pothesize that the influenza-associated encephalopathy patients with poor prognoses could not produce a sufficient amount of TIMP-1, but a large amount of IL-10 strongly stimulated TIMP-1 production.

In summary, an imbalance between MMP-9 and TIMP-1 occurred in influenza virus infection, especially with neurologic complications. The imbalance may damage the blood–brain barrier and promote febrile seizure or encephalopathy.

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PREVALENCE OF NASAL COLONIZATION WITH METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) IN CHILDREN *A MULTICENTER CROSS-SECTIONAL STUDY*

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Abstract: In this cross-sectional multicenter study, we determined the rate of nasal carriage of methicillin-resistant *Staphylococcus aureus* (MRSA) in children admitted to 9 training hospitals in Switzerland during 1 month. From 1337 patients, 1363 nasal swabs were obtained (mean age 6.1 years, median 4.7 years, interquartile range 1.3–10.4 years) and 562 (41.3%) grew *S. aureus*. Only one isolate was MRSA (0.18%) which encoded *mecA* and *femA* genes as well as SCC*mec* type IV, whereas Panton-Valentine leukocidin (PVL) was absent.

Key Words: *Staphyloccus aureus*, MRSA, nasal carriage, children
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Staphylococcus aureus is an important pathogen which causes community-associated and health care-associated infections in all age groups, including children world wide.[1–3] The clinical spectrum of disease comprises infections of the skin, bones and soft tissue, respiratory tract, blood and central nervous system. Resistance to methicillin was first described in *S. aureus* isolates in the early 1960s in England,[4] and has emerged in many countries since.[1] Methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) colonize the skin and mucosae of humans, but the anterior nares is the most frequent site of carriage.[5] Pooled data from 10 studies, mostly from the United States, consisting of 8350 children and adults revealed an overall prevalence of nasal colonization with MRSA of 1.3% and 0.2%, respectively, when persons with health care-associated risk factors were excluded.[3] Some data on the frequency of nasal MRSA colonization are available in children.[3,6–11]

However, this is a dynamic process, and as such ongoing studies are critical in understanding of MRSA epidemiology.

The goal of our study was to determine the carriage frequency of *S. aureus* and MRSA in particular in children admitted to 9 training hospitals in Switzerland, and to evaluate possible risk factors associated with MRSA colonization.

PATIENTS AND METHODS

This was a multicenter, cross-sectional study in children admitted to Swiss children's hospitals during a 1-month surveillance period, March 16 to April 15, 2006. Study sites were 9 children's hospitals representing all geographical areas, and included all 5 university hospitals of Switzerland. Children 4 weeks of age or older admitted for in-patient care were eligible for enrollment. After informed consent had been obtained, a nasal swab was collected within 48 hours of admission and comprehensive patient characteristics were entered into a standardized questionnaire.

Our study was powered to detect an MRSA colonization rate of 1% and this estimate was based on limited previous experience from an area in Switzerland.[12] Taking average numbers of yearly patient admissions at the 9 study sites into account, we aimed for approximately proportional enrollment of patients with 200 patients each in Basel, Bern, Geneva, Lausanne, and Zurich; 150 patients each in Aarau, Lucerne, and St. Gallen; and 50 patients in Bellinzona. The study was approved by each local ethical committee.

One cotton swab was used per patient for both nostrils, placed into the transport medium M40 Transystem (Copan, Brescia, Italy), and was sent overnight to the central bacteriological laboratory in Basel, except on weekends. For culture of *S. aureus*, a chromogenic medium (MRSA ID agar, bioMérieux, Marcy l'Etoile, France), and a selective enrichment broth (brain heart infusion broth with 6% NaCl, Biomedics, Madrid, Spain) were inoculated. After incubation overnight, the broth was subcultured onto another chromogenic agar for *S. aureus* (Chromagar *S. aureus*, Hy Laboratories, Rehovot, Israel). Identification of *S. aureus* was based on various traits such as typical growth on a chromogenic medium and detection of

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Serum levels of matrix metalloproteinase-9 and tissue inhibitors of metalloproteinases 1 in subacute sclerosing panencephalitis

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Abstract

We determined the relationship between the serum concentrations of matrix metalloproteinase-9 (MMP-9) and tissue inhibitors of metalloproteinases 1 (TIMP-1) in 33 patients with subacute sclerosing panencephalitis (SSPE) to investigate the function of the blood-brain-barrier (BBB) in SSPE. Serum MMP-9 and TIMP-1 levels were measured by ELISA. Serum MMP-9 levels and MMP-9/TIMP-1 ratios of SSPE patients in Papua New Guinea ($n=24$), and those in Japan ($n=9$) were significantly higher than the each control (MMP-9, $p=0.0390$, and $p=0.0023$, respectively; MMP-9/TIMP-1, $p=0.0319$, and $p=0.0009$, respectively). Serum MMP-9 levels and MMP-9/TIMP-1 ratios of SSPE patients with Jabbour stage III ($n=13$) were significantly higher than those with Jabbour stage II ($n=18$) ($p=0.003$, and $p=0.0412$, respectively). There were no significant differences of serum TIMP-1 levels between the SSPE patients and controls. High serum MMP-9 and MMP-9/TIMP-1 levels will promote brain invasion through the BBB by immunocompetent cells in the blood. Our findings suggest that the balance of serum MMP-9 and TIMP-1 levels modulate the inflammatory cascade of SSPE.

Keywords: Blood-brain-barrier; MMP-9; Subacute sclerosing panencephalitis; TIMP-1

1. Introduction

Matrix metalloproteinases (MMPs) constitute a family of enzymes that mediate the degradation of extracellular matrix proteins [1]. MMPs play important roles in normal and pathological processes, including embryogenesis, wound healing, inflammation, arthritis, cardiovascular diseases, pulmonary diseases and cancer [2]. MMP-9 is a member of this family, which is capable of degrading collagen IV, a major component of the basement membrane of the cerebral endothelium and promotes the migration of cells through tissue or across the blood-brain-barrier (BBB) [3]. The activity of MMPs is further controlled by specific tissue inhibitors of metalloproteinases (TIMPs) [4]. TIMP-1 has a high avidity for MMP-9 [5].

In multiple sclerosis (MS), which is a chronic inflammatory demyelinating disease of the central nervous system (CNS), MMP-9, TIMP-1, and the balance between MMP-9 and TIMP-1 have been investigated [6–16]. In other CNS disorders, including acute disseminated encephalomyelitis (ADEM), neuroblastoma and cerebral-amyloid-angiopathy-related hemorrhage, it is likely that MMP-9 and/or TIMP-1 play certain roles in the pathogenesis [17–19].

Subacute sclerosing panencephalitis (SSPE) is a rare progressive inflammatory disease of the brain caused by persistent infection by the measles virus. However, the

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immunological pathophysiology of chronic inflammation of SSPE is still unclear. A high incidence of SSPE has been previously reported in Papua New Guinea (PNG) [20]. The annual incidence of SSPE in the Eastern Highlands Province (EHP) of PNG in 1997–1998 was 98 per million of population under 20 years of age, the highest ever reported [21]. The incidence of SSPE was reported to range from 0.1 to 6 cases per million of population in other places [22–24]. Therefore, the incidence of SSPE in the EHP of PNG was more than ten times higher. To investigate the role of MMP-9 and TIMP-1 in the pathogenesis of SSPE, we determined the relationship between serum concentrations of MMP-9 and TIMP-1 in SSPE patients in PNG and Japan. Moreover, the relationship between the data and clinical stage of SSPE (Jabbour stage) was analyzed [25].

2. Materials and methods

Informed consent was obtained from the parents of the patients and controls enrolled in this study. The protocol was approved by the Medical Research Advisory Committee of PNG (MRAC No. 04/01).

2.1. Subacute sclerosing panencephalitis (SSPE)

Serum samples were obtained from 33 children with SSPE (twenty-one males and twelve females, aged from 4 to 17 years; mean, 9.2 years) (24 children at Goroka Base General Hospital in PNG, from October 1997 to June 2004; 9 in Japan, from September 1996 to September 2004). The criteria for the diagnosis of SSPE were (1) progressive neurological disorder, particularly mental or motor deterioration, associated with a positive history or the presence of myoclonic jerks, (2) a positive CSF measles antibody titer determined by enzyme-linked immunosorbent assaying (ELISA), (3) high serum EIA values to an extent comparable to those of cases that fulfil criteria 1 and 2, and (4) periodic synchronous discharges (PSDs) in the EEG (n=26). One patient was included into Jabbour stage I characterized by psychointellectual dysfunction, 18, stage II characterized by convulsive and motor signs, 13, stage III characterized by deterioration of the state of consciousness to coma, and 1, stage IV characterized by mutism. Samples were stored at −70 °C.

2.2. Control subjects

The PNG control subjects for the serum levels of MMP-9 and TIMP-1 were 48 healthy PNG children (29 males and 19 females, aged from 5 to 15 years: mean, 10.7 years). The Japanese control subjects for the serum levels of MMP-9 and TIMP-1 were 33 healthy Japanese children (15 males and 18 females, aged from 2 to 15 years: mean, 6.4 years).

2.3. Determination of MMP-9 and TIMP-1 concentrations

The serum concentrations of MMP-9 and TIMP-1 were determined with sandwich-type ELISA kits (Amersham, Buckinghamshire, England). Assays were performed following the instructions of the manufacturer. The detection limits were 2.5 ng/ml for MMP-9, and 2.4 ng/ml for TIMP-1. The assay of MMP-9 recognizes the pro and active forms of MMP-9. The intra-assay coefficient of variation for serum measurements of MMP-9 has been calculated to be 3.1%.

2.4. Statistical analysis

All values are means±SD. The differences in the results between groups were analyzed using the Mann–Whitney U test.

3. Results

The serum MMP-9 and TIMP-1 levels, and MMP-9/TIMP-1 ratios of the PNG controls were 78.5±57.8 ng/ml, 139.9±84.7 ng/ml, and 0.74±0.60, respectively. The serum

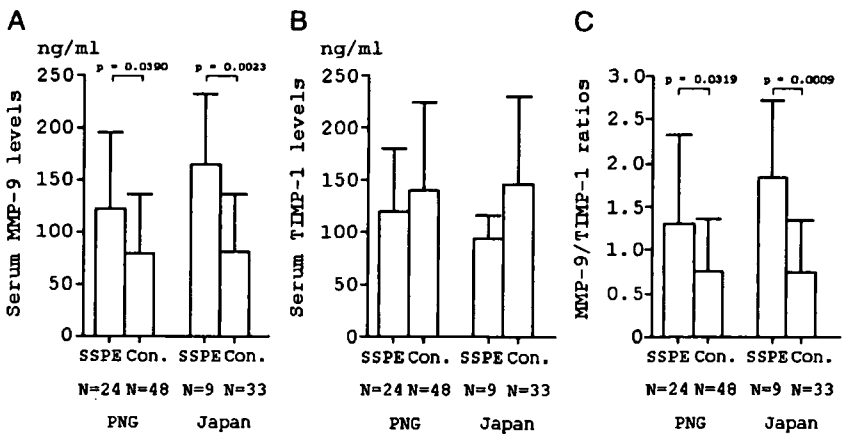

Fig. 1. Serum concentrations of MMP-9 (A), TIMP-1 (B) and the ratios of MMP-9/TIMP-1 (C) in SSPE patients and controls (con.) of PNG and Japan. Data are presented as means+1 SD.

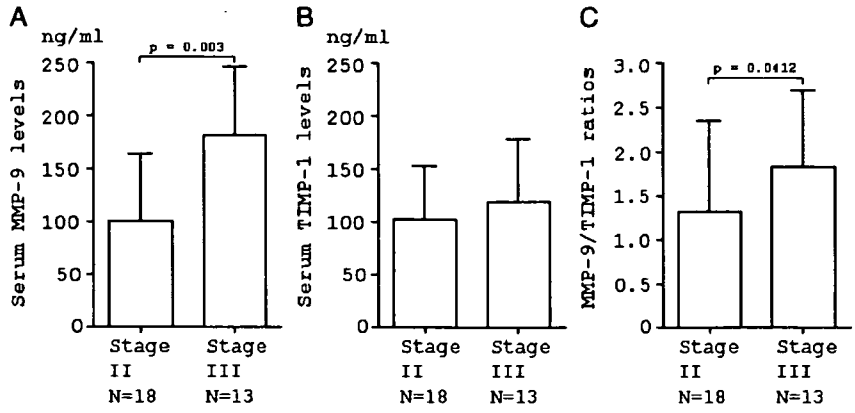

Fig. 2. Serum concentrations of MMP-9 (A), TIMP-1 (B), and the ratios of MMP-9/TIMP-1 (C) in SSPE patients at Jabbour stage II and III. Data are presented as means+1 SD.

MMP-9 and TIMP-1 levels, and MMP-9/TIMP-1 ratios of the Japanese controls were 81.0±54.9 ng/ml, 146.0± 85.4 ng/ml, and 0.75±0.61, respectively. There were no significant differences of the serum MMP-9 and TIMP-1 levels, and MMP-9/TIMP-1 ratios of between controls in PNG and those in Japan. Serum MMP-9 levels and MMP-9/ TIMP-1 ratios of SSPE patients in PNG ($n=24$), and those in Japan ($n=9$) were significantly higher than the each control (MMP-9, $p=0.0390$, and $p=0.0023$, respectively; MMP-9/ TIMP-1, $p=0.0319$, and $p=0.0009$, respectively) (Fig. 1A, B, and C). There were no significant differences of the serum TIMP-1 levels between SSPE patients and controls in PNG or Japan ($p=0.3911$, and $p=0.0598$, respectively). There were no significant differences of the serum MMP-9 and TIMP-1 levels, and MMP-9/TIMP-1 ratios between the SSPE patients in PNG and those in Japan. Serum MMP-9 levels and MMP-9/TIMP-1 ratios of SSPE patients with Jabbour stage III ($n=13$) were significantly higher than those with Jabbour stage II ($n=18$) (MMP-9, 181.4±65.4 ng/ml vs. 100.4±63.6 ng/ml, $p=0.003$; MMP-9/TIMP-1, 1.83± 0.87 vs. 1.32±1.04, $p=0.0412$) (Fig. 2A, B, and C). There were no significant differences of the serum TIMP-1 levels between SSPE patients with Jabbour stage II and III ($p=0.5215$).

4. Discussion

Several studies have reported the cytokine profiles of SSPE [26–31]. We newly analyzed serum MMP-9 and TIMP-1 levels in SSPE. MMPs are produced by a wide variety of cells, such as monocytes/macrophages, T cells, neutrophils, endothelial cells, microglia, astrocytes and oligodendrocytes [1,32]. It is likely that proinflammatory cytokines and MMPs facilitate the migration of T cells and macrophages from the intravascular compartment into the CNS in MS [33,34]. Moreover, it is believed that MMPs induce myelin break-down, proinflammatory cytokine production, and axonal damage in MS [35–37].

It is also likely that MMP-9 and TIMP-1 are related to the inflammatory cascade of SSPE because serum MMP-9 levels and MMP-9/TIMP-1 ratios were elevated in SSPE patients, and were related to the clinical severity of SSPE. Serum MMP-9 levels and MMP-9/TIMP-1 ratios of SSPE patients in Japan tended to be higher than those in PNG with high incidence of SSPE because the Jabbour stage of SSPE patients in Japan (mean, 2.78) was worsened more than that in PNG (mean, 2.29). However, there were no significant differences of the serum MMP-9 and TIMP-1 levels, and MMP-9/TIMP-1 ratios between both groups. It is likely that elevated MMP-9 levels and MMP-9/TIMP-1 ratios indicate the poor function of the BBB. We suggest that the function of BBB in SSPE patients in PNG is similar to that in Japan.

In the brain of SSPE patients, CD4+ T lymphocytes, CD8+ T lymphocytes, and CD20+ B lymphocytes were existed, especially in the perivascular regions [38]. These findings suggest that the immunocompetent cells in the blood invade the brain. Peripheral blood cells can easily invade the CNS through the BBB at high serum MMP-9 levels and MMP-9/TIMP-1 ratios. We suggest that MMP-9 and TIMP-1 are concerned with some aspect of the inflammatory cascade in SSPE.

In summary, serum MMP-9 levels and MMP-9/TIMP-1 ratios were elevated in SSPE, and were related to the clinical severity of SSPE. It is our speculation that MMP-9 and TIMP-1 modulate the inflammatory cascade of SSPE.

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Direct detection of human herpesvirus 6 DNA in serum by the loop-mediated isothermal amplification method

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Abstract

Background: A more rapid and easier method is needed for monitoring human herpesvirus 6 (HHV-6) infections. The loop-mediated isothermal amplification method (LAMP) can detect viral DNA with high specificity, efficiency, and speed under isothermal conditions. LAMP requires only simple equipment that is available in hospital laboratories.
Objectives: We evaluated LAMP as a means of detecting HHV-6 DNA directly from patients' sera.
Results: The sensitivity of the HHV-6 LAMP protocol without heat denaturation was 1000 copies/tube; with heat denaturation 10 copies/tube were detected. Three hundred serum samples from children with fever were analyzed. Using HHV-6 isolation as a definition of HHV-6 infection, the sensitivity, specificity, positive predictive value, and negative predictive value of the HHV-6 LAMP method without DNA extraction were 95.5%, 95.2%, 94.0%, and 96.4%, respectively.
Conclusion: Direct detection of HHV-6 DNA in serum with a modified HHV-6 LAMP could be used for rapid diagnosis of exanthem subitum (ES).

Keywords: HHV-6; LAMP; Serum; Exanthem subitum

1. Introduction

Exanthem subitum (ES) is common febrile illness in infants caused by primary human herpesvirus 6 (HHV-6) B infection (Yamanishi et al., 1988; Asano et al., 1989). Although the disease usually is benign and self-limited (Asano et al., 1994), HHV-6 rarely can cause severe complications, such as encephalitis (Asano et al., 1992; Suga et al., 1993), fulminant hepatitis (Asano et al., 1990), hemophagocytic syndrome (Huang et al., 1990), and myocarditis (Yoshikawa et al., 2001a). As parents' anxiety about febrile infants is great, most patients with ES in Japan visit outpatient clinics. The clinical diagnosis is difficult before appearance of the skin rash, since high fever alone is characteristic of the acute phase of the disease (Asano et al., 1994). Therefore, a rapid laboratory method is needed to diagnose HHV-6 infection during the febrile period of ES. Additionally, reactivation of the virus can manifest in several ways, such as acute graft-versus-host-disease-like illness (Yoshikawa et al., 1991, 2001b, 2002), encephalitis (Drobyski et al., 1994; Singh and Paterson, 2000), bone marrow suppression (Drobyski et al., 1993; Carrigan and Knox, 1994; Ljungman et al., 2000), and interstitial pneumonitis (Carrigan et al., 1991; Cone et al., 1993) in organ transplant recipients. Thus, a rapid diagnostic procedure would be valuable for management of organ transplant recipients.

Abbreviations: HHV-6, human herpesvirus 6; LAMP, loop-mediated isothermal amplification; PBMCs, peripheral blood mononuclear cell; PCR, polymerase chain reaction

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To date rapid diagnosis of HHV-6 infection has proven difficult. Isolation of HHV-6 requires co-cultivation with pre-activated cord blood mononuclear cells, which is difficult to perform in commercial laboratories. Moreover, both viral isolation and serological testing require substantial amounts of time to obtain final results. Although we established the antigenemia assay for HHV-6 immediate early antigen in peripheral blood mononuclear cells (PBMCs) (Nishimura et al., 2005), it is not practical for routine use in small hospital laboratories because of its complexity. Rapid diagnosis using polymerase chain reaction (PCR), in particular real-time PCR, may eventually become a valuable tool for monitoring of active viral infection (Ihira et al., 2002; Sashihara et al., 2002), but it is not a common procedure in small hospital laboratories that lack the required thermal cycler. As most ES patients visit private outpatient clinics or small hospitals, cheap and easy diagnostic procedures for ES would be desirable.

Notomi et al. (2000) reported a novel nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP) that amplifies DNA with high specificity, efficiency, and speed under isothermal conditions. The most significant advantage of the LAMP method is the ability to amplify specific sequences of DNA under isothermal conditions between 63 °C and 65 °C. As a result, it requires only simple and inexpensive equipment available in typical hospital laboratories. We developed the LAMP method for detection of six human herpesviruses (Ihira et al., 2004; Yoshikawa et al., 2004; Okamoto et al., 2004; Enomoto et al., 2005; Kimura et al., 2005; Sugiyama et al., 2005; Suzuki et al., 2006). Furthermore, we found that herpes simplex virus DNA could be detected directly in swab samples by the LAMP method without DNA extraction (Enomoto et al., 2005). Recently, we utilized LAMP after extraction of serum to specifically amplify HHV-6 DNA with high efficiency in order to diagnose active HHV-6 infection (Ihira et al., 2004). We describe below the application of the LAMP method for rapid diagnosis of HHV-6 infection by detecting HHV-6 DNA in serum without DNA extraction.

2. Materials and methods

2.1. Study design and study subjects

In order to detect HHV-6 DNA in serum directly, initial validation analysis was carried out as follows. The effect of a heat denaturation (96 °C for 30 s) step in the HHV-6 LAMP reaction on the lower limit of detection was evaluated by using serial dilutions of the pGEMH6 S12 plasmid in control serum that did not contain HHV-6 DNA. Additionally, in order to determine if serum was inhibitory in the LAMP reaction, serial dilutions of the pGEMH6 S12 plasmid in sterilized water and the control serum were used to determine the lower limit of detection.

A total of 300 febrile children (157 male and 143 female, 11.6 ± 8.7 months), who attended an outpatient clinic in our university hospital or Showa hospital, were enrolled into the study. Informed consent was obtained from the parents of all children. Four milliliters of blood (2 ml EDTA-treated and 2 ml clotted) were collected during the acute phase (febrile period) of the illness.

2.2. Isolation of HHV-6

The procedures for isolation and identification of HHV-6 have been described (Asano et al., 1989).

2.3. LAMP

Primers for HHV-6 LAMP (H6U31BIP; H6U31FIP; H6U31B3; H6U31F3) were designed for the HHV-6 B U31 gene using Primer Explorer V Soft Ware (Ihira et al., 2004). Since additional loop primers increase the amplification efficiency (Nagamine et al., 2002), H6U31LPB and H6U31LPF were also synthesized. The LAMP reaction was carried out using the Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan) according to the manufacturer's instruction. LAMP was carried out with 25 μl of a mixture containing 2.4 μM H6U31FIP and H6U31BIP primers, 0.4 μM of each outer primer (H6U31F3 and H6U31B3), 1.2 μM of each loop primer (H6U31LPF and H6U31LPB), 20 mM Tris–HCl [pH 8.8], 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.8 M betaine, 1.4 mM of each d-NTPs, 0.1% Tween20, 8U of *Bst* DNA polymerase, and 5 μl of template DNA. The mixture was incubated at 63 °C for 60 min, and increased turbidity was monitored by using LA-200 (Teramecs Co., Ltd., Kyoto, Japan) (Mori et al., 2002). In order to increase the sensitivity of the method, a heat denaturation step (96 °C for 30 s) was added as follows: the mixture without *Bst* DNA polymerase containing template DNA (patient's serum) was incubated at 96 °C for 30 s, after which 1 μl (8U) of *Bst* DNA polymerase was added, and this mixture incubated at 63 °C for 60 min. The LAMP products were detected turbidometrically using LA-200. The cutoff value for discrimination between positive and negative samples was 0.1, which was determined by analyzing five virus-negative samples. During the initial analysis of the effects of heat denaturation and inhibitory effects of serum, agarose gel electrophoresis was also used to detect LAMP products. Six microliters of LAMP products were subjected to electrophoresis on a 1.5% agarose gel. After staining with ethidium bromide, the products were detected using UV light.

3. Results

In order to evaluate the effect of heat denaturation on assay sensitivity, the lower detection limits were compared with and without a heat denaturation step. The HHV-6 LAMP protocol with heat denaturation was 100 times more sensitive

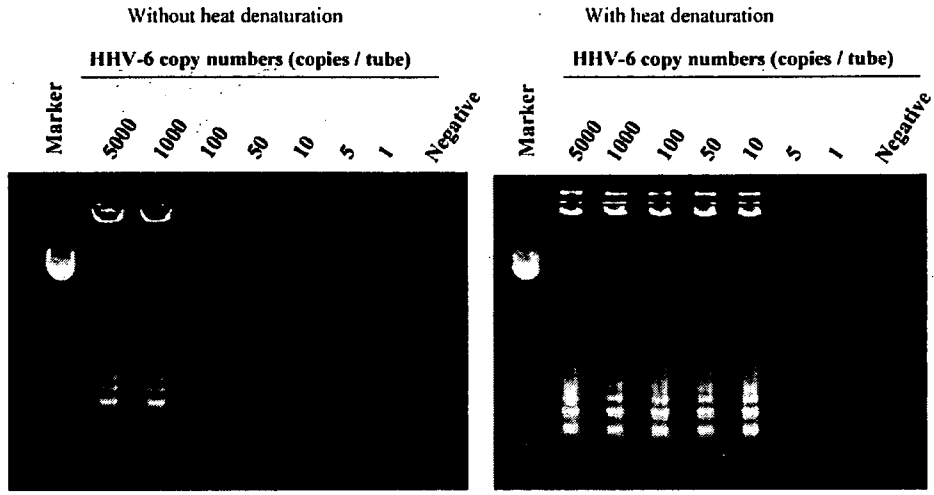

Fig. 1. Evaluation of heat denaturation (96 °C for 30 s) on the sensitivity of HHV-6 LAMP. Serial dilutions of pGEMH6S12 plasmid DNA in control serum with or without heat denaturation were amplified by the LAMP.

(10 copies/tube) than when heat denaturation was omitted (Fig. 1).

Direct detection of HHV-6 DNA in patients' serum by the LAMP method should provide a more rapid and easier diagnostic procedure. In order to evaluate the sensitivity of such an assay, potential inhibitory effects of serum were explored. In reconstruction experiments the lower limit of detection of HHV-6 in sterilized water was 5 copies/tube, while in serum 10 copies/tube were detected, using either the turbidometric or agarose gel electrophoresis assays (Fig. 2). Furthermore, threshold time (time to reach the threshold level of turbidity of 0.1) of each plasmid dilution in sterile water was faster than when serum was present (Fig. 2).

The results of viral isolation and direct serum HHV-6 LAMP were compared to assess the reliability of the direct LAMP method for rapid diagnosis of primary HHV-6 infection. HHV-6 was isolated from 132 (44.0%) of the 300 PBMCs samples collected (Table 1). HHV-6 DNA was detected in 126 (95.5%) of the 132 serum samples collected from the subjects with HHV-6 viremia, while HHV-6 DNA was detected in 8 (4.8%) of the 168 serum samples without HHV-6 viremia. Thus, if virus isolation was defined as standard for active virus infection, sensitivity, specificity, positive predictive value, and negative predictive value of serum HHV-6 LAMP were 95.5%, 95.2%, 94.0%, and 96.4%, respectively. HHV-6 DNA became positive by LAMP in four of the six false negative serum samples after DNA extraction from the serum.

Table 1
Comparison between viral isolation and serum LAMP was performed to evaluate the reliability of direct HHV-6 LAMP method as the diagnostic method for primary HHV-6 infection

Results of HHV-6 LAMP	Isolation of HHV-6	
	Positive	Negative
+	126	8
−	6	160

Sensitivity: 95.5%, positive predictive value: 94.0%, specificity: 95.2%, negative predictive value were: 96.4%.

4. Discussion

It has been demonstrated that the sensitivity of direct amplification of HSV DNA by the LAMP method is slightly less sensitive than when it is applied to DNA first extracted from the clinical sample (Enomoto et al., 2005). Moreover, serum has a slight inhibitory effect on the LAMP reaction (Fig. 2). Thus, it was expected that an increase in assay sensitivity would be required to detect viral DNA in serum without DNA extraction. The addition of a heat denaturation step greatly increases the sensitivity of the LAMP method (Poon et al., 2006), and we found that heat denaturation resulted in assay sensitivity up to 10 copies/tube, as measured by agarose gel electrophoresis (Fig. 1). We concluded that the modified protocol with a heat denaturation step (lower detection limit; 10 copies/tube) should be sufficient for clinical use.

The LAMP method with an initial heat denaturation step was sufficiently sensitive to detect HHV-6 directly from sera from febrile children. The effect of inhibitors in sera was negligible. Furthermore, the use of a turbidometric assay permitted the quick identification HHV-6 DNA-positive sera using equipment available in most hospital laboratories. The method also had a high sensitivity and specificity for the diagnosis of primary HHV-6 infection. As a matter of fact, turbidity of all of the positive samples reached at threshold level (0.1) within 45 min (data not shown) and the turbidity curve of the lower detection limit (10 copies/tube) reached its threshold after 39 min of LAMP reaction (Fig. 2 A). Since this protocol does not require DNA extraction, the final result is obtainable within 60 min after collecting the clinical specimens.

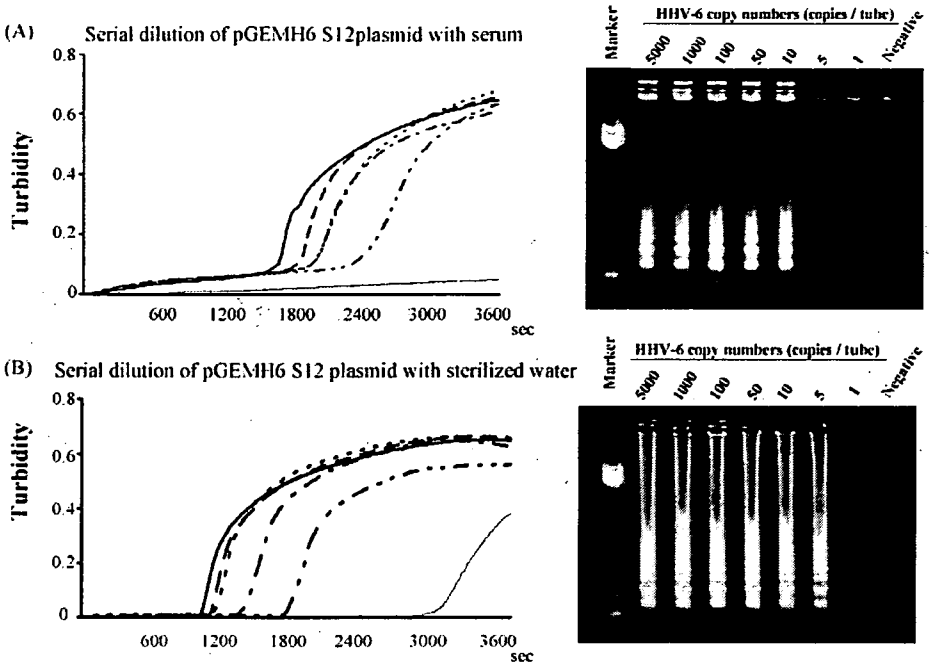

Fig. 2. Evaluation of the inhibitory effect of serum in the LAMP reaction. Serial dilutions of pGEMH6S12 plasmid DNA in either control serum (A) or sterile water (B) were amplified after heat denaturation. The LAMP products were detected by agarose gel electrophoresis and LA-200 (as turbidity). M, marker; Neg., negative; ▬▬, 5000 copies/tube; ▬ ▬, 1000 copies/tube; . . ., 100 copies/tube; ▬ ▪ ▬ 50 copies/tube; ▬ ▪ ▪ 10 copies/tube; —, 5 copies/tube; - - -, 1 copies/tube; ▬ . . ▬ . . ▬, without plasmid DNA.

Some of the false negative samples became positive after DNA extraction. It is possible that these samples contained small amounts of viral DNA that were below the detection limit of the direct HHV-6 LAMP method. Since virus isolation was defined as the marker for viral infection in this study, it is possible that some of the "false positive" results are incorrectly labeled. Serological analysis in combination with viral isolation would be necessary to determine the precise reliability of this method in future experiment.

To our knowledge, this is the first report to demonstrate direct amplification of viral DNA from serum samples without DNA extraction by this molecular biological method. The rapidity of the method permits its use for diagnosis of ES in acutely ill children. It could also be used for management of transplant recipients and could be used for rapid detection of the other pathogens.

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Autoantibodies against Glutamate Receptor ε_2-Subunit Detected in a Subgroup of Patients with Reversible Autoimmune Limbic Encephalitis

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Key Words
Autoantibody · Glutamate receptor ε_2-subunit · Immunotherapy · Limbic encephalitis · N-methyl-D-aspartate glutamate receptor · Paraneoplastic syndrome

Abstract
We investigated the presence of autoantibodies against glutamate receptor (GluR) ε_2 in serum and cerebrospinal fluid (CSF) samples from 12 consecutive patients with acute encephalitis/encephalopathy by immunoblotting using recombinant GluRε_2 as antigen. In 4 patients, IgM autoantibodies against GluRε_2 were detected in CSF in the early phase of the disease but were not detectable after several months. Seizures and psychiatric symptoms were noted during the acute phase of the disease in these 4 patients, who showed various degrees of residual amnesia. Immunotherapy was performed on 3 patients (patients 1, 3 and 4), and they showed marked improvements. Immunohistochemistry using these patients' sera showed that immunoreactivity is specifically detected in the cytoplasm of rat hippocampal and cortical neurons. The clinical features and neuroimaging findings of patients with IgM autoantibodies against GluRε_2 in CSF resemble those of patients with reversible autoimmune limbic encephalitis.

Introduction

There are some reports that indicate the production of autoantibodies in patients with encephalitis [1–3] and encephalopathy [4]. Recently, autoantibodies against the N-methyl-D-aspartate (NMDA)-type glutamate receptor (GluR) ε_2 have been detected in patients with epilepsia partialis continua (EPC) causally related to Rasmussen syndrome [5], nonparaneoplastic limbic encephalitis [6] and acute encephalitis [5, 7]. The NMDA receptor, which is one of the three major ionotropic GluRs, is a heterodimer composed of ε- and ζ-subunit families [8]. There are 4 members in the ε-subunit family (ε_1–ε_4) [9]. The NMDA receptor channel is unique in terms of its functional properties [10, 11]. After birth, the expression of GluRε_2 mRNA becomes restricted to the forebrain, which includes the cerebral cortex and limbic system [12]. GluRε_2 is associated with memory and learning [10, 11]. Therefore, we investigated autoantibodies against GluRε_2 in serum and cerebrospinal fluid (CSF) samples from patients with acute encephalitis/encephalopathy to clarify its clinical features and immunological aspects.

Materials and Methods

We obtained serum and CSF samples from 12 consecutive patients with acute encephalitis/encephalopathy in our department from August 2003 to January 2005 (n = 12; male:female = 6:6; age

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Table 1. Autoantibodies against GluRε_2

Patient	Age/sex	Clinical diagnosis	Anti-GluRε_2 antibody	
			CSF IgM/IgG	serum IgM/IgG
1	45/F	ILE	+/+	+/+
2	62/M	ILE	+/+	–/–
3	53/F	ILE	+/+	+/+
4	30/M	ILE	+/–	+/+
5	22/F	ILE	–/–	–/–
6	68/M	bacterial meningoencephalitis	–/–	–/–
7	57/F	cryptococcal meningoencephalitis	–/–	–/–
8	18/F	MELAS	–/–	–/–
9	59/F	neuro-Sweet disease	–/–	–/–
10	65/M	PLE (anti-Hu antibody-positive)	–/–	–/+
11	26/M	etiology-unknown meningoencephalitis	–/–	+/–
12	56/M	brainstem encephalitis	–/–	–/–

ILE = Idiopathic limbic encephalitis; MELAS = mitochondrial encephalopathy with lactic acidosis and stroke-like episodes; PLE = paraneoplastic limbic encephalitis.

range = 18–68; mean age = 47 years; idiopathic limbic encephalitis, 5; etiology-unknown meningoencephalitis, 1; anti-Hu-antibody-positive paraneoplastic limbic encephalitis, 1; bacterial meningoencephalitis, 1; mitochondrial encephalopathy with lactic acidosis and stroke-like episodes, 1; cryptococcal meningoencephalitis, 1; brainstem encephalitis, 1; neuro-Sweet disease, 1).

Detection of Autoantibodies against GluRε_2

The method used has previously been reported [5]. The supernatants of cell extracts from stable NIH3T3-transformant cell lines expressing full-length GluRε_2 were subjected to SDS-PAGE, and the separated proteins on the gels were transferred to nitrocellulose membranes. The membranes were reacted with diluted sera or CSF and stained with alkaline-phosphatase-labeled secondary antibodies (IgG or IgM; Jackson Immunoresearch, West Grove, Pa., USA). Anti-GluRε_2 autoantibodies were detected as a band corresponding to approximately 180 kDa.

Immunohistochemistry Using Patient's Serum

Under ether anesthesia, adult Sprague-Dawley rats were sacrificed. The cerebrums were immediately removed and frozen in dry-ice powder. Frozen sections (8 μm thick) of the cerebrums were fixed in 4% paraformaldehyde. The sections were incubated with serially diluted serum from a patient or with an anti-NMDAε_2 antibody (1:500; Santa Cruz Biotechnology, USA). Then, the sections were incubated with a goat biotinylated anti-human IgM (μ-chain-specific) antibody (Vector, USA) or a rabbit biotinylated anti-goat IgG (H+L) antibody (Chemicon, USA). After washing, the sections were reacted with a streptavidin-peroxidase complex (Nichirei, Japan). The reactions were finally developed with 3,3′-diaminobenzidine tetrahydrochloride (Wako, Japan) and 0.01% H_2O_2 in PBS. For adsorption tests, frozen sections after blocking were immunostained with a patient's serum (1:2,000) or with the anti-NMDAε_2 antibody (1:500) that was incubated for 24 h with extracts from transformant cells expressing full-length GluRε_2.

Results

Detection of Autoantibodies against GluRε_2

The IgM autoantibody against GluRε_2 in CSF was detected in 4 out of 12 consecutive patients in the early phase of the disease but was not detectable after several months (table 1). The IgG autoantibody against GluRε_2 in CSF was detected in 3 of these 4 patients (patients 1–3). No autoantibodies against GluRε_2 were detected in the CSF of the other patients. In the early phase of the disease, patients 1 and 4 had IgM and IgG autoantibodies against GluRε_2 in their serum. Patient 3 had only the IgM autoantibody but became positive for the IgG autoantibody 2 months later.

Clinical Features

All the patients who had the IgM autoantibody against GluRε_2 in CSF presented with seizures (i.e. partial seizures evolving to secondary generalized seizures) and psychiatric symptoms (i.e. hallucination, behavioral changes and agitation) in the early phase of the disease and developed prolonged consciousness disturbances with status epilepticus (table 2). None of these patients presented with paralysis or disturbances in sensation in the chronic stage. However, all of them presented with various degrees of recent memory disturbance and amnesia. Patient 3 showed residual psychiatric symptoms after treatment. Three patients (patients 1, 3 and 4) received intravenous methylprednisolone pulse therapy and showed improvement in their seizures and consciousness levels.

Table 2. Clinical features of patients with IgM autoantibody against GluRε_2 in CSF

Patient	Age/sex	Clinical diagnosis	Initial symptoms	Sequelae	Steroid treatment
1	45/F	ILE	convulsion, visual and olfactory hallucinations	disorientation, amnesia, recent memory disturbance	responsive
2	62/M	ILE	convulsion, auditory hallucination	disorientation, amnesia, recent memory disturbance	not performed
3	53/F	ILE	convulsion, behavioral changes	disorientation, amnesia, recent memory disturbance, psychiatric symptoms	responsive
4	30/M	ILE	convulsion, behavioral changes	amnesia, recent memory disturbance	responsive

ILE = Idiopathic limbic encephalitis.

Case Presentation

Here, we describe a representative patient with IgM autoantibodies against GluRε_2 in CSF. Patient 1 has been described previously [6].

Patient 4 was a 30-year-old male initially complaining of headaches with fever. Throughout 1 week, he showed no improvement in these symptoms and presented with agitation and behavioral disturbance. He was admitted to a nearby hospital because he suddenly suffered a generalized tonic seizure. After admission to the hospital, he was treated with antiepileptic drugs and intravenous acyclovir. However, he developed consciousness disturbance and status epilepticus. Then, he was transferred to our hospital. He had an unremarkable medical history, a temperature of 37.7°C, a pulse of 74/min and a blood pressure of 105/68 mm Hg. On neurological examination, he exhibited somnolence. There was lateral gaze-evoked nystagmus. The deep tendon reflexes of all four limbs were slightly hypoactive. There were no pathological reflexes. No meningeal signs were observed. After his consciousness level had improved, muscle strength, sensation and coordination became normal. Laboratory tests revealed leukocytosis and elevated levels of myogenic enzymes: white blood cell count, 13.8 × $10^3/\mu l$; creatine kinase level, 3,285 IU/l; aspartate aminotransferase level, 80 IU/l, and lactate dehydrogenase level, 394 IU/l. The serum C-reactive protein level was slightly elevated to 1.89 mg/dl. The serum antinuclear antibody was absent. IgM and IgG autoantibodies against GluRε_2 were present in serum on the day of admission. Analysis of CSF showed 34 cells/mm^3 (mononuclear cells only), 36 mg/dl total protein and 68 mg/dl glucose. He had a mildly elevated IgG index (0.73). IgM and IgG antibodies against herpes simplex virus, cytomegalovirus and varicella-zoster virus were absent in paired sera and CSF tested at 2-week intervals. PCR analysis showed negativity for herpes simplex virus, cytomegalovirus and human herpesvirus 6/7 DNA in CSF. The IgM autoantibody against GluRε_2 was present, but the IgG autoantibody was absent in CSF on the day of admission. EEG revealed diffuse θ-waves with small spikes in the left temporal lobe. Brain MRI revealed no abnormal intensity changes but diffuse cortical edema. ^{99m}Tc-ECD SPECT performed on the 14th day of hospitalization revealed hypoperfusion in the diffuse cerebral cortex and bilateral mesial temporal lobes. After admission, he was treated with phenytoin. However, the seizures were difficult to control and required treatment with anesthetic agents (pentobarbital sodium and midazolam) under respiratory management. He was treated with an intravenous infusion of 1 g of methylprednisolone for 3 consecutive days. He showed improvement in consciousness level and the frequency of the convulsions decreased following the treatment. However, approximately 2 weeks after steroid therapy, his seizures increased in frequency again, and intravenous immunoglobulin (400 mg/kg/day for 5 consecutive days) was administered. His condition slowly improved. On the 36th day of hospitalization, he required no respiratory management. Afterwards, we carried out the administration of intravenous immunoglobulin and steroid pulse therapy, and his seizures disappeared completely. However, he showed disorientation and severe amnesia. Revised Hasegawa Dementia Scale (HDS-R) and Mini Mental State Examination scores determined approximately 3 months after admission were 10/30 and 13/30, respectively. His Revised Wechsler Adult Intelligence Scale (WAIS-R) full-scale IQ was less than 40, and his verbal and performance subscale scores were 51 and less than 45, respectively. All Revised Wechsler Memory Scale (WMS-R) indexes showed significantly low scores (gen-

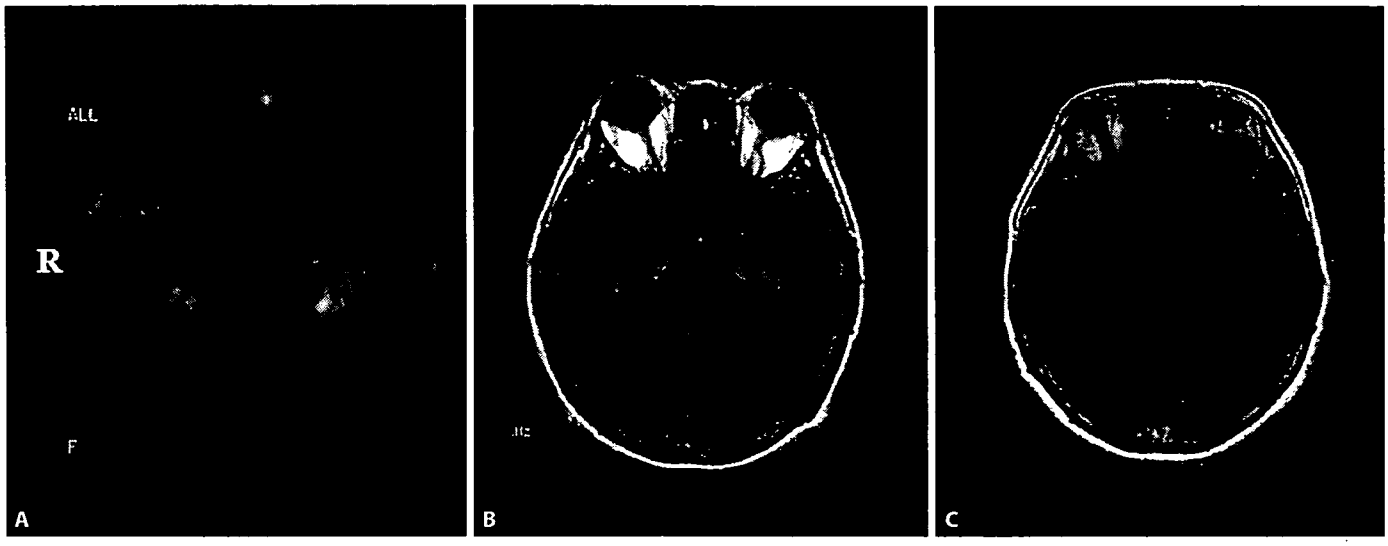

Fig. 1. MR images of patient 1. An axial diffusion-weighted image (**A**) and a fluid-attenuated inversion recovery image (**B**) show hyperintensity in the bilateral mesial temporal lobes in the acute phase (2 days after admission). **C** A follow-up T_1-weighted MR image shows the disappearance of signal abnormalities and the appearance of bilateral hippocampal and mild cortical atrophies (1 year after the disease onset).

Table 3. Laboratory and neuroimaging findings of patients with IgM autoantibody against GluRε_2 in CSF

Patient	WBC n/mm^3	CRP mg/dl	CSF cell n/mm^3	CSF protein mg/dl	IgG index	Other autoantibodies	Initial brain MRI
1	13,800	1.30	81 (M 81)	30	0.53	TPO Ab	hyperintensity in bilateral mesial temporal lobes
2	3,500	0.42	10 (M 9, P 1)	67	0.80	–	hyperintensity in left mesial temporal lobe
3	18,240	<0.05	7 (M 1, P 6)	38	n.e.	ANA	hyperintensity in bilateral mesial temporal lobes, insulae and cingulate gyri
4	17,090	1.89	34 (M 34)	36	0.73	–	normal

WBC = White blood cells; CRP = C-reactive protein; MRI comprised diffusion-weighted, T_2-weighted and fluid-attenuated inversion recovery imagings; ANA = antinuclear antibody; TPO Ab = thyroid peroxidase antibody; M = mononuclear cell; P = polynuclear cell; n.e. = not examined.

eral memory <50; verbal memory = 54; delayed memory <50; visual memory <50; attention and concentration <50). It was difficult for him to immediately recall things related to logical memory after hearing them. He showed no higher functional impairments such as aphasia, apraxia and agnosia. His memory and cognitive state slowly improved, and he was discharged 4 months after admission. Eight months after the disease onset, his memory and cognitive scale scores improved significantly [HDS-R score 25/30, WAIS-R IQ (total IQ, 70; verbal IQ, 63; performance IQ, 87), WMS-R scores (general memory, 53; verbal memory, 57; delayed memory, 60; visual memory, 70; attention and concentration, 61)]. Brain MRI revealed no intensity changes; however, mild cortical atrophy was observed 6 months after the disease onset.

Laboratory Findings

Virological examinations (table 3) showed negativity for IgM and IgG antibodies against the herpes simplex virus, cytomegalovirus and varicella-zoster virus in paired sera and CSF tested at 2-week intervals. PCR analysis showed negativity for the herpes simplex virus and human herpesvirus 6/7 DNA in CSF. Patient 3 had the antinuclear antibody (ELISA: 34.4, normal <20.0) in her se-

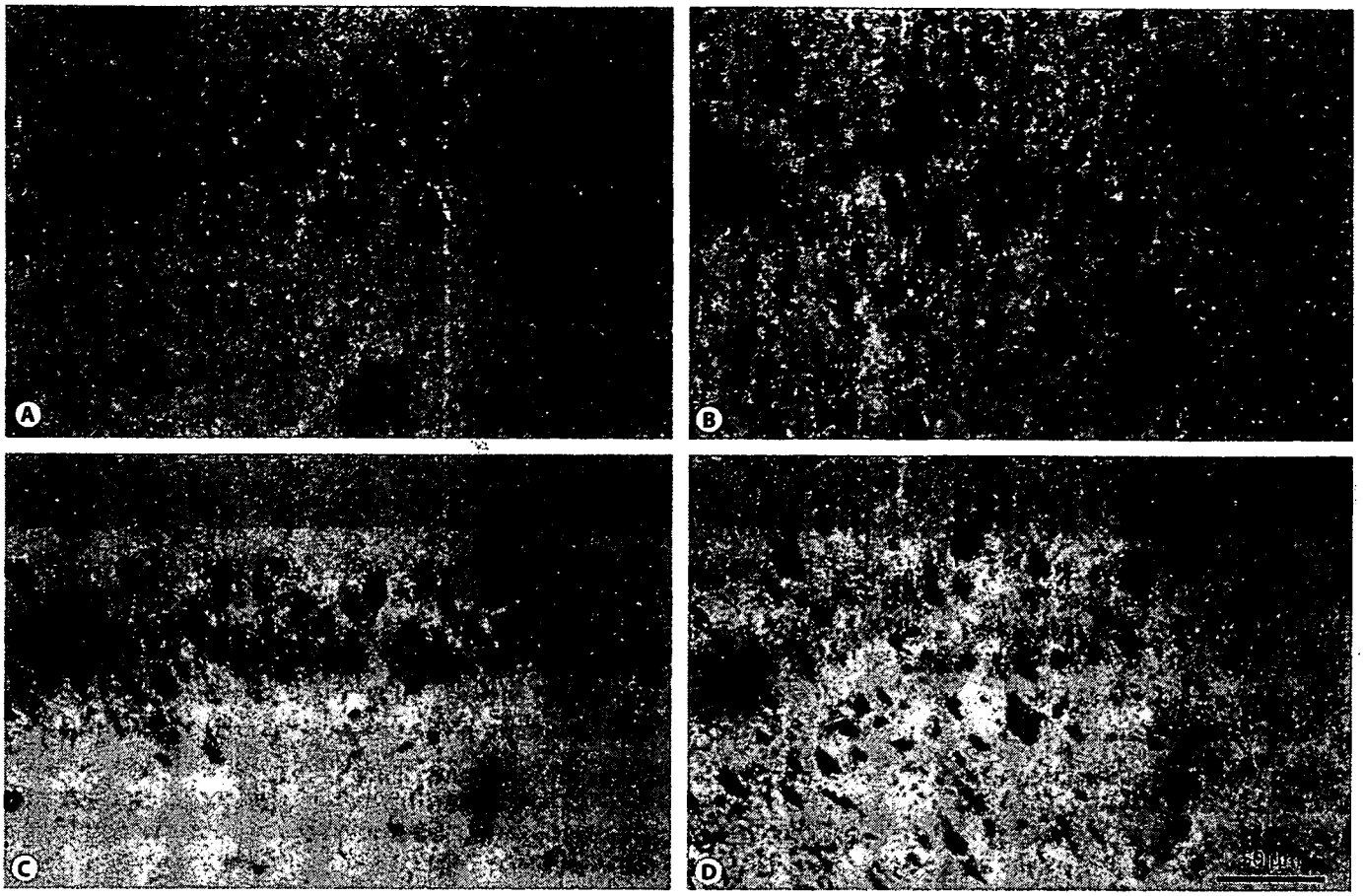

Fig. 2. Immunohistochemistry. **A, B** Coronal sections of rat brain immunoreacted with serum samples from patient 1. **C, D** Coronal sections immunoreacted with serum samples from patient 4. The diluted sera (1:2,000) of these patients reacted with the cytoplasm of rat hippocampal (**A, C**) and cortical (**B, D**) neurons. The same pattern of reactivity was observed for the serum samples from patient 3 and the anti-NMDAε_2 antibody. The sections are slightly counterstained with hematoxylin. Magnification ×400.

rum. Patient 1 showed an elevated level of the antithyroperoxidase antibody in the serum, which was not detected in CSF. We measured the titers of voltage-gated potassium channel (VGKC) antibodies by radioimmunoassay using whole rabbit brain homogenate as described previously [13]. The titers of VGKC antibodies showed normal levels in these 4 patients (patient 1: 0 pM, patient 2: 21 pM, patient 3: 0 pM, patient 4: 18 pM, normal range <100 pM).

Neuroimaging and Physiological Examination

Brain MRI at disease onset showed signal abnormalities in the bilateral mesial temporal lobes in patients 1 and 3 and on one side of the mesial temporal lobe in patient 2. In patient 4, brain MRI showed no signal abnormalities. However, several months (range, 6 months to 1 year) after the disease onset, cerebral atrophy of various degrees, including atrophy in the mesial temporal lobe, was common (fig. 1). SPECT was performed in the acute or subacute phase. All of the patients except patient 1 showed hypoperfusion in the mesial temporal lobe. In patient 1, ^{99m}Tc-HMPAO SPECT performed the day after admission revealed hyperperfusion in the bilateral mesial temporal lobes; however, hypoperfusion was observed 17 months later. All the patients showed irregular diffuse cortical hypoperfusion. In 3 patients, EEG revealed small focal spikes. In all the patients, a mixture of diffuse θ-waves was observed. No tumors were detected in any of the patients using various methods [chest, abdomen and pelvic CTs (all patients), whole-body FDG-PET (patient 1) and gallium scintigraphy (patient 2)].

Immunohistochemical Findings

The diluted serum samples from patients 1, 3 and 4 reacted with the cytoplasm of rat hippocampal and cerebral cortical neurons (fig. 2). The most appropriate dilution of serum for immunohistochemical staining was 1:2,000–4,000. The sections incubated with the anti-NMDAε_2 antibody showed the same pattern of immunoreactivity as those incubated with the patients' sera. The serum of the healthy control did not significantly immunoreact with the sections. The immunoreactivity of sera of the 3 above-mentioned patients to the anti-GluRε_2 antibody was markedly decreased by prior incubation with the supernatants of extracts from stable transformant cells expressing GluRε_2. Patients 1, 2 and 3 were negative for serum paraneoplastic anti-Yo, anti-Hu, anti-Ri, anti-CV2, anti-Tr, anti-Ma-2 and antiamphiphysin antibodies.

Discussion

We presented 4 patients with acute encephalitis who had IgM autoantibodies against GluRε_2 in CSF in the early phase of the disease; however, these antibodies were not detectable after several months. Other acute encephalitis/encephalopathy patients (patients 5–12) had neither the IgG nor IgM autoantibody against GluRε_2 in CSF. Four antibody-positive patients had the characteristic clinical features of reversible autoimmune limbic encephalitis such as intractable convulsion, psychiatric symptoms, recent memory disturbance and sufficient responsiveness to immunotherapy. Concerning the cause of limbic encephalitis, there are many reports in which limbic encephalitis is associated with cancer, most commonly a small-cell carcinoma of the lung [14–20]. The 4 antibody-positive patients had no findings of viral infection or cancer. Several reports have been published concerning the immunotherapy response form of nonparaneoplastic limbic encephalitis [21–26]. Recently, a VGKC antibody has been detected in patients with reversible autoimmune limbic encephalitis [27–32]. However, the titers of VGKC antibodies were normal in our 4 antibody-positive patients' sera. We speculate that reversible autoimmune limbic encephalitis is heterogeneous. Some forms of this disease may be mediated by autoantibodies against antigens such as ion channels or ionotropic receptors in the limbic system.

In our immunohistochemical analysis of the sera of patients with the IgM autoantibody against GluRε_2, immunoreactivity was detected in the cytoplasm of neurons in the hippocampus and cerebral cortex. The immunoreactivity was specifically demonstrated using an immunoadsorption test. The NMDA receptor is one of the ionotropic glutamate receptors essential for excitatory neurotransmission and synaptic plasticity, which underlie memory and learning [10, 11]. An antibody-mediated disturbance of NMDA-type GluR function might influence synaptic plasticity in the hippocampus and cortical neuronal excitability. The clinical response to immunotherapy and the results of immunoblotting and immunohistochemistry suggest that IgM autoantibodies may be related to pathogenesis in a subgroup of patients with reversible autoimmune limbic encephalitis.

In a previous study, autoantibodies against GluRε_2 were detected in patients with chronic EPC causally related to Rasmussen syndrome [7]. There are some reports that these antibodies have also been detected in patients with encephalitides other than chronic EPC [6, 7]. In this study, we detected autoantibodies against GluRε_2 in patients with reversible autoimmune limbic encephalitis. We suggest that autoantibodies against GluRε_2 contribute to the onset of localized encephalitides such as EPC and reversible autoimmune limbic encephalitis [7].

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High prevalence of serum autoantibodies against the amino terminal of α-enolase in Hashimoto's encephalopathy

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Abstract

Recently, we discovered autoantibodies against the amino (NH_2)-terminal of α-enolase (NAE) in patients with Hashimoto's encephalopathy (HE) (83.3%; 5/6) [Fujii, A., Yoneda, M., Ito, T., Yamamura, O., Satomi, S., Higa, H., Kimura, M., Suzuki, M., Yamashita, M., Yuasa, T., Suzuki, H., Kuriyama, M., 2005. Autoantibodies against the amino terminal of α-enolase are a useful diagnostic marker of Hashimoto's encephalopathy. J. Neuroimmunol. 162, 130–136]. We further investigated the anti-NAE autoantibodies in 25 patients who fit the diagnostic criteria for HE, based on the presence of anti-thyroid antibodies and responsiveness to immunotherapy. In this study, we demonstrated a high prevalence (68%, 17 of 25) and high specificity of anti-NAE autoantibodies in patients with HE, and clarified the clinical features of HE. This result demonstrated that anti-NAE autoantibodies, in addition to anti-thyroid autoantibodies, are emphasized as useful serological diagnostic markers of HE.

Keywords: Hashimoto's encephalopathy; Autoantibodies; NAE; Clinical features

1. Introduction

Hashimoto's thyroiditis (HT) is the most common disorder affecting the thyroid gland. In 1966, Brain et al. reported the first case of encephalopathy associated with HT, who presented with recurrent neuropsychiatric symptoms accompanied by serum anti-thyroid antibodies in euthyroid states (Brain et al., 1966). Hashimoto's encephalopathy (HE) therefore was recognized as a nomenclature of new disease, distinct from myxoedema encephalopathy associated with hypothyroidism (Behan et al., 1988; Shaw et al., 1991; Chaudhuri and Behan, 2003; Chong et al., 2003).

Autoimmune mechanism has been proposed as an underlying pathogenesis (Brain et al., 1966; Chaudhuri and Behan, 2003; Chong et al., 2003), and immunotherapy such as steroids, immunosuppressants and/or intravenous injection of immunoglobulin (IVIg)/plasmapheresis was successfully administered (Shaw et al., 1991; Boers and Colebatch, 2001; Chaudhuri and Behan, 2003). Over 100 accumulated cases (Shaw et al., 1991; Chaudhuri and Behan, 2003; Chong et al., 2003) reported mainly by neurologists, emphasized this potentially treatable encephalopathy associated with HT in the differential diagnosis of unknown etiology of encephalopathy, and suggested the risk of under-diagnosis of HE (Ghika-Schmid et al., 1996; Maydell et al., 2002).

Several diagnostic criteria for HE have been proposed based on encephalopathy, the presence of anti-thyroid antibodies and/or responsiveness to immunotherapy including steroids (Shaw et al., 1991; Peschen-Rosin et al., 1999). Endocrinologists however argued against the terminology of HE because of the wide spectrum clinical features in patients with HE and the high prevalence of anti-thyroid antibodies in the normal population, which are usually subclinical (Sawka et al., 2002; Fatourechi, 2005).

To resolve such debates on the nomenclature and nature of HE, more specific diagnostic markers are needed (Chong et al., 2003; Fatourechi, 2005). Very recently, we discovered autoantibodies against the amino (NH_2)-terminal of α-enolase (referred to as NAE) that were highly specific in sera from a limited number of HE patients (83%, 5 of 6 with HE; 11%, 2 of 17 with HT without any neuropsychiatric features; none of controls [50 individuals] including those with other neurological or immunological conditions involving encephalopathy [25 individuals]) (Fujii et al., 2005). Thus, the anti-NAE autoantibodies are a potential tool for the diagnosis of HE and resolving the debate

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over HE described above. We further investigated the prevalence and specificity of anti-NAE autoantibodies, and clarified the clinical features in a large number of patients with HE in this study.

2. Patients and methods

2.1. Patients

In this study, we selected 25 patients with HT, who presented with encephalopathy and fit the diagnostic criteria for HE, based on the presence of anti-thyroid antibodies and responsiveness to immunotherapy such as steroids, immunosuppressants and/or IVIg/plasmapheresis. These 25 patients included our own 8 cases and 17 cases from other institutions. Neurological specialists carefully excluded other possible causes of encephalopathy including infections, other autoimmune conditions, vitamin deficiency, intoxication, cerebrovascular diseases, neoplasms and Creutzfeldt–Jakob disease and so on, and the detailed clinical information of each patient was obtained from the attending physician. The ethics committee of the University of Fukui approved this research, and written permission was obtained from each patient.

The clinical profiles of all patients were summarized in Table 1 and Fig. 1. The sex ratio of patients examined was 7:18 (male:female). The mean age was 60 years old (range: 23 to 83). All patients showed the responsiveness to steroids in variable degrees. We categorized the patients into three clinical forms such as acute encephalopathy (AE)-form, subacute psychiatric (SP)-form and others. AE was the most common clinical form (76%; 19 of 25), SP was much less frequent (16%; 4 of 25), and others were 8% (2 of 25). Four of 25 patients demonstrated recurrence (16%). Five of 25 patients had a history of HT before encephalopathy appeared (20%). All patients carried anti-thyroid antibodies (both of anti-thyroglobulin [Tg] and anti-thyroid peroxidase antibodies [TPO], 48%, 12 of 25; anti-Tg, 32%, 8 of 25; anti-TPO, 20%, 5 of 25). Most of the patients were in euthyroid states (72%, 18 of 25), except for a few patients in mild hypothyroid states treated with thyroxin (16%, 4 of 25) or transient hyperthyroid states (thyrotoxicosis) (12%, 3 of 25). All of the patients showed neuropsychiatric symptoms after recovery from dysthyroid states.

Table 1
Clinical features and anti-NAE autoantibodies in patients with Hashimoto's encephalopathy

Patient	Age/ gender	Clinical form	Anti-thyroid	Immunotherapy (response)	Anti-NAE	Neurological manifestation					Abnormal EEG	Abnormal brain MRI	Elevated CSF/ IgG protein
						C	S	C/P	I	A			
1	44, F	AE	TPO[a]	PSL (excellent)	Positive	+	+	+	Chorea	−	+	−	+
2	34, M	AE	TPO, Tg	mPSL/PSL (excellent)	Positive	+	+	+	−	−	+	−	−
3	71, F	AE[b]	TPO, Tg[a]	mPSL/PSL (excellent)	Positive	+	+	+	−	−	+	−	+
4	45, F	AE	TPO, Tg	mPSL (excellent)	Positive	+	+	−	Tremor	−	*n.d.*	−	−
5	60, F	AE	Tg	PSL (excellent)	Positive	+	−	+	−	−	+	−	+
6	78, F	AE	Tg[c]	PSL (excellent)	Positive	+	+	−	Myoclonus	−	+	−	+
7	32, F	AE (LE)	TPO,Tg	mPSL/PSL (excellent)	Positive	+	+	+	−	−	+	L	−
8	57, F	AE[b]	TPO[a]	PSL (excellent)	Positive	+	+	+	Chorea	+	+	−	−
9	78, F	AE	Tg	PSL (excellent)	Positive	+	−	+	Tremor	−	+	−	+
10	83, F	AE	TPO, Tg	mPSL/PSL (good)	Positive	+	+	−	−	−	+	−	−
11	76, M	AE	TPO, Tg[c]	PSL (good)	Positive	+	+	−	Myoclonus	−	+	−	+
12	79, F	AE	TPO, Tg[c]	PSL (good)	Positive	+	−	+	Chorea	−	+	−	−
13	36, F	AE	Tg	mPSL/PSL (good)	Positive	+	+	+	−	−	+	−	−
14	71, F	AE[b]	Tg	mPSL (fair)	Positive	+	+	+	Myoclonus	−	+	WM	*n.d.*
15	56, M	AE	Tg[d]	mPSL/PSL (good)	Negative	+	+	+	−	−	+	−	+
16	74, M	AE	Tg[d]	mPSL/PSL (good)	Negative	+	+	−	−	−	*n.d.*	−	+
17	69, M	AE (LE)	TPO	mPSL/PSL (good)	Negative	+	+	+	Myoclonus	−	+	L	−
18	58, F	AE (LE)	TPO, Tg[d]	mPSL (good)	Negative	+	+	+	Myoclonus	−	+	L	−
19	61, F	AE (LE)	TPO, Tg[d]	mPSL/PSL (fair)	Negative	+	+	+	−	−	−	L	−
20	69, F	SP	TPO[a]	PSL (good)	Positive	−	−	+	−	−	*n.d.*	−	+
21	60, M	SP	TPO, Tg	PSL (fair)	Positive	−	−	+	−	−	+	−	+
22	69, F	SP	TPO	PSL (fair) IVIg (excellent)	Positive	−	−	+	Myoclonus	−	+	−	*n.d.*
23	61, M	SP	TPO, Tg	PSL (excellent)	Negative	−	−	+	−	+	+	−	+
24	63, F	CJD[b]	Tg[a]	mPSL/DX/azathioprine (excellent)	Negative	+	+	+	Myoclonus	+	+ (PSD)	−	+
25	23, F	IVM	TPO, Tg	PSL (excellent)	Negative	−	−	−	Myoclonus	−	−	−	−

AE, acute encephalopathy form; SP, subacute psychiatric form; LE, limbic encephalopathy-like clinical feature; CJD, Creutzfeldt–Jakob disease-like clinical feature; IVM, involuntary movement dominant-form; TPO, anti-thyroid peroxidase antibodies; Tg, anti-thyroglobulin antibodies; mPSL, methylprednisolone; PSL, prednisolone; DX, dexamethasone; IVIg, intravenous administration of immunoglobulin G; C, consciousness disturbance; S, seizures; C/P, cognitive impairment/psychiatric symptoms; I, involuntary movements: A, ataxia; PSD, periodic synchronized discharge-like paroxysmal discharges; L, limbic lesions; WM, white matter lesions. *n.d.* not determined. Patients 1, 3, 8, 14, 20 and 24 were very briefly reported (Fujii et al., 2005).

[a] Known history of Hashimoto's thyroiditis before the onset of encephalopathy.

[b] Positive history of recurrence.

[c] Transient hyperthyroidism (thyrotoxicosis).

[d] Hypothyroidism treated with thyroxin.

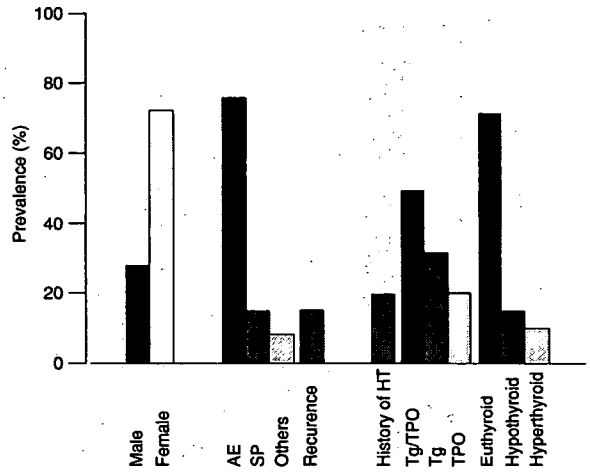

Fig. 1. Clinical profiles of patients. AE, acute encephalopathy form; SP, subacute psychiatric form; HT, Hashimoto's thyroiditis. Tg, anti-thyroglobulin antibodies; TPO, anti-thyroid peroxidase antibodies.

We examined anti-thyroid antibodies (anti-Tg and anti-TPO), steroid-responsiveness, clinical features (consciousness disturbance [C], seizures [S], cognitive impairment/psychiatric symptoms [C/P], involuntary movements [I], ataxia [A]), electroencephalogram [EEG], brain MRI and protein/immunoglobulin G in CSF, and compared these between patients with anti-NAE autoantibodies [referred to as NAE(+)] and without [NAE(−)]. Steroid-responsiveness was evaluated in three degrees: excellent, good, and fair.

2.2. SDS-PAGE and immunoblotting

Anti-NAE autoantibodies were investigated in encephalopathic patients with HT and patients with other disorders including autoimmune conditions or collagen diseases. Immunoblotting analysis of the patient's serum against the NAE expressed in human cultured cells was carried out with 12% sodium lauryl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using a gel electrophoresis system (BE-220, BIO CRAFT, Tokyo, Japan), described previously (Fujii et al., 2005). The proteins on the gel were Western-blotted onto polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham Biosciences, NJ) with a blotting apparatus (KS-8453, Oriental Instrument, Tokyo, Japan) at 0.3 mA/cm^2 for 8 h at 4 °C. For detection of the band specific to NAE, serum was applied to the membrane and incubated in 1% gelatin for 1 h at room temperature, then horseradish peroxidase (HRP)-conjugated anti-human goat IgG Fc (ICN Pharmateuticals, Inc., OH) was applied to the membrane as the secondary antibody, fluoresced, and developed on X-ray films (BioMax, Kodak, NY).

2.3. Statistical analysis

We used χ^2 test to assess the correlation between patients with encephalopathy with HT and anti-NAE antibodies.

3. Results

3.1. Anti-NAE autoantibodies

The clinical profiles of patients and immunological characters were summarized in Table 1 and Fig. 1. Of the 25 patients with encephalopathy with HT examined here, 68% of them were NAE(+) (17 of 25) ($p<0.001$, compared to patients with HT without encephalopathy [10%, 2 of 20], supplemented by additional data from 3 patients of HT without encephalopathy to our previous study) (Fujii et al., 2005). To our knowledge, there

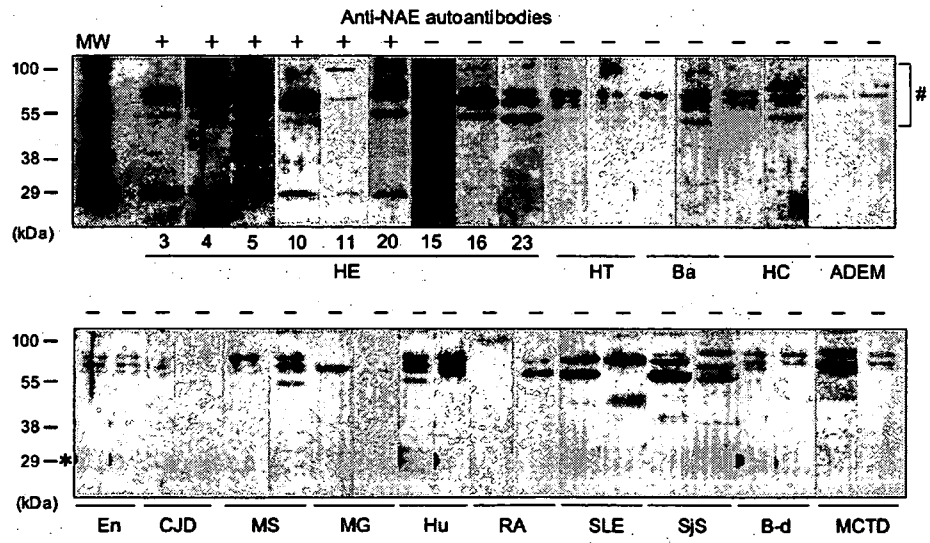

Fig. 2. Immunoblotting of the recombinant NAE with sera from patients with encephalopathy with HT, HT without encephalopathy, Basedow's disease or other neurological disorders including autoimmune conditions, or from controls. HE, Hashimoto's encephalopathy; 3–23, patients 3–23 with HE in Table 1; HT, Hashimoto's thyroiditis without encephalopathy; Ba, Basedow's disease; HC, healthy controls; ADEM, post-infectious acute disseminated encephalomyelitis; En, viral encephalitis; CJD, Creutzfeldt–Jakob disease; MS, multiple sclerosis; MG, myasthenia gravis; Hu, paraneoplastic syndrome associated with Hu-antigen; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SjS, Sjögren syndrome; B-d, Behçet disease; MCTD, mixed connective tissue disease. MW, molecular weight marker. *The position of the recombinant NAE. #Derivatives from human cultured cells for expression, which showed non-specific reactions with sera.