content of ADH in the thymoma. Increased demand of glucocorticoid must have been associated with the thymoma, because an ordinary dose of glucocorticoid was sufficient to keep the patient's serum sodium concentrations normal after the thymectomy.

Paraneoplastic neurologic syndromes are classified into several types depending on their symptoms and signs.[3,4] In the present case, the diagnosis of PNNS corresponded to a combination of limbic encephalitis, opsoclonus/myoclonus, and sensorimotor and autonomic neuropathies. It is not unusual for more than one type of PNNS to occur with a neoplasm.[4] With regard to the cause of PNNS, antibodies against a surface antigen or intracellular protein of the neoplasm may cross-react with similar antigens in the nervous system or muscles and can evoke a variety of neurologic symptoms.[4] Several autoantibodies correlated with neurologic disorders and the tumors have been characterized in PNNS and have proved to be helpful in the diagnosis.[3] In the present case, however, no well-known autoantibodies could be detected. However, detection of unknown antibodies against 48-kD proteins in the adrenal and thymoma (Figure 1) or 850-kD proteins in

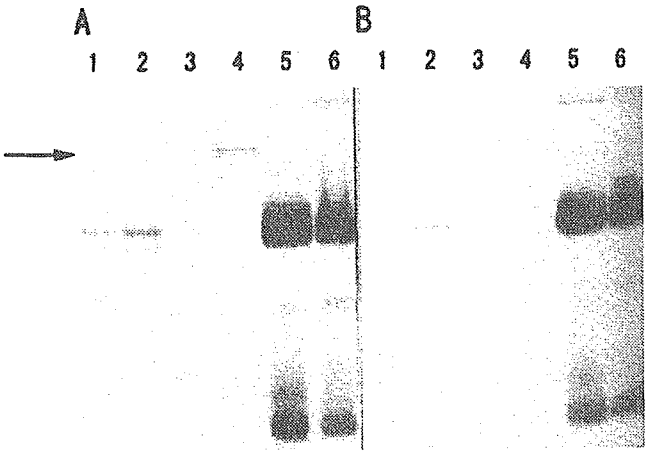

Figure 2. Antibodies against neuronal cells detected by Western blots. Crude homogenates containing 50 μg of proteins obtained from human gray matter (lane 1), white matter (lane 2), mouse cerebellum (lane 3), human neuroblastoma cell line (NB-1) (lane 4), human liver (lane 5), and peripheral nerve (lane 6) were subjected to sodium dodecyl sulfate-7.5% polyacrylamide gel electrophoresis. The blotted nitrocellulose membranes were incubated at room temperature for 1 hour with serum diluted to 1:100 of the patient (A) and a control subject (B) followed by incubation for an hour with biotinylated antihuman IgG diluted to 1:400, and then with avidin-biotin peroxidase complex.[25] The blots were finally incubated with 4-chloro-1-naphthol and hydrogen peroxide for color development. A specific antibody against 85 kD protein indicated by the arrow in lane 4 could be observed in the serum of the patient (A), but not in that of a normal subject (B).

NB-1 cells (Figure 2) may have been involved in the pathogenesis of PNNS.

The exact role of thymoma in the pathogenesis of autoimmune diseases is uncertain. Thymomas are the only tumors that have been shown to generate mature T cells from immature precursors[14] and often generate large numbers of long-lived T cells that appear to be sensitized to self-epitopes in the thymoma.[2,15] In general, however, autoantibodies are not produced within the thymoma, and the autoantigens recognized by the autoantibodies are not expressed in thymomas; only fragments of the proteins or unrelated proteins whose epitopes mimic ones of autoantigens have been identified in neoplastic epithelium.[1,2] Moreover, there are virtually no interactions between autoantigen-specific T cells and autoantibody-producing B cells within thymomas, implying that autoantigen-specific T cells must leave the thymoma to become pathologically relevant.[1] Many thymomas are enriched with autoantigen-specific T cells and alter T-cell subset composition in the peripheral blood, which may contribute to paraneoplastic diseases associated with thymoma.[16]

Other than PNNS, fewer than 10% of thymoma patients have a variety of other autoimmune diseases such as systemic lupus erythematosus, autoimmune thyroid disease, ulcerative colitis, and autoimmune hemolytic anemia.[17–20] However, some of them have been considered to represent only coincidental conditions because so few such patients have been reported.[21] Autoimmune Addison disease is such an example, with only three patients having been reported in the literature.[22–24] No details of the first case except for death caused by myasthenia gravis were described.[22] The second one was a 63-year-old woman with myasthenia gravis who developed Addison disease after excision of a recurrent lymphoepithelial thymoma.[23] The third case was a 46-year-old man with myasthenia gravis and Addison disease associated with an invasive thymoma.[24] The adrenal cortex was severely compressed at autopsy. As far as we know, the present case is the first one exhibiting associated thymoma with autoimmune Addison disease and PNNS other than myasthenia gravis with the latter going into remission after thymectomy.

We here propose the possibility that the thymoma in the present case played an important role in the onset of not only PNNS but also autoimmune Addison disease. In the present case, autoantibodies were present against thymoma, adrenal gland, and neural tissue. According to the hypothesis by Vincent and Willcox,[2] a two-step pathogenetic process is involved in thymoma-associated autoimmunity. The first involves cytotoxic T lymphocytes and HLA class I molecules, and the second step involves helper T lymphocytes, antigen-presenting cells, HLA class II molecules, and B cells leading ultimately to the production of specific autoantibodies. The same hypothesis can be applied to both autoimmune diseases, Addison disease and PNNS.

Two possible mechanisms explaining why thymectomy resulted in remission of PNNS could be the removal of a reservoir in the thymus of specifically primed thymic lymphocytes capable of producing the autoantibodies and/or the elimination of the source of thymic hormones that may stimulate cellular immune reactivity.[20,21] The present patient with PNNS had a complete neurologic response 1 year later, which was more likely due to the thymectomy than spontaneous remission. Such a clinical response to thymectomy is rare, because PNNS other than myasthenia gravis, Lambert-Eaton myasthenic syndrome, and opsoclonus/myoclonus seldom respond to treatment of the associated tumor and immunosuppressive therapy, and the overall prognosis generally depends on the associated neoplasm.[4]

In summary, we report a case of thymoma associated with autoimmune Addison disease and PNNS. This case may provide a clue to the pathogenesis of autoimmune diseases associated with thymomas and may assist in understanding the immunologic mechanisms of thymomas.

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Original Communication

Rapid detection and subtyping of herpes simplex virus DNA in CSF by means of LightCycler PCR

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ABSTRACT

Prompt laboratory diagnosis of Herpes simplex virus (HSV) infection facilitates patient management and the possible initiation of antiviral therapy. In order to assist in arriving at a rapid diagnosis, real-time PCR assays have been developed for the detection of herpes virus DNA in patient specimens. A recently described set of real-time PCR assays using LightCycler technology enabled the parallel detection of DNA from herpes simplex virus by using a single LightCycler program. We set up HSV real-time PCR on the Lightcycler system using the Roche LightCycler HSV 1/2 Detection kit and evaluated this LightCycler assay in regard to the rapid detection and subtyping of herpes simplex virus (HSV) in the cerebrospinal fluid (CSF) of patients with herpes simplex infection of the central nervous system (CNS). We also compared the results with those of the 'in-house' nested PCR. The sensitivity of the LightCycler PCR assay was the same as that of the nested PCR assay. Furthermore, this system enabled the simultaneous identification of HSV-1/HSV-2 through the use of melting curve analysis. The total processing time for the detection and subtyping of HSV was less than 1 hour. Thus, LightCycler PCR has the advantages of rapid amplification and a reduced risk of contamination, and is a suitable method for diagnosis of HSV infection in the CNS.

KEYWORDS: herpes simplex virus, Lightcycler PCR, cerebrospinal fluid

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INTRODUCTION

In central nervous system infections caused by herpes simplex virus (HSV), such as encephalitis and myelitis, PCR represents an important technique for diagnosis and therapeutic planning. PCR is widely utilized by medical institutions and private testing companies. However, because cerebrospinal fluid (CSF) samples collected by lumbar puncture from patients with encephalitis contain very small amounts of HSV DNA, the sensitivity of PCR must be improved [1,2]. Real-time PCR is a recently developed technique that can amplify and detect a target gene quicker than previous PCR techniques [3]. Herein we report that LightCycler PCR is capable of not only detecting HSV DNA with a comparable degree of sensitivity to nested PCR, but also differentiating between HSV types 1 and 2.

LIGHTCYCLER PCR METHODS

Real-time PCR was performed using a LightCycler (Roche Diagnostics, Mannheim, Germany) and a LightCycler HSV 1/2 Detection Kit (Roche Diagnostics, Mannheim, Germany), which contains the necessary primers, fluorescent-labeled probes, Taq DNA polymerase and reaction buffers for Hybri-probe PCR. Using 20–μl reaction solutions, each containing 2 μl of CSF sample that had been boiled at 100°C. PCR was performed with preprocessing, temperature cycle (amplification) and melting curve analysis. Cycling conditions were as follows: initial denaturation /FastStart Taq DNA polymerase activation at 95°C/10 min, 45 cycles of denaturation at 95°C/10 sec, annealing at 55°C/15 sec and extension at 72°C/15 sec. After amplification was complete, melting curve analysis was performed as follows: starting at 40°C

followed by a gradual increase in temperature (transition rate of 0.1°C/sec) to 80°C with continuous fluorescence acquisition. The fragment selected for amplification and detection using the HSV 1/2 Detection Kit includes areas specific to HSV-1 and HSV-2 subtypes and an area common to the two subtypes (the primer region of the DNA polymerase gene is highly conserved for both HSV-1 and HSV-2). Sequence differences between the PCR product and hybridization probes resulted in shifts in the melting temperatures Analysis of the PCR amplification and probe melting curves was accomplished through the use of LightCycler software.

SENSITIVITY AND SELECTIVITY (COMPARISON WITH NESTED PCR)

Using plasmid DNA carrying the HSV DNA polymerase gene that was included in the LightCycler HSV 1/2 Detection Kit as a positive control, serially diluted samples were analyzed. Results showed that fluorescent signals can be detected even at a concentration of 1 copy/tube, and determination was possible up to 10^3 copies/tube (Fig. 1A). Melting analysis was conducted by measuring fluorescent intensity at different melting temperatures after amplifying HSV-1- and HSV-2-positive samples. Fig. 1B shows cumulative fluorescent intensity per unit temperature. Peak melting temperature was 54 °C for HSV-1 and 67 °C for HSV-2, and HSV subtypes could be differentiated based on this difference in melting temperature. Next, CSF samples were analyzed. CSF samples collected from 8 patients with HSV-induced encephalitis or myelitis were examined in the present study. Presence of HSV DNA in CSF was confirmed using nested PCR. HSV types 1 and 2 can be differentiated by restriction fragment length of nested PCR productions [1]. Controls comprised CSF samples collected from 10 patients diagnosed with non-HSV viral meningoencephalitis based on various tests including viral antibody test. HSV DNA was not detected in the 10 control samples. LightCycler PCR detected HSV DNA in all 8 samples in which HSV DNA was detected by nested PCR, suggesting that detection sensitivity of LightCycler PCR is comparable to that of nested PCR. In addition, subtype differentiation based on melting curve analysis matched that based on restriction band pattern of nested PCR products (Table 1). Furthermore, LightCycler PCR did not detect HSV DNA from any of the 10 CSF samples collected from patients with non-HSV viral meningoencephalitis. The process of LightCycler PCR including melting curve analysis took about 50 min to complete.

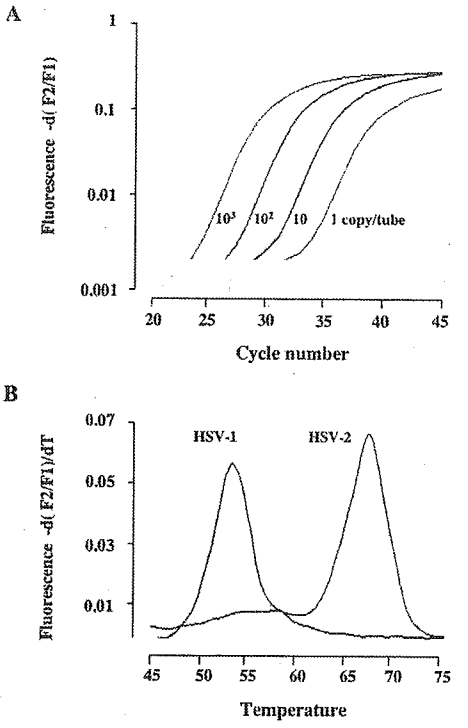

Fig.1. (A) Detection of serially diluted suspensions of HDV plasmid DNA by LightCycler PCR. (B) Melting curve analysis of HSV-1 and HSV-2 .

Table 1. Comparison of conventional PCR and Light Cycler PCR

No.	Diagnosis	Conventional PCR			Light
		Direct	Nested	subtype*	Cycler
1	encephalitis	–	+	HSV-1	HSV-1
2	encephalitis	–	+	HSV-1	HSV-1
3	encephalitis	–	+	HSV-1	HSV-1
4	encephalitis	–	+	HSV-1	HSV-1
5	encephalitis	–	+	HSV-1	HSV-1
6	myelitis	–	+	HSV-2	HSV-2
7	myelitis	–	+	HSV-2	HSV-2
8	myelitis	–	+	HSV-1	HSV-1

Differentiation of HSV types 1 and 2 was made by restriction fragment length of nested PCR productions

DISCUSSION

When diagnosing HSV infection of the central nervous system using PCR, efficiency of HSV DNA detection is crucial. While sensitivity of nested PCR for HSV DNA is high [1], caution must be exercised, since amplification of already amplified PCR products increases the risk for false-positive results due to contamination. Real-time PCR does not require electrophoresis of PCR products, and is more convenient than existing PCR techniques. The TaqMan probe technique is a frequently used real-time PCR technique. Sensitivity is higher than existing PCR techniques, and PCR results can be quantified with a high degree of specificity [4]. The TaqMan probe technique is thus useful in quantifying HSV DNA in CSF samples and diagnosing HSV encephalitis [5,6]. LightCycler PCR has recently been developed as a technique for quick PCR [7]. The Hybri-probe technique employed by the HSV DNA detection system used in the present study reportedly exhibits even higher detection sensitivity [8,9]. The present results show that sensitivity of LightCycler PCR is comparable to that of nested PCR, but studies on detection of leukemia cells using peripheral blood samples have shown that sensitivity of Hybri-probe is either comparable to or an order of magnitude greater than that of nested PCR. The Hybri-probe technique has thus been used for diagnosis of not only infections, but also leukemia [10,11]. Another characteristic of LightCycler PCR HSV DNA detection is that HSV subtypes can be differentiated using melting curve analysis. In general, encephalitis is caused by HSV-1, while myelitis is caused by HSV-2. HSV-2 infection also appears to display a strong element of opportunistic infection. When clarifying the correlation of individual immunity to HSV-induced central nervous system infection, detection of HSV DNA and concurrent identification of HSV subtype should prove useful.

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LETTER TO THE EDITOR

F-WAVE LATENCY IS THE MOST REPRODUCIBLE NCS PARAMETER IN REPEATED STUDIES PERFORMED AT SHORT INTERVALS

Whether nerve conduction studies (NCS) can serve as useful objective markers in multicenter clinical trials of disorders of the peripheral nervous system (PNS) depends on reproducibility between examiners. The results of previous investigations on this aspect have limitations because many were performed in a single laboratory with a small number of examiners, and an important NCS parameter such as minimum F-wave latency was not included for analysis.[2–5,8] Recently, Kohara et al. reported that F-wave latency is the most reproducible measure in NCS in multicenter studies, as have others in recordings from a single institute.[1,7] Since the study by Kohara et al. was performed in 32 laboratories over an interval of 1–4 weeks,[7] many different external factors in each laboratory could have affected reproducibility, such as room temperature, electric shield, and duration of the NCS. Therefore, we undertook NCS measurements over a short interval in a single laboratory with a large number of examiners to clarify the reproducibility of each NCS parameter.

A total of 52 EMG technicians from 23 institutions all over Japan performed NCS in 33 healthy subjects (19 men; mean age, 34.5 years; age range, 25–56). Informed consent was obtained from all subjects. The study was conducted in a single EMG laboratory with six EMG machines on two occasions in October and November 2003. After instruction in standardized NCS techniques, a compound muscle action potential (CMAP) was recorded with the limb temperature between 31 and 34°C from (1) the left abductor pollicis brevis, after stimulation of the median nerve at the wrist (60 mm from the active electrode E1) and elbow, and (2) the left abductor hallucis, with stimulation of the tibial nerve at the ankle (80 mm from E1) and popliteal fossa. Minimum F-wave latencies of the left median and tibial nerves were measured by 20–32 consecutive stimulations at 1 Hz to obtain at least 10 F waves. In most cases, 20 stimuli sufficed to obtain at least 10 F waves. In addition, the left sural sensory nerve action potential (SNAP) was obtained antidromically with surface recording electrodes at the lateral aspect of the ankle with a stimulator placed 140 mm more proximally. The same examiner performed the measurements twice with an interval of 1–2 h between measurements. The electrodes were removed at the end of the first measurement and then replaced for the second study.

To assess the reproducibility (test–retest reliability) of a NCS parameter, the intraclass correlation coefficient (ICC) was calculated (SPSS 11.0; Chicago, IL). The ICC is defined as the proportion of the variance attributable to variability among subjects, from 0 (all variability is experimental error) to 1 (no experimental error). An ICC in the range of 0.9–1.0 is generally recognized as highly reproducible.

Figure 1 shows that minimum F-wave latency was more reproducible than motor CV in both median and tibial nerves. Furthermore, both minimum F-wave latency and motor CV were more reproducible in the tibial than median nerve. As expected from earlier studies, the sural SNAP amplitude was only modestly reproducible.[5] Among the five NCS parameters assessed in the present study, the tibial minimal F-wave latency had the highest reproducibility.[7] The slightly higher ICC in each of the NCS parameters in this study than in an earlier one appears to be due to uniform setting and the short interval between measurements.[7] The high reproducibility of the minimum F-wave latency in the present study was similar to that reported recently by Puksa et al.[9] To our surprise, the reproducibility of motor CV was fairly low, despite the short interval between measurements. The reproducibility of the sural SNAP amplitude was similar to that of the tibial motor CV, but may be lower in patients with peripheral neuropathy because of reduced SNAP amplitudes. Our data clearly show that a NCS parameter obtained through a long nerve segment (i.e., tibial nerve F-wave latency) has superior reproducibility. F-wave measurement has been recommended as a useful objective measure in diabetic polyneuropathy.[6] Another theoretical advantage of determining F-wave minimal latency over motor CV and SNAP amplitude is its relative stability despite changes in body temperature, as the greater part of the nerve segments assessed by F-wave studies is deep in contrast to the distal nerve segments that are prone to limb cooling. In conclusion, the present study employing a large number of ex-

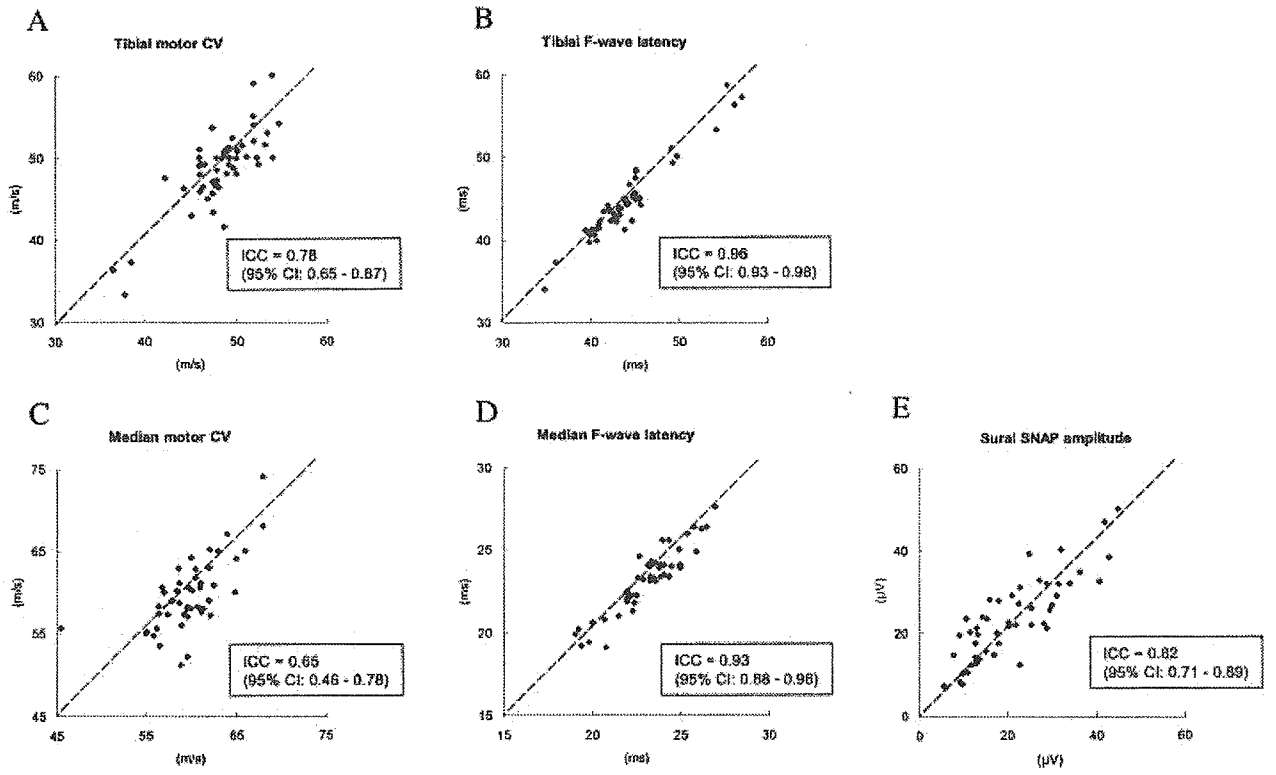

FIGURE 1. The reproducibility of NCS parameters (first attempt: *x*-axis; second attempt: *y*-axis) obtained by 52 examiners shows that the minimum F-wave latency had much higher ICC (i.e., was more reproducible) than motor conduction velocity (CV) of the respective tested nerves and sural SNAP amplitude. Also, both the minimum F-wave latency and motor CV were more reproducible in the tibial than median nerve—hence the tibial minimum F-wave latency was the most reproducible among parameters.

aminers, as encountered in clinical trials, in a uniform testing condition demonstrated that minimum F-wave latency is a more reliable NCS parameter than motor CV and SNAP amplitude. Hence F-wave studies, especially from a lower extremity, should be utilized for longitudinal NCS studies in monitoring progression or improvement of PNS disorders.

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Research Article

Prevalence and clinical characteristics of restless legs syndrome in Japanese patients with Parkinson's disease

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KEYWORDS

restless legs syndrome ·Parkinson's disease ·prevalence ·Pittsburg Sleep Quality Index ·iron

ABSTRACT

To explore the clinical significance of restless legs syndrome (RLS) in Parkinson's disease (PD) and the causal relationship between these two disorders, we made a comparison of both the prevalence of RLS and the severity of sleep disturbance manifested on the Pittsburg Sleep Quality Index (PSQI) between patients with PD (n = 165) and age- and sex-matched control subjects (n = 131). The prevalence of RLS diagnosed by clinical interview was significantly higher in PD patients than in control subjects (12% vs. 2.3%). PSQI score was significantly higher in PD patients with RLS than in both patients without RLS and controls. However, PSQI score was not statistically different between the latter two groups. Among the PD patients with RLS, only 2 had a positive family history of RLS. Only 3 PD patients had requested treatment for the disorder. Our results emphasize the etiological link between RLS and PD in a Japanese cohort, and the existence of RLS is thought to be one of the most important factors aggravating sleep disturbance in PD, despite the low RLS severity. © 2005 Movement Disorder Society

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ARTICLE TEXT

Restless legs syndrome (RLS) is characterized by unpleasant leg sensations and irresistible urges to move the lower extremities, mainly at night,[1] and may cause sleep disturbance. The reported prevalence rate of RLS varies from 0.1% to 15% among different ethnic populations.[2-6] The disorder is well-known to be familial[7] or secondary to other medical conditions, including iron-deficiency anemia, end-stage renal disease, neuropathy, pregnancy, and rheumatoid arthritis.[8-11] It has been widely accepted that dopaminergic drugs show therapeutic efficacy in RLS, and dopamine agonists now represent the first line of treatment for this disorder.[12] Ondo and colleagues[13] have suggested that Parkinson's disease (PD) and RLS may share a common pathogenesis. From this point of view, several studies have examined the possible etiological association between RLS and PD.[14] Previous studies in Caucasians, in which the prevalence of RLS has been estimated at 5 to 15% of the general population,[2-4] have indicated the high association of the two disorders.[13][15] However, a study of Chinese PD patients in Singapore, in which the reported prevalence of RLS in the general population was much lower than that in Western countries,[5]

showed that none of the subjects satisfied diagnostic criteria of RLS.[16] This difference raises the question of whether there is a racial or ethnic difference between the prevalence of RLS not only in the general population but also in patients with PD. Moreover, the clinical significance of RLS secondary to PD remains unresolved. To clarify these issues, we investigated the prevalence and causal risk factors of RLS and its influence on sleep disturbance in Japanese patients with PD.

SUBJECTS AND METHODS

The ethics committees of Tottori University and the Neuropsychiatric Research Institute approved this study, and all subjects gave their informed consent to take part in this investigation. This study investigated 165 consecutive PD patients (67 men, 98 women; mean age 68.8 7 10.3 (SD) years) who visited the outpatient clinic of either the Department of Neurology, Tottori University Hospital, or the Department of Neurology, Fuchu Hospital from 1 May 2003 to 31 October 2003, and 131 controls (50 men, 81 women; mean age 68.3 7 5.8 years), most of whom accompanied the outpatients at the Department of Neurology and were taking care of patients with neurological disorders. They did not report subjective sleep problems. Among the PD patients and controls described above, no subjects reported having conditions that might cause RLS such as pregnancy, diabetes mellitus, iron-deficiency anemia, rheumatoid arthritis, or renal failure. None of the subjects reported taking either antipsychotics or antidepressants at the time of the investigation. Diagnosis of PD was made based on standardized clinical criteria.[17] There was no significant difference in gender distribution or mean age between the two groups. The Pittsburg Sleep Quality Index (PSQI)[18] was measured for each subject to investigate the existence of sleep problems, and the presence of RLS was clinically evaluated by sleep disorder specialists experienced with RLS. Despite the difficulty in discriminating akathisia from RLS, we carefully excluded akathisia by investigating whether patients were medicated with antipsychotics and whether patients showed clear aggravation of symptoms at night, as well as whether symptoms were clearly relieved by movement.

After tremor, dyskinesia, painful neuropathy, and akathisia were carefully excluded, the diagnosis of RLS was made using four major symptoms of RLS developed by the International Restless Legs Syndrome Study Group (IRLSSG): (1) an urge to move the legs, usually accompanied or caused by uncomfortable sensation in the legs; (2) the beginning or worsening of symptoms during periods of rest or inactivity; (3) the partial or total relief of symptoms by movement; and (4) the symptoms being worse in the evening or night than during the day or only occurring in the evening or night. A positive diagnosis of RLS was made when a subject had all of the four symptoms described above. [19] Additional input from spouses or caregivers was specifically allowed in some PD cases for which the patients could not competently answer all the questions.

After making a diagnosis of RLS, the PD patients were divided into two groups: those affected with RLS (PD with RLS) and those without RLS (PD without RLS). To investigate the causal factors for RLS in PD, clinical background data such as age; gender; both course and possible positive family history of RLS; duration of PD morbidity; severity of PD (Hoehn and Yahr grade); the amount of drugs used for the treatment of PD, including levodopa, dopaminergic agonists, droxydopa, amantadine, and anticholinergics such as trihexylphenidyl; and the number of patients who showed a reduction in symptoms in response to dopaminergic treatment were compared between the two groups described above. For ease of comparison, doses of dopaminergic agonists were converted into bromocriptine equivalents, such that 1 mg of pergolide was considered equivalent to 10 mg of bromocriptine, 1 mg of cabergoline to 5 mg of bromocriptine, and 1 mg of talipexole to 3.75 mg of bromocriptine.[20] Serum values of both iron and ferritin at the investigation were also compared between the two groups. In addition, the severity of RLS was evaluated using the Japanese version of the IRLSSG rating scale (IRLS).[21]

Comparisons of continuous variables between each group were made using both analyses of covariance followed by a post hoc test and a Mann-Whitney *U* test when appropriate. A χ^2 test was used to compare the categorical variables. Data are presented as mean 7 SE unless otherwise indicated. Statistical significance was considered to exist at $P < 0.05$ (*SPSS v. 11.5J*, 2002; SPSS, Tokyo, Japan).

RESULTS

A total of 20 (M:F = 8:12) of the 165 PD patients (12%) and 3 (M:F = 0:3) of the 131 controls (2.3%) were diagnosed to have RLS; the prevalence rate of the disorder was significantly higher in the PD patient group compared with the control group ($P < 0.01$, χ^2 test). Among the 20 PD subjects with RLS, possible family history was reported by 2 patients (10%). The age at onset of RLS was 56.2 7 2.7 (SE) years old, and length of RLS morbidity was 6.6 7 1.8 years. All but 1 PD patient with a positive diagnosis of RLS reported that RLS symptoms clearly appeared after the onset of PD, and the mean period between the onset of the two disorders was 6.5 7 1.8 years. The exceptional patient reported to suffer from RLS 2 years before the onset of PD. Except for this single case, previous diagnosis of RLS had not been made in the PD group with RLS, because they had not reported RLS symptoms before the investigation. They also reported to have thought that the RLS symptoms were part of their PD symptom complex. Of 20 PD patients with RLS, 7 (35%) reported asymmetrical appearance of RLS symptoms; however, none of them reported any correlation between the predominantly affected side of RLS and that of PD. The IRLS score was 19.7 7 1.5. Of 20 PD patients with RLS, 11 (55%) reported that the symptoms of RLS appeared almost every day, but only 3 patients requested treatment for the disorder at the interviews.

When the PSQI scores were compared (Fig. 1), a group difference was observed between the values found for PD