OBSERVATION

Postinfectious Myeloradiculoneuropathy With Cranial Nerve Involvements Associated With Human Herpesvirus 7 Infection

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Background: Infection with human herpesvirus 7 (HHV-7) generally results in a febrile illness with accompanying exanthema subitum.

Objectives: To ascertain and describe the role of HHV-7 in a case of acute myeloradiculoneuropathy.

Patient: A previously healthy young man with complaints of motor weakness, dysphasia, and nasal voice.

Methods: Serological examinations were performed with the patient's serum. We also examined virus genome DNA in cerebrospinal fluid by regular and real-time polymerase chain reaction. Moreover, we checked the antiganglioside antibody level in the patient's serum samples by the immunoblot analysis.

Results: Serological studies revealed significant change in titers of antibodies against cytomegalovirus, Epstein-Barr virus, and HHV-7, but only HHV-7 genome was detected in the cerebrospinal fluid, with its disappearance after therapy. No antiganglioside antibody was detected in the patient's serum.

Conclusion: The unique clinical picture of the present patient might be closely related to the reactivation of HHV-7 in the nervous system.

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GUILLAIN-BARRÉ SYNDROME (GBS) has been recognized as a postinfectious autoimmune disorder against the peripheral nervous system, characterized by acute muscle weakness and areflexia.[1] Many GBS cases have antiglycosphingolipid antibodies such as GM1 ganglioside in patients with *Campylobacter jejuni* infection[2] and GM2 ganglioside, which shares common epitopes between the infectious agents and peripheral nerves, in patients with cytomegalovirus (CMV) infection.[3]

Previous studies have shown that one of the most common classes of viral infection that precedes GBS is the family of herpesviruses. Of GBS cases with respiratory insufficiency and cranial nerve involvement, roughly 10% to 13% and 8% to 10% demonstrate serological evidence of recent exposure to CMV and Epstein-Barr virus, respectively.[4] Another group of the herpesvirus family includes human herpesvirus (HHV) 6 and HHV-7. Primary infections with either HHV-6 or HHV-7 generally occur in children and are characterized by exanthema subitum and febrile illness.[5,6] Human herpesvirus 6 is recognized as an opportunistic pathogen that causes limbic encephalitis in persons infected with human immunodeficiency virus.[7] Human herpesvirus 7 has recently been described as a cause of encephalitis and myelitis in immunologically competent adults.[8,9]

We report a case of acute myeloradiculoneuropathy mimicking GBS, with genetic evidence documenting the presence of HHV-7 in the cerebrospinal fluid (CSF).

REPORT OF A CASE

A 26-year-old man was admitted to the hospital with a 2-day history of progressive motor weakness, tingling in the extremities, dysphasia, and nasal voice. He had preceding flu-like symptoms 2 weeks before admission. Initial neurological examination revealed moderate motor weakness in the extremities (score of 3 to 4 of 5 on the Medical Research Council scale), with mild hyperreflexia except for the absence of an Achilles tendon reflex. The plantar response was initially flexor and then temporarily extensor. There was evidence of cranial nerve involvement including the facial, glossopharyngeal, and hypoglossal nerves, and autonomic dysfunctions were manifested as a heart conduction block. Examination results of CSF samples taken at admission were normal, but successive examinations demonstrated an increase in protein (89 mg/dL

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Table. Changes in Serum Virus Titer and Virus DNA in Cerebrospinal Fluid Before and After Treatment

	Before Treatment (Day 1)	After Treatment (Day 20)	Normal Range
Serum virus titer			
Mumps virus	<4	4	<4*
Cytomegalovirus	<4	16	<4*
Coxsackie B1 virus	<4	<4	<4*
Coxsackie B2 virus	<4	<4	<4*
Coxsackie B3 virus	<4	<4	<4*
Coxsackie B4 virus	<4	<4	<4*
Herpes simplex virus	<4	<4	<4*
Varicella-zoster virus	<4	<4	<4*
Epstein-Barr virus			
EBNA IgM†	-	-	-
EBNA IgG†	-	+	-
Human herpesvirus 6 (IgG)	<4	<4	<4‡
Human herpesvirus 7 (IgG)	16	64	<4‡
Virus DNA amplification by PCR in CSF, copies/mL§			
Human herpesvirus 6	0	0	0
Human herpesvirus 7	2800	0	0
Cytomegalovirus	0	0	0
Epstein-Barr virus	0	0	0

Abbreviations: CSF, cerebrospinal fluid; EBNA, Epstein-Barr nuclear antigen; PCR, polymerase chain reaction; + sign, positive for; – sign, negative for.
*The values were obtained with the complement fixation test.
†The values were obtained with the enzyme-linked immunosorbent assay method.
‡The values were obtained with immunofluorescence method.[1]
§The PCR amplification was performed on the supernatant of CSF samples after centrifugation.

[normal level <40 mg/dL]) and IgG (23 mg/dL [normal level <4 mg/dL]) levels that was accompanied by a modest pleocytosis (8 cells/μL [normal level <5 cells/μL]) by day 20. Laboratory evaluation results for evidence of immunological compromise were negative. A nerve conduction study performed on day 2 and day 25 documented a decrease in compound muscle action potential amplitudes with a reduction of the F-wave frequency. Motor nerve conduction velocities and distal latencies were preserved. There were no temporal dispersions or conduction blocks. Sensory nerve conduction study results were normal. Auditory brainstem response as well as magnetic resonance imaging results of the brain and the spinal cord with gadolinium enhancement appeared normal and, therefore, did not support a diagnosis of brainstem encephalitis. We tentatively diagnosed the patient as having acute myeloradiculoneuropathy, and we treated him with a high dosage of intravenous immunoglobulin (400 mg/kg per day) for 5 days. After treatment, complete recovery of cranial nerve dysfunction was noted within a week, and motor weakness recovered gradually, with pronounced hyperreflexia in the extremities without pathological reflexes. Eight months after the onset of neurological symptoms, his muscle strength returned to subnormal levels (score of 4 to 5 of 5 on the Medical Research Council scale).

VIROLOGICAL AND SEROLOGICAL STUDIES

We performed serological testing and found a significant change in the serum titers of antibody against CMV and Epstein-Barr virus (**Table**) during the 2-week interval without evidence of a recent *C jejuni* infection, although DNA of neither virus was detected in the CSF by polymerase chain reaction, suggesting cross-reacting (heterologous) antibody responses to CMV and Epstein-Barr virus. We further investigated HHV-6 and HHV-7 DNA in the CSF on day 1 and day 20 (after treatment) by real-time polymerase chain reaction,[5] and we found a significant decrease in the amount of HHV-7 DNA (2800 copies/mL to 0 copies/mL), although no HHV-6 or HHV-7 genomes were detected in the serum sample. Fluorescent antibody testing of serum samples on day 1 and day 20 demonstrated an increase in anti–HHV-7 titers from 1:16 to 1:64 (Table).

ANTIGANGLIOSIDE ANTIBODY

To evaluate the patient's serum for the presence of antiganglioside antibodies, mixtures of gangliosides (GM1, GM2, GM3, GD1a, GD1b, GT1b, GQ1b, and asialo GM1) processed by thin-layer chromatography (using a solvent of chloroform, methanol, and 0.02% calcium chloride in a 55:45:10 vol/vol/vol ratio) were blotted onto a polyvinylidene difluoride membrane by an electrothermal blotter (ATTO Co Ltd, Tokyo, Japan). This polyvinylidene difluoride membrane was probed using patient sera taken on day 1 and day 20 (×1000 dilution) in blocking buffer (2% nonfat milk in the wash buffer, which was phosphate-buffered saline containing 0.5% Nonidet P-40 [Nakarai Tesque Inc, Kyoto, Japan]). After treatment with the second antibody, a positive band was sought using an enhanced chemiluminescence reagent (New England Nuclear, Boston, Mass). A band was present in the positive control (anti-GM1–antibody positive), but no band was detected using the patient's serum samples (**Figure**).

COMMENT

Human herpesvirus 6 can be silently harbored in the human brain following primary infection.[11] Detection of vi-

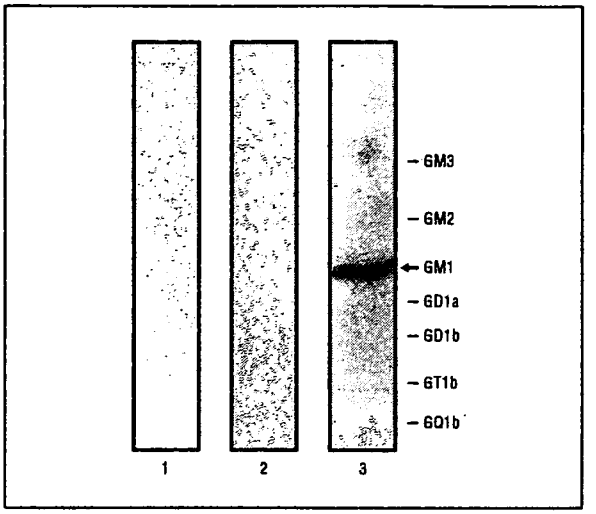

Figure. Electrothermal blotting of gangliosides following immunoblot analysis with serum. Various ganglioside subspecies (GM3, GM2, GM1, GD1a, GD1b, GT1b, GQ1b, and asialo GM1) were electrothermally blotted onto a polyvinylidene difluoride membrane. The membrane was probed with serum from the present 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,[3] 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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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.

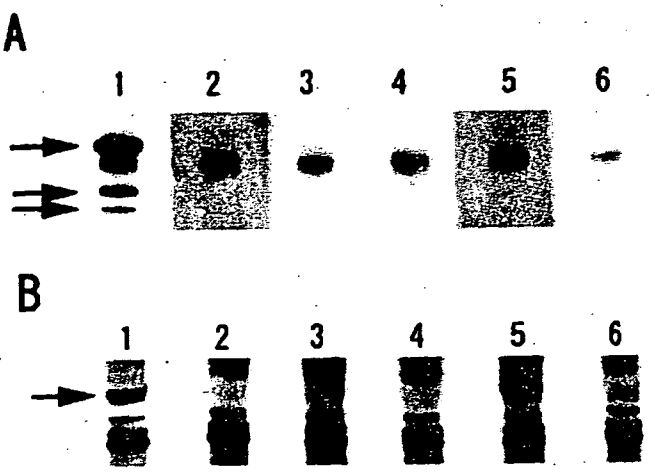

Figure 1. Antiadrenal autoantibodies (A) and antithymoma autoantibody (B) detected by Western blots. Thymoma of the patient and a human adrenal (obtained with informed consent at surgery from a patient with renal carcinoma) were homogenized in lysis buffer. The homogenates were centrifuged at 3000 × 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

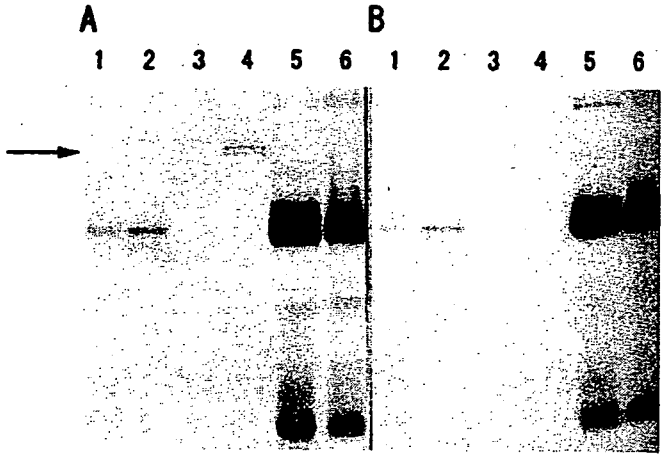

Figure 2. Antibodies against neuronal cells detected by Western blots. Crude homogenates containing 50 μg of proteins obtained from human gray matter (lane 1), white matter (lane 2), mouse cerebellum (lane 3), human neuroblastoma cell line (NB-1) (lane 4), human liver (lane 5), and peripheral nerve (lane 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.

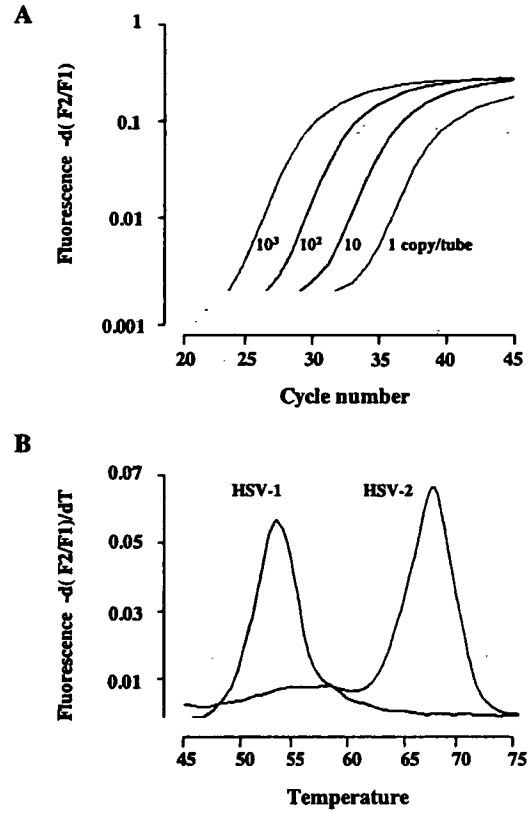

Fig.1. (A) Detection of serially diluted suspensions of HDV plasmid DNA by LightCycler PCR. (B) Melting curve analysis of HSV-1 and HSV-2 .

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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Original Article

Apoptosis under hypercytokinemia is a possible pathogenesis in influenza-associated encephalopathy

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Abstract

Background: Influenza-associated encephalopathy is reported to be frequent in Japan and East Asia. No evaluating markers except interleukin (IL)-6 and tumor necrosis factor (TNF)-α and no likely pathological mechanism for the disease have yet been elucidated.

Methods: In this study, influenza-associated encephalopathy was defined by clinical symptoms, and the use of an anti-influenza antibody test and/or influenza antigen detection kits, as well as computed tomography and/or magnetic resonance imaging. The levels of proinflammatory cytokines, acute phase proteins, endothelial markers and cytochrome c were compared in sera from 11 patients with and 42 without encephalopathy.

Results: Cytochrome c concentration in sera from patients with encephalopathy was markedly increased compared with that from patients without encephalopathy and normal controls. Although levels of several other proinflammatory cytokines and acute phase proteins such as TNF-α and IL-8 were also elevated in patients with influenza virus infection, the difference between those with and without encephalopathy, though significant, was less dramatic. The mean serum concentration of cytochrome c in 11 patients with encephalopathy, consisting of four deceased, four with and three without residual central nervous system sequelae, was 26.7 ± 19.5 ng/mL on admission. In contrast, cytochrome c levels in 42 patients without encephalopathy were 0.3 ± 0.7 ng/mL.

Conclusion: The present results indicate that cytochrome c is a useful marker to follow patients with influenza-associated encephalopathy and suggest that an apoptosis of cells in several organs including the cerebrum and liver under the influence of hypercytokinemia is a possible mechanism of the disease.

Key words apoptosis, cytochrome c, hypercytokinemia, influenza-associated encephalopathy.

Frequently seen during the winter season are children suffering from influenza infection who exhibit complications including typical febrile convulsion, decreased sensorium, delirium, and finally deep, irreversible coma. Cases of such patients with encephalopathy are reported to be more frequent in East Asia than in the west.[1–3] In Japan, the total number of fatal cases of influenza encephalitis/encephalopathy in the 1997/1998 seasons was estimated at 100–200.[4] Togashi *et al.* reported that 64 infants and children developed encephalitis/encephalopathy during five influenza seasons in Hokkaido, the northern island of Japan.[5]

Although several types of influenza-associated encephalopathy have been reported clinically,[6] the pathological

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mechanism of influenza-associated encephalopathy still remains to be clarified. Current treatment of patients with the disease is restricted to early administration of viral inhibitors such as amantadine, zanavimir and oseltamivir and occasional plasma exchange and/or mild hypothermia therapy.[7] This is because the evaluation of effectiveness of these therapies is difficult and no appropriate marker has been found to monitor the severity or the progression of the disease.

With this in mind, we compared several proinflammatory cytokines, acute phase proteins, endothelial markers and also cytochrome c to find out pathogenic and clinical markers. Although cytochrome c is an intramitochondrial protein in intermembrane spaces and is involved in the electron transport system for oxidative phosphorylation, it also triggers the execution of apoptosis. In our retrospective study, we found that the serum level of cytochrome c in addition to tumor necrosis factor (TNF)-α and interleukin (IL)-8 is apparently elevated in patients with influenza-associated encephalopathy as compared with those without it. The elevation of serum cytochrome c reflects the severity of the disease and, furthermore, suggests that apoptosis of tissues including those of the cerebrum occurs in patients with influenza-associated encephalopathy under the influence of hypercytokinemia.

Materials and methods

Patients

All 53 patients involved in this study were diagnosed with influenza virus infection with influenza antigen detection kits (Directigen Flu A, Becton Dickinson, USA; FIU OIA, BIOSTAR, Australia; or Influ A and B-AD, Denka Seiken, Japan). Elevation of hemoaglutination inhibition antibody (HI-Ab) was also confirmed in some patients.

In this study, we defined the patients as suffering from influenza-associated encephalopathy if they showed: (i) clinical symptoms and signs compatible with acute encephalitis/encephalopathy, and exclusion of bacteria or fungal infection and all other neurological, vascular, metabolic, endocrine, toxic, and drug-induced disorders; (ii) isolation of influenza virus from the throat, or a fourfold increase in the antibody titer determined by means of the hemagglutination inhibition test and/or virus antigen detection in the throat with Kits; and (iii) the presence of cerebral edema, bleeding or acute necrotizing encephalopathy (ANE) confirmed by computed tomography (CT) or magnetic resonance imaging (MRI).

The profiles of 11 patients with influenza-associated encephalopathy are listed in Table 1. Most patients with encephalopathy showed multiorgan dysfunction to varying degrees. Patients 2, 3, 6, 7 and 11 suffered from hepatic and other organ dysfunction in which aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were also elevated. Renal dysfunction was found in patients 1, 3, 4, 5, 6, 7 and 8. Thrombocytopenia was found in patients 1, 2, 3, 4, 6, 7 and 10. Rhabdomyolysis was found in patient 7. The sera of seven patients (1, 2, 3, 4, 5, 10 and 11) were sequentially stocked for more precise analysis.

All the 42 remaining patients with influenza infection were admitted for several days at hospitals in Kumamoto city with symptoms of high-grade fever and/or febrile convulsion. They were 20 boys and 22 girls and were 2 years 6 months ± 2 years 5 months old. They had no apparent biochemical abnormalities and no residual neurological sequelae on discharge.

All the sera were stored at –20°C on admission and subsequently with parental consent.

Excluded patients

In this study, we excluded four deceased patients (a 1-year-old female, a 1-year and 3-month-old male, a 2-year-old female and a 3-year-old male), even though they were suspected to have had an influenza-associated encephalopathy, because they were dead on arrival and biochemical data were limited, without CT and MRI findings.

Methods

Cytokine levels in sera were detected by the the following enzyme-linked immunosorbent assay (ELISA) kits: soluble E-selectin, sE-selectin ELISA ver2 (Bender med System); sTM, TM test (Teijin; Teijin Diagnostics); TNF-α, Human TNF-α Cytoscreen Immunoassay Kit (Bioscorce International); sFas, sFas ELISA Kit (Medical and Biological); sFas-L, sFas Ligand ELISA Kit (MBL), and IL-6 and IL-8 (Biotrak) IL6 and IL-8 human ELISA system (Amersham Pharmacia, NJ, USA). A normal range of these markers is shown in Table 2, and the values were reconfirmed by normal volunteers.

For serum cytochrome c assay, we developed a sandwich enzyme immunoassay (EIA) system.[8] A total of 50 μL of serum samples were placed in a polystyrene plate that had been coated with mouse antihuman cytochrome c monoclonal antibody (Fujisawa pharmaceutical Co. Ltd, Osaka, Japan). After 1 h incubation at room temperature, the plate was washed with buffer and incubated further for 1 h with rabbit anticytochrome c antibody IgG Fab fragment conjugated with horseradish peroxidase. Reaction buffer containing 2,2'-Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid and H_2O_2 was added to all wells and incubated at room temperature for 1 h. The reaction was terminated with NaN_3 and absorbance at 405 nm of the reduced products was determined. The serum cytochrome c level of non-infected controls was

Table 1 Profile of the patients with influenza-associated encephalopathy

	Age	Sex	Onset days	Detection Kits	HI-Ab	JCS	CT/MRI	Outcome	Cyto c ng/mL	AST IU/L	Cr mg/dL	Plat 10^4/mL	CK U/L
1	3 years 2 months	M	1	FIU OIA		> 200	Diffuse	Died	50	53	1.2	2.9	–
2	2 years	M	1	Influ B		100–200	Diffuse	CNS sequela	56	6830/9520	ND	4.9	361/2250
3	2 years	F	1	Influ B		100	Diffuse	Improved	46	13 884/14 909	1.4	4.9	580
4	1 year 8 months	F	2		H3N2	300	Diffuse	Died	35	126	1.4	+	–/516
5	4 years	M	17 h	PCR/Influ A		300	ANE	Improved	32	102/695	1	–	–
6	3 years 2 months	F	1	PCR/Influ A	H3N2	30–300	Diffuse	Died	30.5	53 340	Renal failure	1.8	1139
7	6 years	M	0	Influ A	H3N2	3–200	Diffuse	Died	24	1174/6168	1.4	10.7	124 600
8	7 years	M	1	Influ A	H3N2	300	Lobar	CNS sequela	11	37	1.3	18.3	89
9	9 years	M	1	Influ A	H1N1	300	Lobar	CNS sequela	5.8	28/48	0.5	–	n.d.
10	1 month	M	0		H3N2	300	Mild edema	CNS sequela	2.4	51/33	0.3	+	n.d.
11	2 years	F	7 h	Influ A		300	ANE	Improved	1.2	102/695	0.7	–	n.d.

'Onset' means time between appearance of symptom and admission. FLU OIA and Influ A or B are influenza virus detection kits. Biochemical data are shown as level on admission/maximum during the course.

ANE, acute necrotizing encephalopathy; AST, aspartate aminotransferase (IU/L); CK, creatinine phosphokinase (U/L); CNS, central nervous system; Cr, creatinine (mg/dL); Cyto c, cytochrome c (ng/mL); F, female; HI-ab, hemoagglutination-antibody; H3N2 and H1N1, type of influenza viruses; JCS, Japanese coma score; M, male; –, normal; ND, not done; Plat, platelet (+ or –, with or without thrombocytopenia; X 104/μL).

less than 0.05 ng/mL. That of patients suffering from influenza infection was stable (0.1 ± 0.2 ng/mL). The level of serum cytochrome c was reproducible even after several freezing and thawing procedures.

Statistical procedures

The comparisons between the groups were performed by the Mann–Whitney rank sum test. The 0.05 level of probability was used as a significance criterion.

Results

We measured the serum levels of proinflammatory and acute phase proteins from 11 patients with influenza-associated encephalopathy and 42 other influenza patients. The statistical evaluation of serum concentrations of soluble E-selectin, sTM, TNF-α, sFas, sFas-ligand, IL-6, IL-8, and cytochrome c in the patients with and without influenza-associated encephalopathy is shown in Table 2. It is apparent that the level of cytochrome c was markedly elevated in the patients with influenza-associated encephalopathy as compared with the normal range and that of other influenza patients. The levels of TNF-α and IL-8 were also significantly elevated in the patients with influenza-associated encephalopathy. The *P*-values for these are, however, less significant, indicating that elevation of cytochrome c is the most reliable marker among the serum proteins tested for estimating the severity of influenza-associated encephalopathy.

As shown in Table 1, the level of serum cytochrome c in patients with influenza-associated encephalopathy correlated well with their clinical outcomes. Among the seven patients whose serum contained more than 20 ng/mL cytochrome c on admission, four died and one had severe central nervous system sequelae. Only two patients who received high dose gamma globulin therapy (patient 5) and/or cyclosporinee therapy (patient 3) survived. The concentration of cytochrome c in sera from the surviving patients decreased during treatment. Three patients (8, 9 and 10) with a greater than 2 ng/mL cytochrome c concentration developed severe neurological sequelae.

Cytochrome c levels of several patients who were subsequently analyzed are shown in Table 3. It appears that levels remained high in patients 1 and 4, who died later, but decreased after admission in patients 2, 3 and 5 who subsequently survived. In patient 10 who showed CNS sequelae, the level was slightly high on admission, but increased to 14 ng/mL on the third day of admission. Those of patient 11 remained at low levels as shown in Table 3. In patient 1, serum cytochrome c levels were assayed twice a day (with permission), they stayed high for the first 2 days and then decreased for 3 days and rose again after the sixth day of admission.

Table 2 Statistical analysis of various markers in sera from patients with and without influenza-associated encephalopathy

Markers	Normal	without IE	with IE	*P*-value
Cytochrome c	< 0.05 ng/mL	0.3 ± 0.7 (42)	26.8 ± 19.5 (11)	< 0.001
TNF-α	0 pg/mL	0.9 ± 3.7 (17)	11.6 ± 17.1 (5)	0.003
IL-8	1.2–16.7 pg/mL	17.3 ± 16.1 (16)	339.8 ± 400.2 (8)	0.016
IL-6	0–149 pg/mL	49.8 ± 156.7 (17)	55.5 ± 125.7 (6)	0.35
Soluble TM	11–20 ng/mL	1.4 ± 0.8 (27)	17.4 ± 19.8 (6)	0.39
Soluble Fas	1–3.9 ng/mL	1.4 ± 0.8 (29)	1.7 ± 0.5 (6)	0.52
Soluble E-selectin	20–60 ng/mL	76.6 ± 43.7 (42)	133.7 ± 0.5 (6)	0.99

The Mann-whitney *U*-test was used to determine significant differences in each marker between the patients with or without encephalopathy ($P < 0.05$).

IE, influenza-associated encephalopathy; IL, interleukin; Patients, number of patients examined; TNF-α, tumor necrosis factor-α.

Table 3 Time course of cytochrome c concentration after admission of patients who were analyzed over time. Serum cytochrome c levels were assayed twice a day (morning and evening) with permission in the case of patient 1

Patients	Days after admission									
	1	2	3	4	5	6	7	8	9	< 10
1	50/68	62/80	9.4/10.5	5.5/9.8	13/14.5	96	110/90	96		
2	56				6	5.8		5.3		3.3
3	46	37							6.2	8.1
4	35							98		66
5	32		0				0			0
10	2.4		14				9		3	
11	1.2	2.7	0	0			0			0

Discussion

We showed in this paper that serum concentration of cytochrome c was extremely high in the patients with influenza-associated encephalopathy. The serum levels of cytochrome c well reflect the severity of the disease and clinical outcome of the patients. The four excluded patients who were dead on arrival after influenza infection also showed high levels of serum cytochrome c (75, 58, 42 and 28.5 ng/mL). All patients had more than 2 ng/mL cytochrome c on admission or subsequently had severe sequelae, except the two who had received high dose gamma globulin therapy (patient 5) and/or cyclosporinee therapy (patient 3) for alleviation of hypercytokinemia. In contrast, two out of the 42 patients without influenza-associated encephalopathy who had more than 2 ng/mL of cytochrome c in the serum on admission developed persistent convulsions a few months later.

In patients 2, 3, 6 and 7, but not in the others, AST, ALT, and creatinine phosphokinase (CK) were elevated as well as cytochrome c. The other markers (creatinine, platelets, creatine phosphokinase etc.) were also elevated in some but not others, as in Table 1. From the view points of cytokine and biochemical markers, cytochrome c appeared to be a good marker for evaluating the clinical severity of influenza-associated encephalopathy.

At present, the following evidence has been reported about the relation between apoptosis and influenza virus infection: (i) an increase of mitochondrial permeability was reported in Reye's syndrome[9] which was supposed to be associated with influenza infection and aspirin side-effects; (ii) during apoptosis of the cell, cytochrome c triggers the execution phase of apoptosis by massive translocation into the cytoplasm;[10] (iii) in our study, serum cytochrome c on admission was elevated in the patients with but not without influenza-associated encephalopathy; (iv) in the biopsy specimens from patients 2 and 3, DNA breakage cells were detected at the early stage of hepatic failure by the TUNEL staining method (DeadEnd Colorimetric TUNEL System, Promega, Tokyo, Japan); and (v) it had already been confirmed that serum cytochrome c levels increased in MODS to MOD,[8] but did not increase in patients with other viral infections such as, acute hepatitis, upper respiratory viral infections, and enterovirus 71-rhomb encephalitis (H. Nunoi, unpubl. obs., 1998). Based on the evidence reported and that obtained in the present investigation, we believe that apoptosis is induced in several tissues of patients with influenza-associated encephalopathy, resulting in the release of cytochrome c from mitochondria.

We speculate that some cytokines may have induced apoptosis of the cells and organs in these patients. As serum TNF-α and IL-8 were significantly elevated as shown in

Table 2, it is possible that TNF-α is one of the inducers of apoptosis in this disease because TNF-α plays an important role in the apoptosis of influenza-infected cells *in vitro*[11] and serum TNF-α shows considerable correlation with the severity of the disease. In addition, pharmacokinetic studies have revealed that TNF-α has a rapid clearance rate with a half-life of 15–30 min,[12] so that the levels were supposed to be influenced by the stage of the disease. Although it was proposed that IL-6 had a predictive value for patients with influenza-associated encephalopathy,[6,13] we did not observe a significant difference in the levels of IL-6 between those with and without the disease ($P = 0.35$). It is still uncertain which cytokine is causative and pathogenic in the disease and how they are induced by influenza virus infection.[13–16]

Of certainty, is that the present results indicate that the serum level of cytochrome c is a reliable indicator for estimating the severity of influenza-associated encephalopathy. A follow-up survey of serum cytochrome c in patients with the disease is important for the prediction of their clinical outcome.

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Original article

Delirious behavior in children with influenza: its clinical features and EEG findings

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Abstract

The clinical characteristics and electroencephalogram (EEG) findings in delirious behavior in children with influenza were studied in order to differentiate it from influenza-associated encephalitis/encephalopathy. Fifteen consecutive children with delirious behavior associated with influenza were investigated. Their clinical courses were investigated using medical records. EEG was obtained during the delirious behavior, when possible. The body temperature during the delirious behavior was 39.0 °C or higher in 13 children. A subtle reduction of consciousness was observed in 10 children. Seizures were observed in five children. EEG revealed some mildly abnormal findings in 13 children, including mild slowing of the background activity, insertion of semirhythmic high voltage slow waves, and appearance of relatively high voltage semirhythmic theta waves. The EEG findings normalized after the delirious behavior had disappeared. EEG revealed transient and mild abnormalities in children with delirious behavior but without encephalitis/encephalopathy, and thus might be useful for diagnostic evaluation in such condition.

Keywords: Delirium; Influenza; Electroencephalogram

1. Introduction

Encephalitis and encephalopathy subsequent to influenza have been important problems in Japanese children [1,2]. The Collaborative Study Group on Influenza-Associated Encephalopathy in Japan identified 148 patients with influenza-associated encephalitis/encephalopathy during the winter of 1998–1999. The rates of mortality and neurological sequleae were 31.8 and 27.7%, respectively [2]. The major signs included loss of consciousness, seizures, and vomiting. In addition, the Annual Report of the National Research Committee on Influenza-Associated Encephalopathy in 2001 stated that abnormal behavior was observed during the early period of the disease in 30 of 70 children who died of influenza-associated encephalitis/encephalopathy. On the other hand, delirium is sometimes seen among children without encephalitis/encephalopathy. Infection or fever is known to be a common cause of delirium among children [3], and it is often observed in patients with Reye syndrome or metabolic disorders. However, the difference between 'simple' febrile delirium and delirium due to malignant diseases such as influenza-associated encephalopathy is not fully understood.

We studied the delirious behavior in children with influenza in order to differentiate from influenza-associated encephalitis/encephalopathy. We turned our attention to electroencephalograms (EEGs) because we considered that

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EEG findings might help us to distinguish febrile delirium from encephalopathy.

2. Patients and methods

We identified 25 consecutive children who were admitted to the Departments of Pediatrics of four Nagoya University-affiliated hospitals and National Mie Hospital because of delirious behavior associated with febrile illness during the winters of 1999–2000, 2000–2001, and 2001–2002. Among them, 16 were diagnosed as having influenza infection. The diagnosis of influenza was made when a child fulfilled either or both of the following criteria; (a) influenza virus antigen was detected in a throat swab, and (b) the antibody titers against influenza virus in paired serum samples were elevated more than 4-fold. One of these, 16 children was later diagnosed as having encephalopathy on the basis of clinical manifestations such as semicoma lasting for 60 h. EEG in this patient demonstrated generalized and remarkable slowing during the delirious behavior. This patient was excluded from this study because the aim of the study was to elucidate the clinical features of delirious behavior among children without encephalopathy.

A brief outline of the delirious behavior is given in Table 1. The clinical courses of the patients were investigated using medical records. EEG was obtained during the delirious behavior, when possible. When EEG could not be recorded during the delirious behavior, it was obtained as soon as possible afterwards. Sedatives or antiepileptic drugs were not used during the EEG recordings. EEGs were evaluated by well-trained pediatric neurologists in each institute.

3. Results

3.1. Characteristics of the patients (Table 1)

There were 10 boys and 5 girls. The average age of the children was 6.5 years (range 1.8–14.3 years). Eleven children were infected with influenza A and four with influenza B.

In 14 children, delirious behavior appeared on the day of the onset of fever or on the next day. The body temperature at the beginning of the delirious behavior was 39.0–39.9 °C in eight children and 40.0 °C or higher in five. In one child, the body temperature at the beginning of the delirious behavior was uncertain, because it was observed when the body temperature began to decrease after the administration of acetaminophen. The duration of the delirious behavior was within 1 h in two children, 1–6 h in four, 7–12 h in two, and 13–24 h in seven. A subtle reduction of consciousness such as somnolence was observed in 10 children, whereas it was not seen in the other five. Other neurological abnormalities were not observed in any children. Seizures were observed in five children. Delirious behavior preceded a seizure in one child. Three children exhibited delirious behavior soon after seizures. In the remaining one child, delirious behavior was seen several hours after a seizure. The clinical manifestations of seizures were compatible with those of simple febrile convulsions, that is, isolated generalized symmetric convulsions for a few minutes.

Brain CT and/or MRI were performed in all but one child. Abnormal findings were not recognized in any children. Blood chemistry revealed elevation of AST in five children, ALT in three, LDH in six, and CK in two.

Table 1
Characteristics of the patients

Age (years)	Sex	BT (°C)	Duration of DB (h)	Reduction of consciousness	Sz	EEG findings	Delirious behavior
6.9	F	>39	18	Subtle	No	SL	Visual hallucination such as 'Angels are flying'
4.4	M	>40	8	Subtle	No	SL	Meaningless speech, laughing alone.
12.9	M	Unknown	14	None	No	Normal	Meaningless speech such as 'A giraffe is dying'
8.5	M	>39	18	None	No	SL, INS	Meaningless speech such as 'My body moves involuntarily', shouting loudly
6.3	M	>39	18	Subtle	No	SL	Unresponsiveness, blank eyes
6.4	F	>40	22	Subtle	No	SL, HVT	Visual hallucination, meaningless speech
7.6	M	>39	5	Subtle	No	Normal	Meaningless speech
8.4	M	>39	15	None	No	SL, INS	Meaningless speech, fearful response
2.3	F	>39	20	Subtle	Yes	SL, INS	Staring unresponsiveness, tremulous movement of the upper extremities
5.9	M	Normal	<1	None	No	SL, INS	Irritability, shout loudly
4.9	F	>39	6	Noen	No	SL, INS	Fearful response, searching for her mother although her mother sat beside her
14.3	F	>39	6	Subtle	Yes	SL, HVT	Meaningless speech, disorientation
1.8	M	>40	<1	Subtle	Yes	SL, HVT	Meaningless speech, blank eyes
4.7	M	>40	12	Subtle	Yes	SL, INS	Visual hallucination such as 'Mummy grows a beard'
2.5	M	>40	5	Subtle	Yes	SL, HVT	Fearful response, crying periodically

BT, body temperature; DB, delirious behavior; Sz, seizures. EEG findings: SL, mild slowing of the basic rhythm; INS, insertion of semirhythmic high voltage slow waves; HVT: appearance of relatively high voltage semirhythmic theta waves.

However, most of these elevated values were within 1.5–2 fold of the upper normal limits.

3.2. EEG findings

EEG revealed some mildly abnormal findings in 13 children, whereas it was normal in the remaining two children. The EEG abnormalities included mild slowing of the background activity, insertion of semirhythmic high voltage slow waves, and appearance of relatively high voltage semirhythmic theta waves (Fig. 1). These findings were occipital-dominant in 12 children, whereas frontal-dominant semirhythmic theta waves were seen in the other child. However, no patients exhibited generalized or predominantly unilateral slowing. The differentiation between awake and sleep background EEG activities was preserved. Paroxysmal discharges were not observed in any children. Follow-up EEGs were obtained 5–14 days after the delirious behavior had disappeared in 12 of the 13 children with abnormal findings in the initial one. The EEG was normal for all of them (Fig. 1).

4. Discussion

Delirium associated with fever or infection in children is a well-known condition. However, its pathogenesis and clinical characteristics have not been sufficiently elucidated. Delirium is also observed in children with encephalitis/encephalopathy. Fever is often present in such patients. Therefore, the distinction between 'simple' febrile delirium and delirium caused by encephalitis/encephalopathy is very important, because early recognition of that with encephalitis/encephalopathy is necessary in order to start intensive treatment early and thereby to improve the prognosis. We focused on children with influenza, because influenza-associated encephalopathy has been an important problem since the large epidemic during the winter of 1998–1999.

In this study, EEG showed mild slowing of the background activity, insertion of semirhythmic high voltage slow waves, and appearance of relatively high voltage semirhythmic theta waves. Although there have been few reports on EEG findings in delirious behavior among children, these findings are similar to those in the previous studies on delirium among the elderly. EEG in elderly

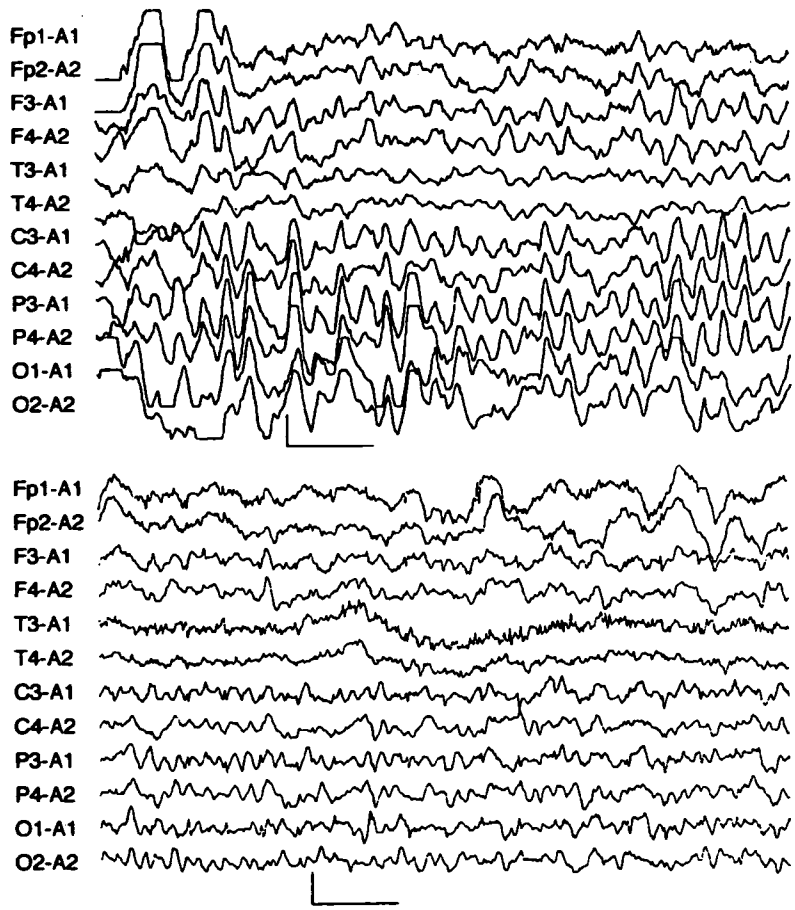

Fig. 1. EEG findings during wakefulness. The patient was a 2-year-old girl. Upper, EEG during the delirious behavior. Semirhythmic high voltage theta activities are observed over the bilateral parieto-occipital regions. Lower, EEG 6 days later. Theta activities are not present and a normal background activity is recognized. Calibration, 1 s, 100 μV.

patients with delirium shows slowing or dropout of the posterior dominant rhythm, and poor organization of the background rhythm [4,5]. It is noteworthy that none of the present patients exhibited generalized or predominantly unilateral slowing, which are characteristic EEG abnormalities in children with acute encephalitis/encephalopathy [6]. The EEG findings are clearly different between those in the case of 'simple' febrile delirious behavior and acute encephalitis/encephalopathy. Therefore, we consider that EEG is useful for differentiating febrile delirium from encephalitis/encephalopathy and that EEG should be performed as soon as possible when we experience febrile children with delirious behavior. Some previous studies on delirium among the elderly revealed that quantitative EEG is also useful for the diagnosis of delirium and the distinction from dementia, and provides a quantitative measure of the severity of the delirium [5,7]. The usefulness of quantitative EEG has not yet been determined in children with delirious behavior. Further studies on conventional and quantitative EEGs in children will be necessary in order to completely establish their usefulness.

There have been few studies on the clinical features of delirious behavior in patients with influenza with or without encephalitis/encephalopathy. The Annual Report of the National Research Committee on Influenza-Associated Encephalopathy in 2001 stated that children with influenza-associated encephalopathy exhibited various forms of delirious behavior including visual hallucinations, fearful responses, meaningless speech or laughter, singing songs, and an oral tendency. These symptoms of delirious behavior in patients with influenza-associated encephalopathy are not different from those in our patients, who did not have encephalopathy. Kashiwagi et al. [8] also reported that there was no difference between delirious behavior associated with encephalopathy and that without encephalopathy. They reported that abnormal neurological signs and disturbed consciousness might be warning factors for encephalopathy [8]. This is consistent with our finding that no patients had neurological abnormalities except for a subtle reduction of consciousness. These facts may enhance the importance of EEG when we must differentiate encephalothic patients from non-encephalopathic ones.

In conclusion, we could delineate the clinical features and EEG findings among children with delirious behavior associated with influenza but without encephalitis/encephalopathy. EEG is thus useful for diagnostic evaluation of such patients.

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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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