Table 1

	Right globus pallidum		Left globus pallidum	
	8 years	9 years	8 years	9 years
MRI				
UBO in T2-WI	++	+	+	–
MRS				
NAA/Cr (1.80±0.17)	1.34	1.66	1.42	1.85
Cho/Cr (0.89±0.12)	0.60	0.65	0.61	0.83
Lactate/lipid	+	–	–	–

between clinical symptoms in NF1 and UBO has rarely been reported. Our patient showed left hemiconvulsion in parallel with the appearance of UBO in the right globus pallidum. In a previous study, 5–6% of NF1 patients showed seizures including complex partial seizure, but there were no alternations of UBO on MR imaging [7]. Pathologically, UBO may represent increased fluid within the myelin associated with hyperplastic or dysplastic glial proliferation. Therefore, it may indicate cognitive deficits including seizures [4]. In our patient, left hemiconvulsion may be caused by neuronal change in the right globus pallidum. ^{1}H-MR spectroscopy (^{1}H-MRS) has been used to investigate the classification and pathophysiology of various neurological conditions including neoplasms, viral infections, ishemia and some of the dementias. In the previous study, reduction of *N*-acetylaspartate (NAA), and elevation of choline (Cho) were indicated, however there was no lactate/lipid peak demonstrated with ^{1}H-MRS in UBO [8–10]. In our patient, the ratios of NAA/creatine(Cr) and Cho/Cr were slightly decreased in UBO, and those are gradually improved in parallel with the disappearance of UBO. However, the lactate/lipid peak was visible in newly identified UBO, but was not shown in the same lesion one year later, even though T2-prolongation persisted on MR images. Jones et al. described that the ^{1}H-MRS findings with UBO of NF1 patients could be speculated into two groups of lesions. One consisted of lesions with less significant changes relative to normal brain, demonstrating only slight reductions in all metabolite ratios. The other group showed significant increase in Cho and decrease in NAA, similar to findings for astrocytoma [8]. ^{1}H-MRS of UBO in our patient demonstrated the former finding. NAA is located almost exclusively in neurons and axons, while Cho appears to contain contributions from phosphorylcholine and glycerophosphorylcholine, which are precursors of cell membrane and breakdown products of cell membrane, respectively. Therefore, NAA and Cho may be prognostic markers, either UBO are clinically silent, or can act as precursors of neoplasm [8,9]. In addition, ^{1}H-MRS of UBO in our patient showed a lactate/lipid peak during the early stage accompanied by hemiconvulsion, which had not previously been reported. Elevation of the peak has previously been demonstrated in ischemic brain damage and acute multiple sclerosis, and was attributed to an increase in products of myelin break down [11]. UBO are thought to represent an abnormal configuration of myelin [4,8]. Elevation of lactate/lipid peaks in multiple sclerosis is also a transient phenomenon, and further evaluation of developing UBO by ^{1}H-MRS may contribute to elucidating the pathogenesis and relation of these lesions to seizures [11].

In this study, we examined serial MR images and ^{1}H-MRS of UBO with a NF1 patient. UBO has not been considered associated with neurological symptoms, however, hemiconvulsion developed simultaneously with the appearance of new UBO in our patient. The association between the seizures and the findings on neuroimaging remained unclear in our patient, however, multiple chemical metabolites with ^{1}H-MRS might represents paroxysmal neuronal tissue changes of UBO. Serial follow up using MR image and MR spectroscopy is expected to be useful in unraveling the pathophysiology and clinical symptoms of UBO in children with NF1.

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Case report

A case of acute encephalitis with refractory, repetitive partial seizures, presenting autoantibody to glutamate receptor Gluε2

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Abstract

An 11-year-old male was admitted to our hospital because of high-grade fever, repetitive seizures, and prolonged impairment of consciousness (Glasgow coma scale E1, M5, V1). His seizures were repetitive complex partial seizures that expanded from the unilateral face to the corresponding side of the body. He sometimes developed secondary generalized seizures. While most seizures lasted 1 or 2 min, intractable seizures also frequently (about 5 times/h) occurred. We diagnosed him as encephalitis/encephalopathy, and treated him with artificial respiration, thiamylal sodium, mild hypothermia therapy, steroid pulse therapy, massive γ-globulin therapy, etc. Afterwards, he had sequelae, such as post-encephalitic epilepsy (same seizures continued to recur), hyperkinesia, impairment of immediate memory, change in character (he became sunny and obstinate), dysgraphia, and mild atrophy of the hippocampus, amygdala, and cerebrum. However, he could still attend a general junior high school. He was diagnosed as acute encephalitis with refractory, repetitive partial seizures (AERRPS). In this case, he was positive for autoantibody to glutamate receptor Gluε2 IgG or IgM in an examination of blood and spinal fluid, and we presumed that this may have influenced his sequelae. In this case, a combination of mild hypothermia therapy, steroid pulse therapy, and massive γ-globulin therapy was effective.

Keywords: Autoantibody to glutamate receptor Gluε2; Acute encephalitis with refractory, repetitive partial seizures (AERRPS); A peculiar type of post-encephalitic/encephalopathic epilepsy; Mild hypothermia therapy; Massive γ-globulin therapy; Steroid pulse therapy

1. Introduction

A peculiar type of post-encephalitic/encephalopathic epilepsy was first reported by Awaya et al. [1]. It is characterized by epilepsy with the same repetitive intractable partial seizures from the acute phase to the convalescence phase. However, it is not known when epileptogeneity is acquired. Soon thereafter, Shiomi et al. reported a similar case of encephalitis accompanied by frequent seizures in Japan. Sakuma et al. proposed the terminology acute encephalitis with refractory, repetitive partial seizures (AERRPS), which satisfied the following five criteria: (1) a prolonged acute phase of more than 2 weeks, (2) partial seizures with the same symptoms persisting from the acute phase to the convalescence phase, (3) seizures frequently evolving into status convulsivus, especially during the acute phase, (4) marked intractability of seizures, and (5) exclusion of related disorders such as known viral encephalitis or metabolic disorders [2], based on these two previous reports. On the other hand, it was reported that autoantibody to glutamate receptor Gluε2 was often positive in Rasmussen's encephalitis [3] and in acute encephalitis/encephalopathy. It is possible that autoantibody to glutamate receptor Gluε2 may cause persistent excitation of glutamate receptor Gluε2 and may be associated with seizures and impairment of the central nervous system. We report here a case of AERRPS, presenting autoantibody to glutamate receptor Gluε2. To the best of our knowledge,

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this is the first report of AERRPS presenting autoantibody to glutamate receptor Gluε2. It is possible that autoantibody to glutamate receptor Gluε2 may be associated with the pathophysiology of AERRPS.

2. Case report

An 11-year-old male was admitted to our hospital because of high-grade fever, repetitive seizures, and prolonged impairment of consciousness (Glasgow coma scale E1, M5, V1). His seizures were repetitive complex partial seizures that expanded from the unilateral face to the corresponding side of the body. He sometimes developed secondary generalized seizures. While most seizures lasted 1 or 2 min, intractable seizures also frequently (about 5 times/h) occurred. The family history and past history were not marked. On admission, he showed no abnormal neurological findings except for impairment of consciousness and intractable seizures. On blood examination, he showed no abnormality except for FDP and ALT (16 μg/ml (1–12 μg/ml), 53 U/l (5–40 U/l), respectively). On spinal fluid examination, leukocyte count was 25 mm^3. Brain computed tomography (CT) and magnetic resonance imaging (MRI) with T2-weighted imaging (T2-WI) showed no abnormality (Fig. 1(A)). There was no significant increase in any virus antibody titer. His clinical course after admission is described in Fig. 2. On day 1 of admission, he was administered glycerol (5 ml/kg × 4 times/day), acyclovir (5 mg/kg × 3 times/day), γ-globulin (250 mg/kg/day for 3 days), steroid pulse therapy (methylprednisolone 25 mg/kg/day for 3 days), and midazolam (0.1 mg/kg/h) for his encephalitis and seizures. On day 2, since he had repetitive seizures, the dose of midazolam was increased and he was administered lidocaine hydrochloride. On day 3, artificial respiration was begun along with thiamylal sodium at 3 mg/kg/h because of intractable seizures. Afterwards, we treated him with thiamylal sodium at 8 mg/kg/h because of intractable seizures and mild hypothermia therapy. He was given an intravenous injection of phenytoin (5 mg/kg × 2 times/day) during treatment with thiamylal sodium, but this was not effective. Interictal electroencephalogram (EEG) on day 8 showed slow spike and wave predominantly in the frontal and central region (Fig. 3(A)). Ictal EEG on day 8 showed rhythmic spikes in the left frontal–central–temporal region with antecedent spikes (Fig. 3(B)). At this time, he was treated with thiamylal sodium at 6 mg/kg/h because of repetitive seizures. Interictal EEG on day 10 showed a burst-suppression pattern and spikes were present during the burst phase (Fig. 3(C)). He was then treated with mild hypothermia therapy and thiamylal sodium at 8 mg/kg/h, and the seizures stopped. On day 10, the leukocyte count was 3 mm^3 and IgG was 10.2 mg/dl (reference value, 0.2–0.6 mg/dl) on spinal fluid examination. Since we thought that a mechanism of abnormal immunity may be involved in his encephalitis because of the increase in IgG on spinal fluid examination, massive γ-globulin therapy (400 mg/kg/day over 5 days) was performed again on day 12. On day 12, we discontinued treatment with thiamylal sodium and began treatment with massive phenobarbital suppository therapy (20 mg/kg/day) because of an impairment of liver function on blood examination. In association with a decrease in thiamylal sodium, his EEG findings worsened. However, EEG spikes almost disappeared following treatment with

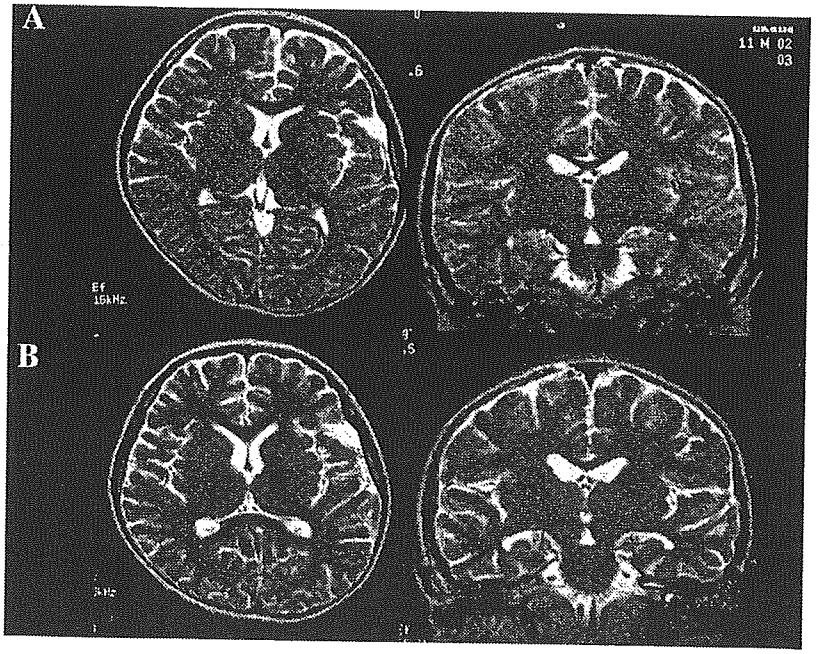

Fig. 1. (A) Brain MRI T2-WI on admission showed no abnormality. (B) Brain MRI on day 36 showed mild atrophy of the hippocampus, amygdala, and cerebrum.

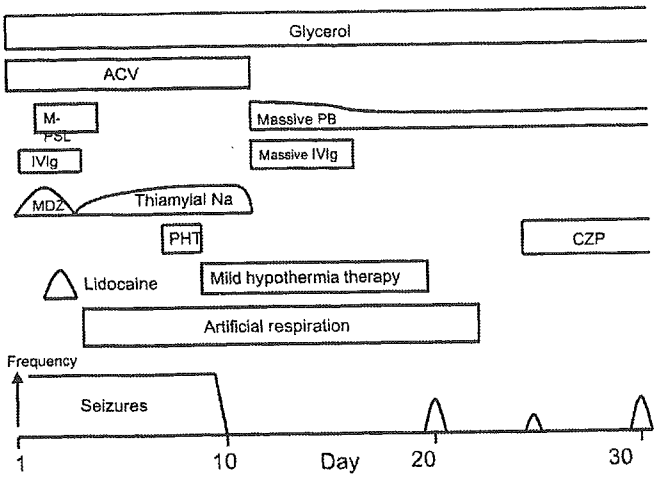

Fig. 2. Clinical course after admission. Abbreviations: ACV, acyclovir; M-PSL, methylprednisolone (steroid pulse therapy); IVIg, intravenous γ-globulin; PB, phenobarbital; MDZ, midazolam; PHT, phenytoin; CZP, clonazepam.

massive γ-globulin therapy and massive phenobarbital suppository therapy. On day 12, acyclovir was also stopped because polymerase chain reaction of herpes simplex virus DNA was negative on spinal fluid examination. On day 20, we stopped mild hypothermia therapy and on day 22 he was extubated. After extubation, his level of consciousness gradually improved. However, since the same seizures appeared several times per week, we started clonazepam (0.075 mg/kg/day) on day 24. The frequency of seizures then gradually decreased. Brain MRI on day 36 showed mild atrophy of the hippocampus, amygdala, and cerebrum (Fig. 1(B)). EEG on day 39 showed a disappearance of spikes. However, the same seizures continued to recur at about once per month under the oral administration of phenobarbital and clonazepam. Afterwards, he had sequelae, such as post-encephalitic epilepsy (same seizures continued to recur), hyperkinesia, impairment of immediate memory, change of character (he became sunny and obstinate), and dysgraphia. However, he could still attend a general junior high school. He was positive for autoantibody to glutamate receptor Glue2 IgG or IgM in an examination of blood and spinal fluid on day 10, but negative on day 80.

3. Discussion

For the treatment of seizures in AERRPS, barbiturate and benzodiazepine are often effective in the acute phase, and phenytoin, zonisamide, and potassium bromide in addition to barbiturate and benzodiazepine are often effective in the convalescence phase [2]. In this case, midazolam, lidocaine hydrochloride, and phenytoin were not effective, and a complete suppression~burst suppression pattern on EEG and thiamylal sodium at 8 mg/kg/h were necessary to stop his seizures; thus, he showed inveterate epileptogeneity. Massive phenobarbital suppository therapy (20 mg/kg/day)

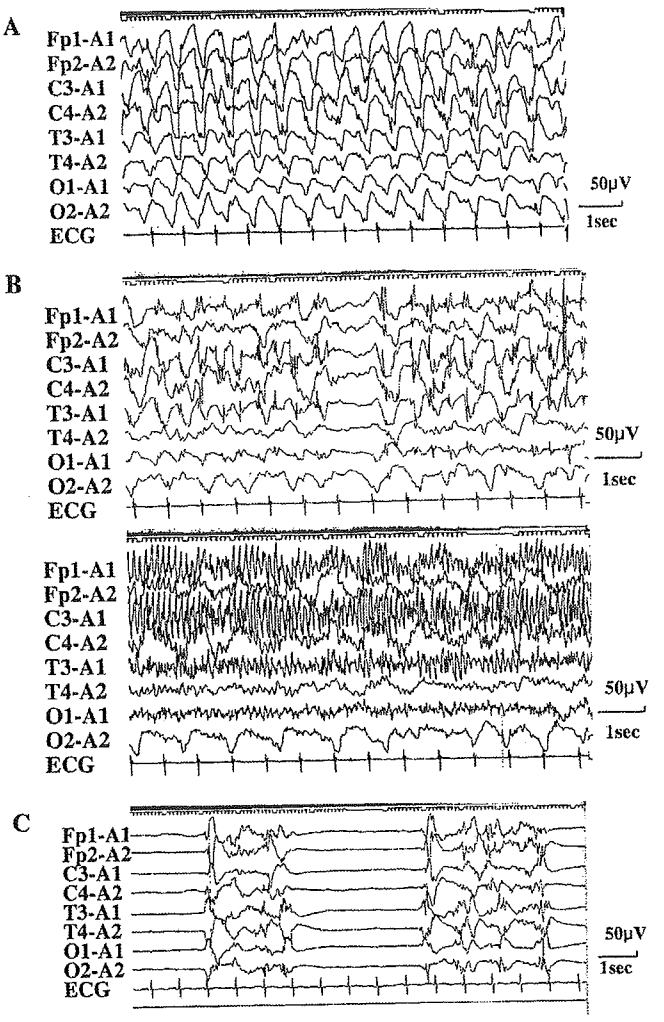

Fig. 3. (A) Interictal EEG on day 8 showed slow spike and wave predominantly in the frontal and central lobe. He was treated with thiamylal sodium at 6 mg/kg/h because of repetitive seizures. (B) Ictal EEG on day 8 showed rhythmic spikes in the left frontal–central–temporal region with antecedent spikes in the same region. (C) Interictal EEG on day 10 showed a burst-suppression pattern and spikes were present during the burst phase. He was treated with mild hypothermia therapy and thiamylal sodium at 8 mg/kg/h, and the seizures stopped.

was partly effective and made it possible to break away from thiamylal sodium. Sakuma et al. reported that massive phenobarbital therapy or phenytoin was useful for the discontinuation of barbiturate [2]. In our case, phenobarbital was most effective at stopping seizures after the early phase of treatment. Sakuma et al. reported that phenobarbital was effective in 2/15 cases in the acute phase, and in 4/12 cases in the convalescence phase [2]. Hamano et al. reported a case of AERRPS that showed the transient disappearance of seizures with the occurrence of choreo-ballistic involuntary movements [4]. That case showed secondary generalized seizures that originated in the face. It was thought that epileptic discharge from the lateral motor cortex was transmitted to basal ganglia or the brain stem, and resulted in secondary generalized seizures. Thus, it is possible that

impairment of the basal ganglia associated with involuntary movement may have blocked epileptic discharge from the motor cortex, and convulsions decreased accompanied by a worsening of involuntary movement [5]. It is possible that hyperexcitability in the subcortex may have blocked epileptic discharge from the cortex [4]. These results may be useful for the treatment of AERRPS.

For the treatment of encephalitis/encephalopathy in AERRPS, we used steroid pulse therapy, massive γ-globulin therapy, and mild hypothermia therapy. A combination of mild hypothermia therapy with steroid pulse therapy is recommended for encephalopathy [6,7]. In encephalitis, since cytokine [8] and neopterin [9] are both increased on spinal fluid examination, it is thought that inflammation and immunoreaction are present in the central nervous system. The aims of hypothermia therapy and steroid pulse therapy are (1) to suppress brain edema, (2) to suppress secondary impairment of nerve cells due to the transmission of excitatory amino acids and neurotoxic materials, and (3) to suppress an abnormal increase in cytokine [6]. In this case, the aims of massive γ-globulin therapy were (1) to immunize against contagions, (2) to suppress an abnormal increase in cytokine, and (3) to suppress abnormal immunity and the generation of antibody (autoantibody to glutamate receptor Gluε2, etc.) because of an increase in IgG on spinal fluid examination. Sandstedt et al. reported that γ-globulin therapy was more effective in cases with a high level of IgG on spinal fluid examination in intractable post-encephalitic epilepsy [10].

In this case, autoantibody to glutamate receptor Gluε2 was positive on blood and spinal fluid examination. It has been reported that autoantibody to glutamate receptor is often positive in Rasmussen's encephalitis [3] and acute encephalitis/encephalopathy [11]. On the other hand, patients with West syndrome or Lennox–Gastaut syndrome, or control subjects are negative for autoantibody to glutamate receptor [11]. In acute encephalitis/encephalopathy, it has been speculated that severe cases tend to generate autoantibody to glutamate receptor Gluε2 [11]. Patients with status convulsivus in the acute phase generated autoantibody to glutamate receptor Gluε2 significantly more often than those without status convulsivus [11]. Furthermore, patients who were positive for autoantibody to glutamate receptor Gluε2 had sequelae such as developmental delay, motor paralysis, or epilepsy significantly more often than those who were negative for autoantibody to glutamate receptor Gluε2 [11]. It is possible that autoantibody to glutamate receptor may persistently adrenalize glutamate receptor, and this may be associated with the generation of seizures and impairment of the central nervous system. Also, since the glutamate receptor plays an important role in the genesis of memory and learning in the hippocampus, it has been speculated that autoantibody to glutamate receptor may be associated with atrophy of the hippocampus and impairment of memory. To the best of our knowledge, there has been no previous report in which autoantibody to glutamate receptor Gluε2 was positive in AERRPS. In general, AERRPS shows a neurologically poor prognosis, and the appearance of autoantibody to glutamate receptor also reflects a neurologically poor prognosis. If it can be demonstrated that an abnormal immune state is involved in AERRPS based on an examination of autoantibody to glutamate receptor Gluε2, it may be possible to reduce the incidence of sequelae through the use of immunotherapy. In this case, the patient could attend a general junior high school. These results suggest that active therapy such as with a combination of mild hypothermia therapy, steroid pulse therapy, and γ-globulin therapy may be effective in AERRPS.

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Puffer Fish Poisoning, Guillain–Barré Syndrome and Persistent Sodium Channels

Puffer fish poisoning has been a unique complication of the oriental cuisine for centuries. It is intoxication caused by tetrodotoxin, a sodium channel blocker, and is a medical emergency presenting with acute development of perioral paresthesia and general weakness that frequently involves respiratory muscles. Similarly, Guillain–Barré syndrome is a condition that needs prompt recognition and treatment because of acutely developing weakness. It is classified into the demyelinative form (acute inflammatory demyelinating polyneuropathy or AIDP) and the axonal form (acute motor axonal neuropathy or AMAN), and the latter is often associated with elevated titers of anti-GM1 antibodies. The mechanism of acute weakness has been ascribed to demyelinative conduction block in cases of AIDP, whereas that for AMAN is less clear. The pathological findings in AMAN are mostly axonal degeneration,[1] but the response to treatment can be rapid, and complete recovery is possible if appropriate therapy is instituted early. Electrophysiological findings in AMAN include greatly increased threshold for excitation and normal conduction velocities in motor nerves, whereas sensory conduction remains intact.[2] The selective motor nerve involvement and electrical inexcitability led to the hypothesis that anti-GM1 antibodies may bind specifically to motor nerves and interfere with axonal sodium channels.[3]

In this issue, Kiernan and colleagues[4] presented evidence that nerve excitability changes in puffer fish poisoning are distinct from those in AMAN.[2] Conventional nerve conduction studies in the former demonstrate marked conduction slowing, consistent with reduced sodium current which prolongs internodal conduction time, and concurrent involvement of sensory nerves, whereas AMAN is associated with normal velocities and sparing of sensory fibers. Their study clearly indicates that conduction failure in AMAN cannot be explained by external blockage of the channels by a tetrodotoxin-like substance.

What causes the steeply increased threshold for excitation in AMAN? The threshold for excitation of a nerve is determined by various factors, including membrane potential, input impedance, and sodium channels. For instance, membrane hyperpolarization could increase the threshold. Membrane potentials however turned out to be normal in AMAN.[2] The input impedance may affect the threshold, as has proved to be the case in hereditary demyelinating neuropathy.[5] The lack of pathological and physiological findings for primary demyelination makes this unlikely in AMAN.

Sodium channels are the final common path to the firing of a nerve. It has been increasingly recognized that the sodium channels are diverse on both molecular and physiological bases. They open upon membrane depolarization, and the increased number of open channels further facilitates depolarization by inward flow of sodium ions. This chain reaction is ignited by threshold or persistent sodium channels, and terminated by self inactivation of the sodium channels. These persistent channels show slow inactivation and affect refractoriness and threshold.[6]

Recent studies in neurons revealed that persistent sodium channels are in fact an altered state of voltage-gated or transient sodium channels, mediated through phosphorylation of an amino acid residue of their inactivation gate by protein kinase C (PKC).[7] It is yet to be confirmed that persistent channels in the peripheral nerve are similarly modulated by phosphorylation, but this novel finding could provide a clue to the understanding of conduction failure in AMAN. Interestingly, sensory fibers have more persistent sodium channels than motor fibers.[6] If a disease affects both fibers evenly, motor fibers could be more vulnerable than sensory fibers because of their limited functional reserve of the persistent channels. Although anti-GM1 antibodies may rarely block conduction through demyelination,[8] local mediators such as tumor necrosis factor or nitric oxide cause conduction block in a large proportion of nerves.[9] These are released from phagocytes or lymphocytes, which are found in pathological specimens of AMAN.[1] Inflammatory mediators could affect energy-dependent PKC and other metabolic pathways in axons, and thereby reduce the excitability through sodium channel modulation. If these molecular and physiological views converge, we may be able to gain deeper insights into the functional consequence of not only Guillain–Barré syndrome but also the inflammation in the nervous system in general.

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N-METHYL-D-ASPARTATE RECEPTORS PLAY IMPORTANT ROLES IN ACQUISITION AND EXPRESSION OF THE EYEBLINK CONDITIONED RESPONSE IN GLUTAMATE RECEPTOR SUBUNIT δ2 MUTANT MICE

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Abstract—Classical eyeblink conditioning has been known to depend critically on the cerebellum. Apparently consistent with this, glutamate receptor subunit δ2 null mutant mice, which have serious morphological and functional deficiencies in the cerebellar cortex, are severely impaired in delay paradigm. However, these mutant mice successfully learn in trace paradigm, even in '0-trace paradigm,' in which the unconditioned stimulus starts just after the conditioned stimulus terminates. Our previous studies revealed that the hippocampus and the muscarinic acetylcholine receptors play crucial roles in 0-trace paradigm in glutamate receptor subunit δ2 null mutant mice unlike in wild-type mice, suggesting a large contribution of the forebrain to 0-trace conditioning in this type of mutant mice. In the present study, we investigated the role of *N*-methyl-D-aspartate receptors in 0-trace eyeblink conditioning in glutamate receptor subunit δ2 null mutant mice. Mice were injected intraperitoneally with the noncompetitive *N*-methyl-D-aspartate receptor antagonist (+)MK-801 (0.1 mg/kg) or saline, and conditioned with 350-ms tone conditioned stimulus followed by 100-ms periorbital shock unconditioned stimulus. Glutamate receptor subunit δ2 null mutant mice that received (+)MK-801 injection exhibited a severe impairment in acquisition of the conditioned response, compared with the saline-injected glutamate receptor subunit δ2 null mutant mice. In contrast, wild-type mice were not impaired in acquisition of 0-trace conditioned response by (+)MK-801 injection. After the injection solution was changed from (+)MK-801 to saline, glutamate receptor subunit δ2 null mutant mice showed a rapid and partial recovery of performance of the conditioned response. On the other hand, when the injection solution was changed from saline to (+)MK-801, glutamate receptor subunit δ2 null mutant mice showed a marked impairment in expression of the pre-acquired conditioned response, whereas impairment of the expression was small in wild-type mice. Injection of (+)MK-801 had no significant effects on spontaneous eyeblink frequency or startle eyeblink frequency to the tone conditioned stimulus in either glutamate receptor subunit δ2 null mutant mice or wild-type mice. These results suggest that *N*-methyl-D-aspartate receptors play critical roles both in acquisition and expression of the conditioned response in 0-trace eyeblink conditioning in glutamate receptor subunit δ2 null mutant mice. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: classical conditioning, learning, hippocampus, cerebellum, MK-801.

Classical conditioning of the eyeblink response is a type of motor learning that depends critically on the cerebellum in rabbits (McCormick et al., 1982) and mice (Chen et al., 1996). Using a standard delay paradigm, in which the unconditioned stimulus (US) is delayed, overlaps and coterminates with the conditioned stimulus (CS), the essential neural circuit that resides in the cerebellum and brainstem has been extensively studied in rabbits. Thus this learning task has become a model system that is suitable for analysis of the neural substrates of learning and memory (Thompson et al., 1997). In addition to these essential brain regions, the forebrain including the hippocampus and the medial prefrontal cortex plays an important role in trace paradigm, in which a long trace-interval (a stimulus-free period of time) intervenes between the CS and US (Solomon et al., 1986; Kronforst-Collins and Disterhoft, 1998; McLaughlin et al., 2002; Takehara et al., 2002, 2003). Although this important basic framework for learning mechanism in classical eyeblink conditioning has been established in rabbit, mouse is becoming another important model because of its current progress in gene-manipulating techniques.

We have studied the eyeblink conditioning of mutant mice that lack glutamate receptor subunit δ2 (GluRδ2) (Kishimoto et al., 2001b,d; Takatsuki et al., 2002, 2003). GluRδ2 mRNA is expressed predominantly in Purkinje cells of the cerebellum whereas its expression in the forebrain is hardly detectable (Araki et al., 1993; Lomeli et al., 1993). Within Purkinje cells, GluRδ2 proteins are localized exclusively at parallel fiber-Purkinje cell synapses (Takayama et al., 1996; Landsend et al., 1997). GluRδ2 null mutant mice (GluRδ2$^{-/-}$ mice) have several morphological and functional impairments in the cerebellar cortex such as a deficit in long-term depression (LTD) at parallel fiber–Purkinje cell synapses, a 50% reduction in the number of these synapses, persistent multiple innervation of Purkinje cells by climbing fibers, and abnormal oscillating Purkinje cell activity (Kashiwabuchi et al., 1995; Kurihara

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Abbreviations: APV, 2-amino-5-phosphonovaleric acid; CR, conditioned response; CS, conditioned stimulus; EMG, electromyogram; GluRδ2, glutamate receptor subunit δ2; LTD, long-term depression; NMDA, *N*-methyl-D-aspartate; US, unconditioned stimulus.

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et al., 1997; Yoshida et al., 2004). Impairments in motor coordination (Kashiwabuchi et al., 1995) and vestibular compensation (Funabiki et al., 1995) were also found in GluRδ2$^{-/-}$ mice.

Apparently consistent with the hypothesis that the cerebellum and synaptic plasticity in the cerebellar cortex play a crucial role in classical eyeblink conditioning (Ito, 1989; Thompson and Krupa, 1994), GluRδ2$^{-/-}$ mice exhibited a severe impairment in a delay paradigm with a 252-ms interstimulus interval between the 352-ms CS and 100-ms US (Kishimoto et al., 2001d), as did other cerebellar LTD-deficient mice (Aiba et al., 1994; Shibuki et al., 1996; Miyata et al., 2001; Kishimoto et al., 2001a; Koekkoek et al., 2003). However, in trace paradigm with a 500-ms trace interval between the CS end and US onset, GluRδ2$^{-/-}$ mice successfully acquired the eyeblink conditioned response (CR) (Kishimoto et al., 2001d). Further analysis using various temporal intervals between the CS and US revealed that GluRδ2$^{-/-}$ mice learned as successfully as wild-type mice in trace paradigms with a 50-, 100-, and 250-ms trace interval, and even in the '0-trace paradigm' in which the US starts just after termination of the CS (Kishimoto et al., 2001b). However, in a delay paradigm with a 352-ms interstimulus interval that is equal to that of the 0-trace paradigm with a 352-ms CS, GluRδ2$^{-/-}$ mice exhibited a severe learning impairment. These results suggest that another learning mechanism, one that does not require the cerebellar LTD, underlies the ability of GluRδ2$^{-/-}$ mice to learn in eyeblink conditioning paradigms in which the US does not overlap with the CS. Eyeblink conditioning in another cerebellar LTD-deficient mouse lacking the phospholipase Cβ4 subunit also showed similar results (Kishimoto et al., 2001a).

In the course of discovering this cerebellar LTD-independent learning mechanism, we found that ablation of the hippocampus (Takatsuki et al., 2003) or blockade of muscarinic acetylcholine receptors by systemic administration of scopolamine (Takatsuki et al., 2002) severely impaired acquisition of the CR during 0-trace conditioning in GluRδ2$^{-/-}$ mice. These results suggest that the forebrain including the hippocampus contributes to the successful learning of GluRδ2$^{-/-}$ mice in 0-trace conditioning. In wild-type animals, the hippocampus has an ability to modulate acquisition during a standard delay conditioning (Salafia et al., 1979; Prokasy et al., 1983; Solomon et al., 1983) and is required in more difficult paradigms such as long-trace paradigm (Solomon et al., 1986; Moyer et al., 1990; Weiss et al., 1999; Tseng et al., 2004) and discrimination reversal paradigm (Berger and Orr, 1983). The medial prefrontal cortex also plays an important role in acquisition (Kronforst-Collins and Disterhoft, 1998; Weible et al., 2000; McLaughlin et al., 2002) and retention (Takehara et al., 2003) in long-trace conditioning paradigm. Therefore, contribution of the forebrain seems to increase when the task demand on the animal is raised. Based on this consideration, we have hypothesized that contribution of the forebrain might also increase in GluRδ2$^{-/-}$ mice because the several deficiencies in the cerebellar cortex will make it difficult for the mutant mice to learn the short-trace eyeblink conditioning.

N-methyl-D-aspartate (NMDA) receptors are abundantly expressed in the forebrain (Watanabe et al., 1993) and many studies using NMDA receptor antagonists or gene-targeted mutant mice have revealed that NMDA receptors play important roles in several kinds of learning that depend on the forebrain. In eyeblink conditioning, Thompson and Disterhoft (1997) have demonstrated that the noncompetitive NMDA receptor antagonist (+)MK-801 slows the rate of acquisition in delay paradigm and completely blocks acquisition in 500-ms trace paradigm in rabbits. This drug is also effective in mice, in which the impairment with (+)MK-801 administration is greater in trace paradigm with a longer trace interval (Takatsuki et al., 2001). In addition, acute injections of the competitive NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (APV) into the dorsal hippocampus severely impaired acquisition during 500-ms trace conditioning in mice (Sakamoto et al., 2005). Consistent with the effects of pharmacological blockade, those mutant mice that lack the NMDA receptor subunit ε1 (NR2A) exhibit a mild impairment in delay paradigm and a severe impairment in long-trace paradigm (Kishimoto et al., 1997, 2001c). These results suggest that blockade of NMDA receptors greatly affects the role of the forebrain in eyeblink conditioning in mice.

In the present study, we investigated the effect of (+)MK-801 on 0-trace eyeblink conditioning in GluRδ2$^{-/-}$ mice. As described above, these mutant mice critically depend on the hippocampus to learn the 0-trace eyeblink conditioning. Therefore, blockade of NMDA receptors would result in impairment of learning as reported in long-trace eyeblink conditioning in wild-type animals.

EXPERIMENTAL PROCEDURES

Animals

Previously-developed GluRδ2-mutant mice were repeatedly backcrossed with C57BL/6 mice to yield heterozygous GluRδ2$^{+/-}$ mice with a more than 99.99% C57BL/6 genetic background (Kashiwabuchi et al., 1995; Kishimoto et al., 2001d). These heterozygous mice with highly homogeneous genetic background were crossed with each other to obtain homozygous GluRδ2$^{-/-}$ mice. Both male and female mice were used and randomly assigned to saline-injected control group and (+)MK-801-injected group. No differences were detected between the results obtained from male and female mutant mice. We also investigated the effect of (+)MK-801 in wild-type C57BL/6 male mice obtained from a breeder (Japan SLC, Hamamatsu, Shizuoka, Japan). These animals were kept on a 12-h light/dark cycle with *ad libitum* access to food and water and weighed 18–27 g at the time of surgery. All experiments were performed in accordance with the guidelines established by the Institutional Animal Investigation Committee at the University of Tokyo and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the use of animals and to optimize their comfort.

Surgery

Surgical procedures were the same as those described previously (Takatsuki et al., 2001). Under anesthesia with ketamine (80 mg/kg, i.p.; Sankyo, Tokyo, Japan) and xylazine (20 mg/kg, i.p.; Bayer, Tokyo, Japan), four Teflon-coated stainless steel wires

(No. 7910, A-M Systems, Carlsborg, WA, USA) were implanted under the left eyelid. Two of these wires were used to record electromyograms (EMG) and the remaining two to deliver the US. These wires were soldered to connector pins, which were secured to the skull with dental acrylic resin and stainless steel screws. After surgery, the animals were returned to their home cages and their recovery was monitored.

Drug treatments

(+)MK-801 (0.1 mg/kg, Tocris Cookson, St. Louis, MO, USA) or saline was administered by i.p. injection 10 min before the second adaptation session to the experimental apparatus (spontaneous recording day 2) and the subsequent daily conditioning sessions. (+)MK-801 was diluted in saline and injected in a volume of 5 ml/kg body weight. Control mice were injected with saline (5 ml/kg). The dose and the timing of injection used in the present study is sufficient to cause a severe impairment in a hippocampus-dependent long-trace eyeblink conditioning in wild-type mice as revealed in our previous study (Takatsuki et al., 2001), in which we also found higher dose (0.2 mg/kg, i.p.) reduced the startle eyeblink responses to the tone CS.

Conditioning procedure

On a day (spontaneous recording day 1) three to five days after the surgery, spontaneous eyeblink frequency was recorded for approximately 50 min without the US, CS, or drug injection. On the next day (spontaneous recording day 2), spontaneous eyeblink frequency was recorded in the same way as the day before except that (+)MK-801 or saline was injected 10 min before the start of spontaneous eyeblink recording. The eyeblink conditioning began the next day. A daily conditioning session consisted of 100 trials divided into 10 blocks, which had nine CS-US paired trials followed by a CS-alone trial. Trials were separated by a variable intertrial interval, randomized between 20 and 40 s. The CS was a 350-ms tone (1 kHz, 85 dB) with a 5-ms rise and a 5-ms fall time. The US was a 100-ms periorbital shock (100 Hz square pulses) and was adjusted daily to elicit an eyeblink/head-turn response in each animal. We used '0-trace paradigm,' in which the US starts just after the CS terminates. (+)MK-801 or saline was injected 10 min before the daily conditioning. All experiments were carried out during the light phase of the light/dark cycle.

Data analysis

EMG activity was band-pass filtered between 0.15 and 1.0 kHz and fed into a computer with a sampling rate of 10 kHz. These data were analyzed off-line. The maximum amplitude of the EMG signals during the time period of $t\pm1$ ms was calculated and designated the "EMG amplitude" at t. Then, the mean+S.D. of the EMG amplitude data for 300 ms before the CS onset in 100 trials was defined as the threshold, which was then used in the analysis below. In each trial, the average values for the EMG amplitude above threshold were calculated for a period of 300 ms before CS onset (pre-value), 30 ms after CS onset (startle-value), and 200 ms before the US onset (CR-value). When both the pre-value and startle-value were less than 10% of threshold, the trial was considered a valid trial. Among the valid trials, a trial was assumed to contain the CR if the CR value was larger than 1% of threshold and exceeded two times the pre-value. For the CS-alone trials, the period for CR-value calculation was extended to the presumptive US termination. The frequency of CRs in the valid trials (CR%) was expressed as mean±S.E.M. To evaluate the effects on the startle eyeblink response to the tone CS, the number of trials whose startle value exceeded 10% of threshold during the acquisition session was computed and its ratio to the number of trials was calculated (after removal of those trials whose pre-value exceeded 10% of threshold). Statistical significance was determined by an ANOVA or t-test. $P<0.05$ was considered significant.

RESULTS

Effect of (+)MK-801 on acquisition of the CR

Using the noncompetitive NMDA receptor antagonist (+)MK-801, we investigated the role of NMDA receptors in 0-trace conditioning paradigm in GluRδ2$^{-/-}$ mice. Our previous study revealed that these mutant mice require an intact hippocampus to acquire the eyeblink CR during 0-trace conditioning, while wild-type mice do not (Takatsuki et al., 2003). GluRδ2$^{-/-}$ mice and wild-type mice received an i.p. injection of (+)MK-801 (0.1 mg/kg) or saline 10 min before daily conditioning for 7 days. Fig. 1A shows the CR% for the saline-injected control group ($n=8$) and the (+)MK-801-injected group ($n=8$) of wild-type mice. Both of them readily acquired the CR. A two-way repeated-measures ANOVA revealed that the drug had no significant effect ($F_{1, 14}=2.35$, $P>0.1$). In GluRδ2$^{-/-}$ mice, the CR% of the saline-injected control group ($n=12$) gradually increased to around 80% by the 7th day of conditioning (Fig. 1B). In contrast, the (+)MK-801-treated GluRδ2$^{-/-}$ mice ($n=12$) exhibited a severe impairment in their acquisition of the CR. Their CR% reached only 30% after 7 days of conditioning. A two-way repeated-measures ANOVA confirmed a significant interaction between sessions and the drug ($F_{6, 132}=10.54$, $P<0.001$). These results indicate that (+)MK-801 at this dose greatly impairs learning during 0-trace eyeblink conditioning in GluRδ2$^{-/-}$ mice, but not in wild-type mice.

Effect of (+)MK-801 on spontaneous eyeblink frequency and startle eyeblink frequency

To confirm that the severe impairment of learning in GluRδ2$^{-/-}$ mice was not due to deficits in basic sensory or motor performance, the effects on spontaneous eyeblink frequency measured during adaptation sessions and on startle eyeblink frequency to the tone CS were assessed (Fig. 2). Statistical analysis using a two-way repeated-measures ANOVA indicated no significant differences in spontaneous eyeblink frequency between the saline-injected control group and the (+)MK-801-injected group in wild-type mice ($F_{1, 14}=4.12$, $P>0.05$) and GluRδ2$^{-/-}$ mice ($F_{1, 22}=2.12$, $P>0.1$). The frequency of startle eyeblink to the tone CS during conditioning also did not differ significantly between control group and MK-801 group in wild-type mice (t-test, $P>0.1$) and GluRδ2$^{-/-}$ mice (t-test, $P>0.1$).

Effect of (+)MK-801 on expression of the pre-acquired CR

Because the impairment in the mutant mice was so severe during the acquisition sessions, we also checked the possibility that expression of the CR might be being suppressed by the (+)MK-801 treatment during acquisition. Among the mice that received a saline injection during the 7 days of acquisition sessions with 0-trace conditioning (Fig. 1), we selected those mice whose average CR% over the last three days of conditioning exceeded 60%. All the wild-type mice ($n=8$) and nine of 12 GluRδ2$^{-/-}$ mice met

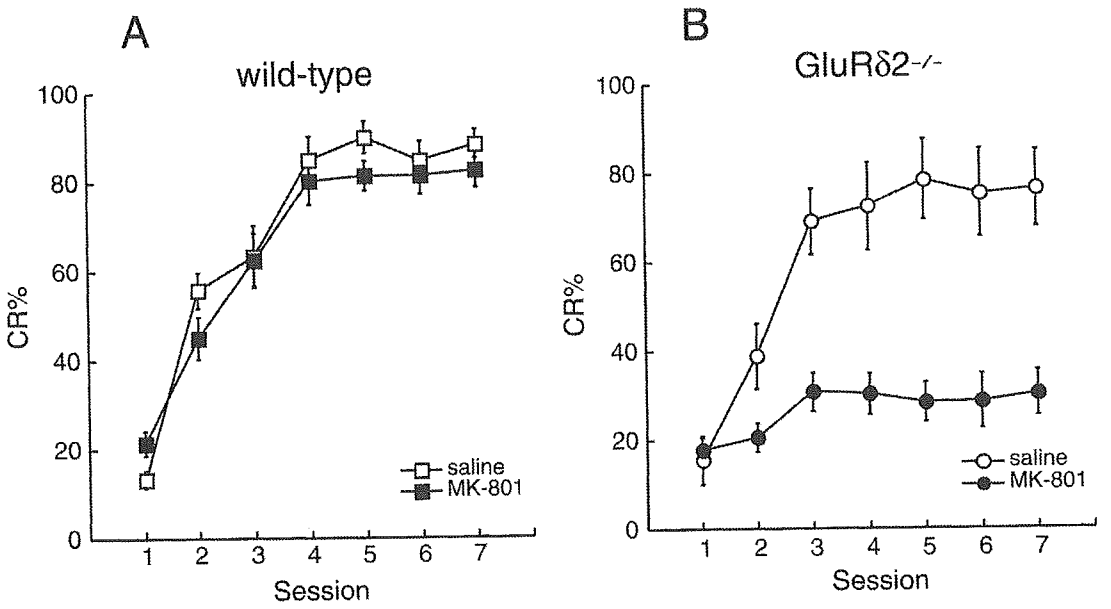

Fig. 1. Effects of (+)MK-801 on acquisition of the CR during 7 days of 0-trace conditioning in wild-type mice (A) and GluRδ2$^{-/-}$ mice (B). Saline or (+)MK-801 (0.1 mg/kg) was intraperitoneally injected 10 min before daily conditioning in wild-type mice (n=8 for saline and MK-801 group each) and GluRδ2$^{-/-}$ mice (n=12 for each group). The error bar indicates the standard error of the mean.

this criterion, and they received three additional days of conditioning (the expression sessions) with (+)MK-801 injections (0.1 mg/kg, i.p.). When the injection solution was switched from saline to (+)MK-801, the CR% of the GluRδ2$^{-/-}$ mice decreased markedly, while that of the wild-type mice decreased only marginally (Fig. 3A). A paired t-test revealed a significant difference between the average CR% over the last three days with saline injections and the CR% on the first day with (+)MK-801 injection both in GluRδ2$^{-/-}$ mice (P<0.001) and wild-type mice (P<0.01).

To further confirm the inhibition of CR expression by (+)MK-801, we looked at the recovery in CR expression after changing the injection solution from (+)MK-801 to saline. The mice that received injections of (+)MK-801 during the 7 days of acquisition sessions (eight wild-type

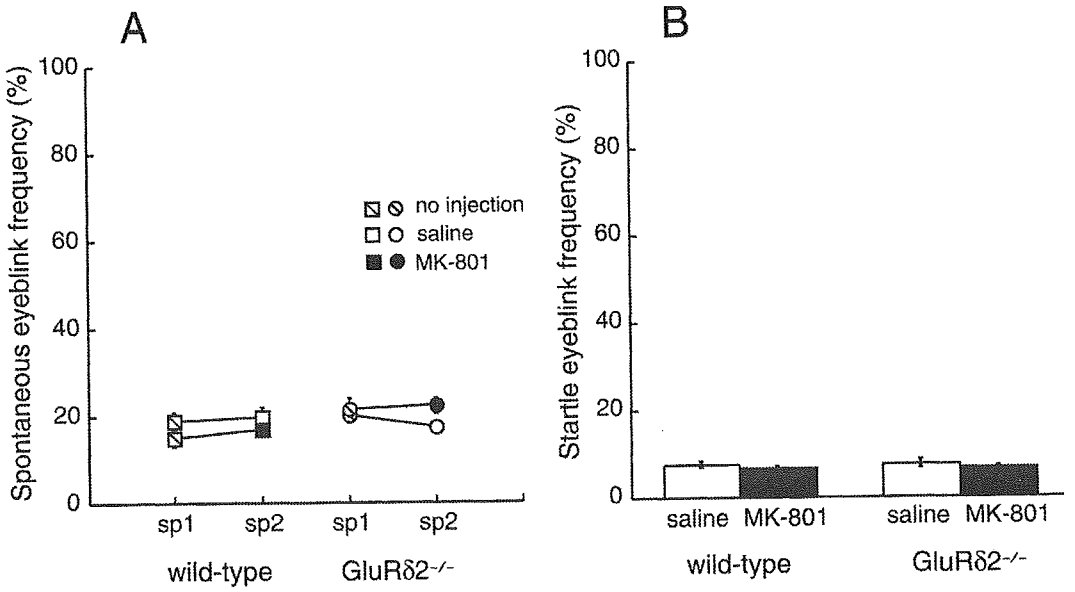

Fig. 2. Effects of (+)MK-801 on the spontaneous eyeblink and the startle eyeblink to the tone CS. (A) Effect on spontaneous eyeblink frequency during adaptation sessions, which were performed before the 7-day acquisition sessions. Eyeblink frequency was recorded using the same protocol as that used for the acquisition sessions except that no stimuli were presented. On the first day (sp1), spontaneous eyeblink frequency was recorded without drug injection. On the second day (sp2), saline or (+)MK-801 (0.1 mg/kg) was intraperitoneally injected 10 min before the adaptation session. (B) Effect on startle eyeblink frequency to the tone CS during conditioning. The data were collected from 7 days of conditioning. The error bar indicates the standard error of the mean.

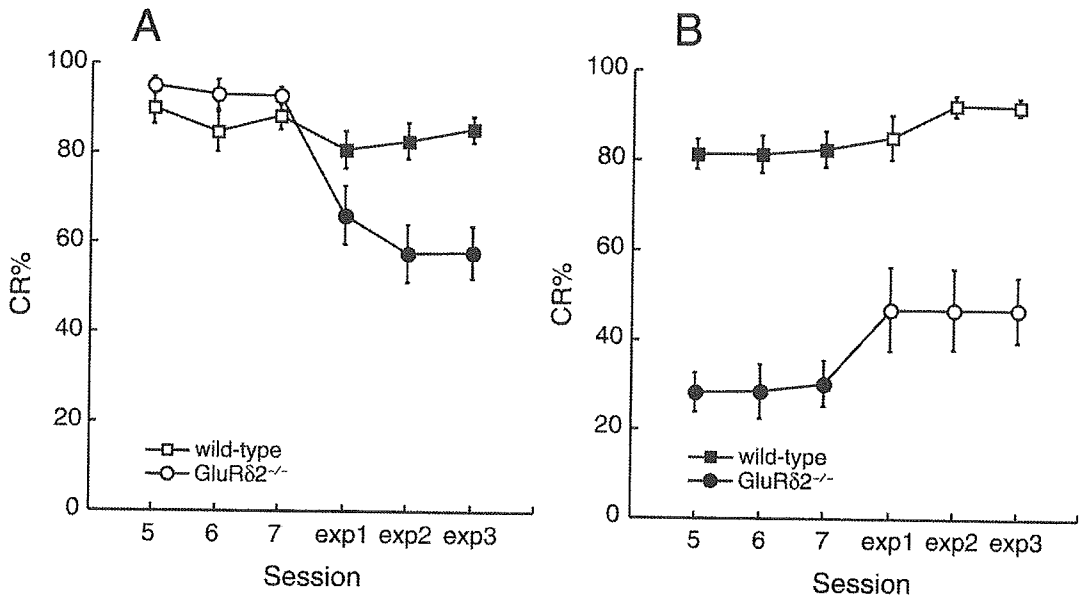

Fig. 3. Effects of (+)MK-801 on expression of the CR. After 7 days of conditioning (shown in Fig. 1), the injection solution was switched from saline to (+)MK-801 (A) or from (+)MK-801 to saline (B). The data during the last three days of conditioning (5th–7th days of conditioning) and the subsequent three days of expression sessions (exp1–3) are shown. (A) Inhibitory effect on expression of the pre-acquired CR. The wild-type mice (n=8) and the GluRδ2$^{-/-}$ mice (n=9), whose average CR% over the last three days with saline injection had exceeded 60%, received additional three days of conditioning with (+)MK-801 injection. (B) Recovery from inhibition by (+)MK-801. The wild-type mice (n=8) and the GluRδ2$^{-/-}$ mice (n=12), which were conditioned with (+)MK-801 injection for 7 days, received additional three days of conditioning with saline injection. Empty and filled symbols indicate saline and (+)MK-801 injection groups, respectively. The error bar indicates the standard error of the mean.

mice and 12 GluRδ2$^{-/-}$ mice used in the experiment shown in Fig. 1) received an additional three days of conditioning with saline injections. When the injection solution was switched from (+)MK-801 to saline, the CR% of the GluRδ2$^{-/-}$ mice rapidly increased, although the CR% did not fully recover during these additional days of conditioning (Fig. 3B). In contrast, the CR% of the wild-type mice showed almost no change on the first day. A paired t-test between the average CR% over the last three days with an (+)MK-801 injection and the CR% on the first day with a saline injection confirmed that there was a significant difference in GluRδ2$^{-/-}$ mice (P<0.05), whereas there were no significant differences in wild-type mice (P>0.1).

DISCUSSION

In the present study, we investigated the effect of the noncompetitive NMDA receptors antagonist (+)MK-801 on the learning ability of GluRδ2$^{-/-}$ mice, which have a deficient cerebellar cortex, in '0-trace' eyeblink conditioning. We found that systemic application of (+)MK-801 (0.1 mg/kg, i.p.) severely impaired acquisition of the CR in GluRδ2$^{-/-}$ mice, while it did not in wild-type mice. Expression of the pre-acquired CR was also markedly impaired in GluRδ2$^{-/-}$ mice, whereas only a marginal impairment of CR expression was observed in wild-type mice. These results indicate that 0-trace conditioning in GluRδ2$^{-/-}$ mice depends largely on NMDA receptors both for acquisition and expression of the CR.

NMDA receptors are extensively expressed over the forebrain, including the prefrontal cortex and the hippocampus (Watanabe et al., 1993), which play critical roles in acquisition during long-trace eyeblink conditioning (Solomon et al., 1986; Kronforst-Collins and Disterhoft, 1998; McLaughlin et al., 2002). In addition to these areas, NMDA receptors are also expressed in the cerebellum (Watanabe et al., 1994a) and brainstem (Watanabe et al., 1994b). Consistent with this, microinfusion of the competitive NMDA receptor antagonist APV into the deep cerebellar nuclei severely impairs acquisition during delay eyeblink conditioning in rabbits (Chen and Steinmetz, 2000), suggesting that some of the effect of (+)MK-801 might be attributable to its effect on the cerebellum. However, in the present study, acquisition was not significantly impaired during 0-trace conditioning in wild-type mice (Fig. 1A) and the impairment of CR expression was minimal (Fig. 3). These results suggest that the effect of (+)MK-801 on the role of the cerebellum and brainstem in mouse 0-trace conditioning must be relatively weak under the present condition.

In contrast to the small effect in wild-type mice, (+)MK-801 severely impaired acquisition in GluRδ2$^{-/-}$ mice (Fig. 1). These results are consistent with our previous work that 0-trace conditioning depends largely on the hippocampus in GluRδ2$^{-/-}$ mice but not in wild-type mice (Takatsuki et al., 2003). Similar effects of (+)MK-801 have been observed in the hippocampus-dependent long-trace eyeblink conditioning in rabbits (Thompson and Disterhoft, 1997) and mice (Takatsuki et al., 2001). Therefore, GluRδ2$^{-/-}$ mice might learn 0-trace eyeblink conditioning using a similar mechanism to that used in long-trace conditioning in wild-type animals. However, in addition to the severe impairment during acquisition sessions, (+)MK-801 also partially inhibited expression of the pre-acquired CR in

GluRδ2$^{-/-}$ mice (Fig. 3A). Thus, a part of the severe impairment in GluRδ2$^{-/-}$ mice during acquisition sessions is attributable to inhibition of the CR expression. In contrast to the large effect in GluRδ2$^{-/-}$ mice, (+)MK-801 had only a little effect on CR expression in wild-type mice (Fig. 3A). Similarly, CR expression in long-trace conditioning was not significantly inhibited by (+)MK-801 in wild-type mice, while its acquisition was severely impaired (Takatsuki et al., 2001). Therefore, (+)MK-801 differently affects 0-trace conditioning in GluRδ2$^{-/-}$ mice and long-trace conditioning in wild-type mice, though both heavily depend on the hippocampus for successful learning.

This impairment of CR expression by (+)MK-801 after sufficient learning was unexpected, because hippocampal lesions made after sufficient learning in 0-trace conditioning paradigm did not affect CR expression in GluRδ2$^{-/-}$ mice, whereas a lesion made before the training did severely impair learning (Takatsuki et al., 2003). Therefore, the site of action of (+)MK-801 to inhibit the CR expression might be outside the hippocampus. At present, there are three candidates for this site. One is the cerebellum. Chen and Steinmetz (2000) have reported that some rabbits that were given APV injection to the deep cerebellar nuclei after sufficient learning showed impairment of CR expression, although most of the animals they examined did not show such impairment. Therefore, it is possible that GluRδ2$^{-/-}$ mice, which have several deficiencies in the cerebellar cortex, preferentially use the expression mechanism that depends on the NMDA receptors in the cerebellar deep nuclei, while wild-type mice use an NMDA receptor-independent mechanism for CR expression. The second candidate site is the medial prefrontal cortex, which plays an important role together with the hippocampus in the long-trace conditioning paradigm (Kronforst-Collins and Disterhoft, 1998; Weible et al., 2000; Takehara et al., 2003) and which also has an abundance of NMDA receptors (Watanabe et al., 1993). If GluRδ2$^{-/-}$ mice depend much more than wild-type mice on the medial prefrontal cortex for CR expression after sufficient conditioning, then blockade of the NMDA receptors in this region might result in an impairment of CR expression. The medial prefrontal cortex plays a critical role when the hippocampus becomes inessential, which occurs one month after completion of long-trace conditioning in rats (Takehara et al., 2003). Therefore, it is plausible that the medial prefrontal cortex also plays an important role in GluRδ2$^{-/-}$ mice, in which the hippocampus is no longer required after 7 days of conditioning (Takatsuki et al., 2003). Finally, the hippocampus cannot be excluded as a candidate, because it is possible that an abnormal hippocampus (caused by blockade of NMDA receptors in our study) could be much more detrimental than its absence (Solomon et al., 1983).

Impairment of CR expression by (+)MK-801 also has been reported in rabbits that received an i.v. injection of (+)MK-801 (Cox et al., 1994). The impairment of CR expression (as well as acquisition) in their study was much more profound than in other reports that adopted s.c. (Thompson and Disterhoft, 1997) or i.p. (Takatsuki et al., 2001) injection of (+)MK-801, suggesting that the i.v. administration may have selectively affected an area that participates in CR expression and that is susceptible to blockade of NMDA receptors in rabbits. Therefore, it is likely that wild-type mice also have a mechanism for CR expression that largely depends on the normal functioning of NMDA receptors.

The present results suggest that GluRδ2$^{-/-}$ mice may utilize an additional mechanism for expression of the CR and possibly for its acquisition as well to overcome the deficiencies in the cerebellar cortex. This mechanism may be present intrinsically but is not dominant in wild-type mice. Several characteristics of 0-trace conditioning in GluRδ2$^{-/-}$ mice (such as the dependence on the hippocampus, muscarinic acetylcholine receptors and on NMDA receptors) are more similar to the features of long-trace conditioning than to those of delay conditioning in wild-type animals (Solomon et al., 1986; Kaneko and Thompson, 1997; Thompson and Disterhoft, 1997). Consistent with the effects of hippocampal lesion in various classical conditioning paradigms, the case of GluRδ2$^{-/-}$ mice suggests that the forebrain may be more likely to be recruited if the task is more difficult for the cerebellum to perform because of deficiencies in the cerebellar cortex. Future study with microinfusion of APV into the cerebellum, the hippocampus and into the medial prefrontal cortex should provide more information about the mechanism underlying hippocampus-dependent eyeblink conditioning in GluRδ2$^{-/-}$ mice and in wild-type mice as well.

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Case report

Progressive multifocal leukoencephalopathy in an HTLV-I carrier

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Abstract

This report describes a previously 28-year-old healthy woman, identified as an asymptomatic human T-lymphotropic virus type I (HTLV-I) carrier, who developed both progressive multifocal leukoencephalopathy (PML) and Pneumocystis jiroveci pneumonia. For diagnostic confirmation of PML, stereotactic brain biopsy demonstrated multiple demyelinating lesions with the presence of JC viral antigen. Intramuscular α-interferon therapy for 2 weeks brought considerable neurologic improvement. Three years later, the patient developed lymphoma-type of adult T-cell leukemia, suggesting that HTLV-I carrier might be one of the underlying diseases of PML.

Keywords: PML; HTLV-I; JC virus; ATL; Immunodeficiency; Pneumocystis jiroveci pneumonia; α-Interferon

1. Introduction

Progressive multifocal leukoencephalopathy (PML) is a fatal demyelinating disease of the central nervous system resulting from JC virus (JCV) infection of oligodendrocytes [1]. JCV is a polyomavirus, which commonly causes PML in the immunocompromised hosts. Although PML was previously considered to be a very rare disease, prevalence of the disease has increased steadily over recent years, in association with acquired immunodeficiency syndrome (AIDS). In contrast, PML associated with human T-lymphotropic virus type I (HTLV-I) infection is extremely rare, and their relationship is still controversial [2]. This report details information about an HTLV-I carrier with PML, who developed adult T-cell leukemia (ATL) 3 years after the onset of PML.

2. Case report

In early October 2001, a previously healthy 28-year-old woman noted weakness in her right hand. The weakness progressed gradually, with the eventual development of a cough and low-grade fever. Because her neurologic and respiratory symptoms continued to worsen, she was sent to our hospital on November 21.

Physical examination revealed body temperature of 37.4 °C, respiration frequency of 22 times a minute, no palpable lymph nodes and no abnormal rales. On neurologic examination, she appeared fully alert and oriented, although the examiner noted decreased attention and inappropriate laughter. Hasegawa's dementia score-revised (HDS-R) [3], a popular scale for assessing dementia or cognitive impairment in Japan, was normal at 26 points (normal range, 20–30 points). Right hemiparesis was evident, and was more severe in the upper than the lower limb. Deep tendon reflexes were brisk on the right, and right Chaddock reflex was positive. The patient was able to walk unassisted, although she exhibited a mild gait disturbance secondary to her right hemiparesis. Sensory examination was normal.

With the exception of an erythrocyte sedimentation rate (ESR) of 60 mm/h and CRP of 0.58 mg/dl (normal range, <0.2 mg/dl), laboratory results were normal. Complete blood count revealed neutrophilia (6160 cells/μl) and mild lymphocytopenia (1232 cells/μl), with a WBC of 7700 cells/μl without atypical or abnormal lymphocytes. Erythrocyte and

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platelet cell count were normal. Immunologic tests, including immunoglobulin, complements and various autoantibodies (anti-nuclear antibody, anti-DNA antibody, anti-SS-A and SS-B antibodies, anti-neutrophil cytoplasmic antibody and anti-cardiolipin antibody), were normal. Lymphocyte subsets analyzed by flow cytometry revealed normal absolute CD8 count (379 cells/μl; normal range, 110–1066), relative CD4 (36.5%; normal range, 25–56%) and CD8 (41.1%; normal range, 17–41%) counts and the CD4/CD8 ratio (0.89, normal; 0.6–2.9) except for decreased absolute CD4 count (337 cells/μl; normal range, 344–1289). Lymphocyte blastoid formation test stimulated by PHA and Con A was normal. Tuberculin skin test was negative, and natural killer cell activity was normal (19%; normal range, 18–40%). Anti-human immunodeficiency virus type I (HIV-1) antibody, serology for syphilis and cytomegalovirus antigenemia were all negative. The most prominent finding was an elevated anti-HTLV-I antibody titer in serum (1:640 by particle agglutination test), although Southern blot analysis from the peripheral blood did not demonstrate monoclonal or polyclonal integration of HTLV-I proviral DNA. Serum soluble interleukin-2 receptor was elevated to 2420 U/ml (normal range, 145–519). Cerebrospinal fluid (CSF) analysis showed normal cell count and protein level, and no anti-HTLV-I antibody was detected in the CSF. Bone marrow tap was normal. Chest X-ray and thoracic CT scan showed infiltrated shadows expanding diffusely in the bilateral lung fields. Transbronchial lung biopsy confirmed the radiologic suspicion of Pneumocystis jiroveci pneumonia. Cranial MRI T2-weighted and FLAIR images demonstrated multiple non-gadolinium enhancing high-intensity lesions in the subcortical white matter (Fig. 1a and b).

JCV DNA in the CSF was undetectable via nested PCR analysis [4]. On December 25, in order to rule out such disease entities as malignant lymphoma, stereotactic brain biopsy was performed focusing on the left frontal subcortical area. Histologic examination of the biopsied brain tissue revealed multiple small demyelinating lesions containing reactive astrocytes without infiltrating mononuclear cells (Fig. 2a and b). Homogeneously hyperchromatic and enlarged nuclei were found in oligodendrocytes immunostained with antibody against JCV (polyclonal rabbit anti-JCV antibody, Dako Co., Japan) (Fig. 2c) [5]. These findings are characteristic for PML. Malignant cells, such as lymphoma cells, were not found.

Treatment with sulfamethoxazole and trimethoprim for Pneumocystis jiroveci pneumonia immediately reversed her respiratory symptoms and normalized the infiltrating shadows on chest X-ray and thoracic CT scan. By contrast, the patient's right hemiparesis, dysarthria, dysphagia and urinary disturbance continued to progress, gradually culminating in akinetic mutism at the beginning of January 2002. The patient's HDS-R score fell to zero points. For treatment of PML, daily intramuscular injections of 3 million units of α-interferon was initiated. On day 10 of α-interferon, improvement was noted in the patient's neurologic findings. She recovered the ability to speak a few words and eat liquid food, and HDS-R score improved to 13 points. α-Interferon therapy was discontinued on day 14 due to the development of side effects including leukocytopenia and thrombocytopenia. However, even after stopping interferon injections, the patient continued to improve and was eventually transferred to another hospital for rehabilitation on 1 May 2002. In September 2004, 35 months after the onset of PML, she was able to communicate by spoken language, read books, operate a wheelchair, walk 20 m with support and eat a regular diet unassisted, although right hemiparesis and mental disturbance remained (HDS-R score, 17 points). Cranial MRI showed diffuse brain atrophy; no new lesions were noted, indicating no recurrence of PML (Fig. 1c). In October 2004, 3 years after the onset of PML, she noticed abrupt swelling of her tonsils and cervical lymph nodes, and marked elevation of serum soluble IL-2 receptor (7250 IU/ml) and

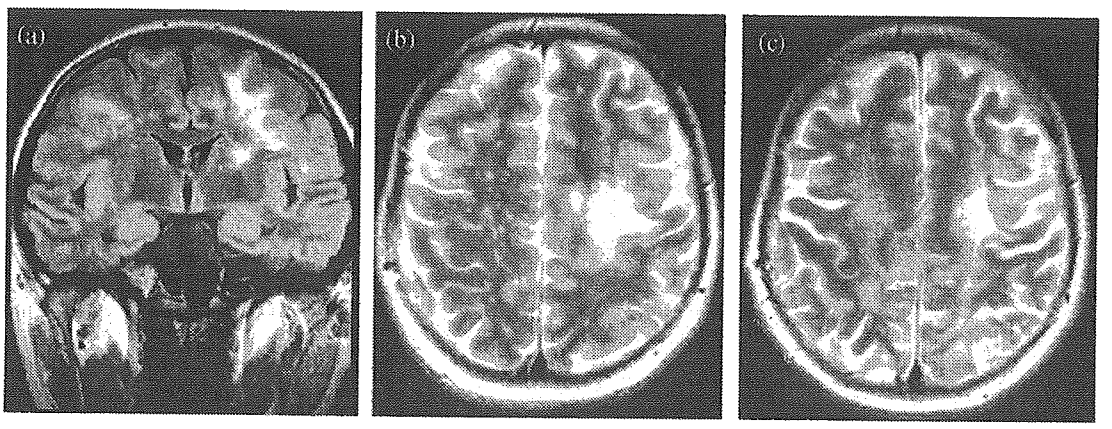

Fig. 1. Cranial MRI (a and b, on November 2001; c, on April 2003). (a) Coronal FLAIR image, showing large high-intensity lesion in the subcortical white matter of the left frontal lobe. (b) Axial T2 weighted image, showing multifocal high-intensity lesions in the bilateral frontal lobes. (c) Following α-interferon therapy, axial T2 weighted image demonstrating no new lesions that might indicate recurrence of PML.

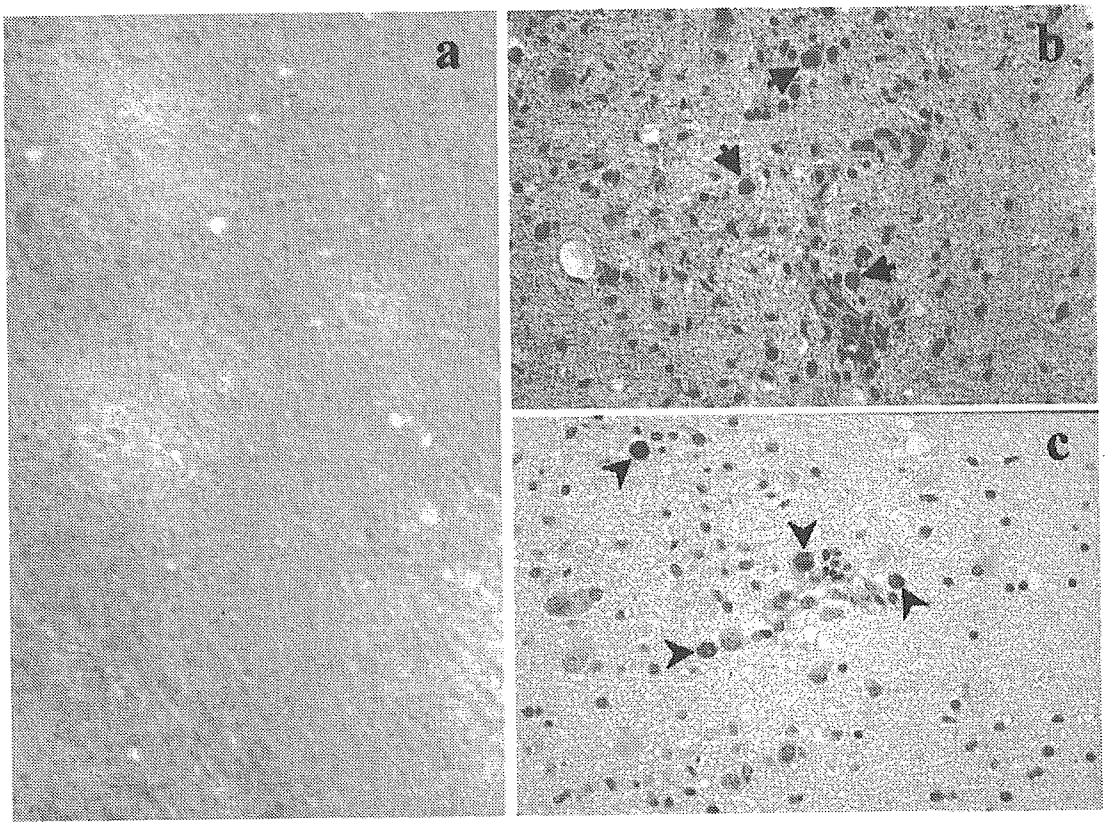

Fig. 2. Histologic finding of biopsied brain. (a) Multifocal demyelinated lesions in the white matter (Luxol fast blue stain, ×20). (b) Reactive astrocytes and inclusions in oligodendrocyte nuclei in the demyelinated lesions (arrows) (hematoxylin and eosin, ×200). (c) These inclusions were intensely stained with anti-JCV antibody (arrowheads (immunohistochemistry, ×200).

appearance of atypical lymphocytes of the peripheral white blood cells (6%) were observed. Lymphoma-type of acute ATL was confirmed by cervical lymph node biopsy, which demonstrated diffuse proliferation of the atypical lymphocytes consisting of CD3, CD4, CD43 and CD45RO-positive T-cells. Southern blot analysis from the peripheral blood demonstrated monoclonal integration of HTLV-I proviral DNA.

3. Discussion

Our report details the case of a previously healthy, asymptomatic HTLV-I carrier, who developed both PML and Pneumocystis jiroveci pneumonia. The diagnosis of PML was confirmed by the presence of JCV antigen recovered from the biopsied brain tissue, as well as radiologic and histopathologic findings compatible with PML.

Although both Pneumocystis jiroveci pneumonia and PML are opportunistic infections, extensive immunologic examinations did not reveal the patient's immunosuppressed state with the exception of a negative tuberculin skin test and the decreased absolute CD4 count. The patient had no underlying diseases that might prompt immunodeficiency, such as malignancy, collagen diseases or AIDS. It is known that asymptomatic HTLV-I carriers demonstrate a cellular immunodeficiency state, involving impairment of T-cell control of Epstein-Barr virus-infected cells and suppression of tuberculin skin reaction [6]. In Japan, there are at least 20 published case reports detailing the presence of opportunistic infection in patients with asymptomatic HTLV-I carrier status [2,6,7]. These reports might support the notion that even HTLV-I carriers exhibit some degree of latent immunodeficiency. Remarkably, 8 cases of 21 (38%) developed ATL subsequently, including our patient. We speculate that our patient's immunosuppressed state might be secondary to her HTLV-I carrier status. In addition, our patient demonstrated that HTLV-I carrier might be one of the underlying diseases of PML.

Recently, an interesting report by Okada et al. showed a relationship between HTLV-I and JCV [8]. They proved the activation of JCV promoter by HTLV-I tax protein in an NF-κB-dependent manner in human neuronal cells in vitro. These results provide new insights into the understanding of the activation of JCV, suggesting a possible role for HTLV-I infection in the pathogenesis of some cases of PML.

In regard to the treatment of PML, α-interferon, β-interferon and cidofovir have been used [1,9]. However, these

drugs have been effective in only a subset of PML patients. Most PML patients have died within 1 year of disease onset, despite receiving these drugs. For PML associated with AIDS, highly active antiretroviral therapy (HAART) is relatively effective [1]. Although there have been no large clinical trials of treatment for PML without AIDS, we selected α-interferon for our patient's therapy due to its anti-viral and immune-regulating functions and its known effectiveness in treating HTLV-I associated myelopathy (HAM) and ATL. It is known that survival in PML patients without AIDS is generally longer, ranging from 9 to 18 months [1], and that only a few patients with PML demonstrated prolonged survival and partial recovery of more than 30 months [10]. Finally, we could not neglect the possibility of the spontaneous remission of PML, but we believe α-interferon therapy brought our patient considerable neurologic improvement from the akinetic mutism, allowing her to leave hospital and live at home. We suggest that α-interferon therapy might be considered for patients with PML associated with HTLV-I infection.

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ORIGINAL INVESTIGATION

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Rapid detection of herpes simplex virus DNA in cerebrospinal fluid: comparison between loop-mediated isothermal amplification and real-time PCR

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Abstract Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method that amplifies DNA with high specificity, efficiency, and speed under isothermal conditions. To evaluate the usefulness of LAMP for diagnosing central nervous system infection with herpes simplex virus (HSV), we compared the LAMP method with real-time PCR, using samples that were previously tested by nested PCR. We examined 69 cerebrospinal fluid (CSF) samples from patients suspected of having HSV infection of the central nervous system. The results of the real-time PCR analysis and nested PCR assay were in complete accord. When nested PCR was regarded as standard, the sensitivity of LAMP was 81%, the specificity was 100%, the positive predictive value was 100%, and the negative predictive value was 90%. Although further improvement is necessary for the wide spread use, the LAMP method might be applicable to diagnosis of HSV infection of the central nervous system.

Keywords HSV · LAMP · Real-time PCR · CSF · Encephalitis

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Introduction

Herpes simplex viruses (HSV) are neurotropic viruses comprising type 1 (HSV-1) and type 2 (HSV-2) [23]. Both HSV-1 and HSV-2 cause central nervous system diseases in humans. Detection of HSV DNA in the cerebrospinal fluid (CSF), most commonly by PCR, is the gold standard for diagnosis of central nervous system infection by HSV [1, 3, 11–13, 17, 18, 23].

Recently, Notomi et al. [21] reported a novel nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP). The LAMP reaction requires a DNA polymerase with strand displacement activity and a set of four specially designed primers, termed inner and outer primers, which improve specificity. As a first step, a stem-loop DNA structure, in which the sequences of both DNA ends are derived from the inner primer, is constructed as the starting material. Subsequently, one inner primer hybridizes to the loop on the product in the LAMP cycle and initiates strand displacement DNA synthesis, yielding the original stem-loop and new stem-loop DNA with a stem twice as long. The final products are stem-loop DNAs with several inverted repeats of the target DNA and cauliflower-like structures with multiple loops, amplifying the amount to 10^9 copies of the target [21]. The most significant advantage of LAMP is its ability to amplify specific sequences of DNA at 63–65°C, without thermocycling. Therefore, the technique requires relatively simple and cost-effective equipment, making it amenable for use in hospital laboratories.

This method has been used for the rapid diagnosis of various infectious diseases, including HSV [4–7, 9, 22, 24]. In this study, we examined the use of a type-specific HSV LAMP method for the diagnosis of HSV infection of the central nervous system. To evaluate the usefulness of LAMP, we tested CSF samples from patients with HSV encephalitis using the LAMP method, and compared the results with those obtained using the real-time PCR assay.

Methods

Samples

A total of 69 CSF samples obtained from 50 patients were analyzed. Clinically, all the patients were suspected of having HSV infection of the central nervous system. There were 43 samples from adults and 23 from neonates. In some patients, samples were obtained sequentially after the initiation of acyclovir therapy. We had previously performed nested PCR to detect HSV DNA in these samples; HSV DNA was detected in 26 of the 69 CSF samples from suspect cases using the nested PCR assay [10]. Restriction fragment length polymorphism of amplified products indicated that 11 samples contained HSV-1 DNA and 15 samples contained HSV-2 DNA [2, 15].

Real-time PCR

The real-time quantitative PCR assay was carried out using a TaqMan PCR kit (Applied Biosystems, Foster City, USA), as described previously [8, 10, 14, 15], using the primers and fluorogenic probe based on the *UL30* gene sequence [8]. DNA was extracted from 200 µl of CSF, using a QIAamp Blood kit (QIAGEN, Hilden, Germany), and eluted in 50 µl of distilled water. Five microliters of the DNA extracted from each sample were used for the real-time PCR assay, which can detect both HSV-1 and HSV-2 with almost equal efficiency. Following 5 cycles of 20 s at 97°C and 1 min at 58°C, 45 cycles of 20 s at 96°C and 1 min at 58°C were carried out using a Model 7700 Sequence Detector (Applied Biosystems). Real-time fluorescence measurements were made, and threshold cycle value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit (10 times the standard deviation for the base line). Standard curves were constructed using the values obtained from serially diluted positive control plasmids that contained *UL30* gene. The values from clinical samples were plotted on the standard curve, and the copy number was calculated by automatically using Sequence Detector v1.6 (Applied Biosystems). To determine a detection limit, a serially diluted plasmid control containing either HSV-1 or HSV-2 was used. A minimum of five copies of either HSV-1 or HSV-2 DNA per assay can be detected [10].

All experiments were done in duplicate. For borderline samples that were estimated as low copies or discordant with the results of LAMP, tests were repeated, and all the repeated results were in accord with the original ones. Sometimes, estimated copy numbers were lower than the detection limit of the real-time PCR assay. The reason was unclear, but inhibitors, which could not be eliminated from clinical samples by the DNA extraction, may influence on PCR reaction and underestimate the copy number [16].

LAMP method

The LAMP reactions were conducted as described previously [4]. The LAMP method requires a set of four primers (B3, F3, BIP, FIP) that recognize a total of six distinct sequences within the target DNA [21]. Primers for the HSV-1- and HSV-2-specific LAMP assays were designed based on the HSV-1 gG and HSV-2 gG gene sequences, respectively. Primer BIP for the gG genes of HSV-1 and HSV-2 contained the B1 direct sequence and B2 complementary sequence, each specific for the respective strains. Primer FIP for the gG genes of HSV-1 and HSV-2 contained the F1 complementary sequence and the F2 direct sequence. Primers B3 and F3 for the gG genes of HSV-1 and HSV-2 were located outside the F2-B2 regions. As additional loop primers increase the amplification efficiency [20], loop primers specific for the HSV-1 gG and HSV-2 gG genes were also used. The primer sequences and binding locations have been described elsewhere [4]. The LAMP reaction was performed using a Loopamp DNA amplification kit (Eiken Chemical, Tochigi, Japan). The 25-µl reaction mixtures contained 1.6 µM each of the FIP and BIP primers, 0.8 µM of each outer primer (F3 and B3), 0.8 µM of each loop primer, 12.5 µl of 2X reaction mix, 1 µl of *Bst* DNA polymerase, and 5 µl of the extracted DNA solution. The mixture was incubated at 63°C for 45 min. The turbidity derived from magnesium pyrophosphate formation, due to release of pyrophosphate during polymerization, was detected on a TERAMECS LA200 real-time turbidimeter (Teramecs, Kyoto, Japan) and used as an indicator of the formation of amplified products. The turbidity of five negative control samples was 0.01 ± 0.02; we thus defined 0.1 as the cut-off value for discrimination between positive and negative samples. To determine a detection limit, a serially diluted plasmid control containing either HSV-1 or HSV-2 was used. A minimum of 500 copies of HSV-1 or 1,000 copies of HSV-2 DNA per assay was detected by the HSV type-specific LAMP method [4]. To avoid contamination between samples, different rooms were used for DNA extraction and LAMP set up, using filter-containing pipette tips for aerosol protection.

Results

We used both real-time PCR and a type-specific HSV LAMP method to detect HSV DNA in the 69 previously tested CSF samples. The results of real-time PCR analysis were completely in accordance with those of the previously conducted nested PCR assay. On the other hand, 5 samples that were positive by nested PCR were negative by LAMP, although there were no false positive results (Table 1). When nested PCR was regarded as standard, the sensitivity of LAMP was 81%, the specificity was 100%, the positive predictive value was 100%, and the negative predictive value was 90%.

Table 1 Comparison between nested PCR and loop-mediated isothermal amplification (LAMP) techniques for detection of herpes simplex virus DNA in cerebrospinal fluid

LAMP	Nested PCR		
	Positive	Negative	Total
Positive	21	0	21
Negative	5	43	48
Total	26	43	69

The samples that were positive by any test are listed in order of the number of copies of HSV DNA detected (Table 2). The copy number measured by the real-time PCR assay ranged from 13 to 3,100,000 copies/ml of CSF. All 5 LAMP-negative samples had low HSV DNA copy numbers (23 to 190 copies/ml of CSF), as determined by real-time PCR. Three of the five samples were obtained during acyclovir therapy. None of the samples that had more than 200 copies/ml of HSV DNA were negative by LAMP. The differentiation of HSV types by LAMP was in complete accordance with those of restriction fragment length polymorphism. Although 4 out of 5 LAMP-negative samples were HSV type 1, there was no statistical difference of sensitivity between type 1 and type 2. A recent study indicates that the time required for the turbidity to exceed a cut-off value is dependent of initial template DNA [19]. The time required for the turbidity in each sample is shown in Table 2. There was no correlation between the copy number measured by real-time PCR and the required time by LAMP.

Discussion

LAMP is a novel technique for amplification of specific DNA sequences, and has several advantages over PCR [20, 21]. First, the specificity of LAMP is high because the LAMP method uses multiple primers, recognizing six distinct sequences in the target DNA. Second, the method is both rapid and simple; only 45 min are needed to amplify the target sequences. Third, the cost of the equipment is inexpensive compared with PCR; equipment cost remains one of the major reasons why PCR diagnostics have not been more widely utilized. With these advantages, LAMP has the potential to become adopted for widespread use in hospital laboratories.

Recently, we established a LAMP-based HSV type-specific DNA amplification method and applied it to swab samples collected from patients with vesicular skin lesions and gingivostomatitis [4]. We showed that the HSV type-specific LAMP is sensitive enough for the detection of viral DNA in swab samples, which contain large quantities of HSV DNA. In this study, we applied the type-specific HSV LAMP method to detect HSV

Table 2 Characteristics of cerebrospinal fluid samples that were positive for herpes simplex virus (HSV) DNA

No.	Age	Sex	Diagnosis	Acyclovir therapy	HSV type	Real-time PCR (copies/ml)	LAMP		
							Type 1	Type 2	Time required* (min)
1	< 1 month	F	Neonatal herpes	No	2	13	−	+	30
2	**61 year**	**M**	**Encephalitis**	No	**1**	**23**	−	−	**45 <**
3	66 year	M	Encephalitis	No	1	40	+	−	28
4	**< 1 month**	**M**	**Neonatal herpes**	Yes	**1**	**96**	−	−	**45 <**
5	**< 1 month**	**F**	**Neonatal herpes**	Yes	**2**	**120**	−	−	**45 <**
6	**1 year**	**F**	**Encephalitis**	Yes	**1**	**160**	−	−	**45 <**
7	**34 year**	**M**	**Encephalitis**	No	**1**	**190**	−	−	**45 <**
8	< 1 month	F	Neonatal herpes	Yes	1	230	+	−	33
9	71 year	M	Encephalitis	No	1	250	+	−	38
10	10 year	F	Meningitis	No	2	500	−	+	37
11	15 year	M	Encephalitis	Yes	1	630	+	−	30
12	15 year	M	Encephalitis	No	1	830	+	−	26
13	< 1 month	F	Neonatal herpes	No	2	1200	−	+	34
14	< 1 month	F	Neonatal herpes	Yes	2	2100	−	+	33
15	< 1 month	M	Neonatal herpes	Yes	2	2200	−	+	28
16	< 1 month	F	Neonatal herpes	Yes	2	4500	−	+	32
17	< 1 month	F	Neonatal herpes	Yes	2	5500	−	+	30
18	10 year	F	Meningitis	No	2	5900	−	+	37
19	1 year	F	Encephalitis	No	1	6500	+	−	29
20	34 year	M	Encephalitis	Yes	1	11000	+	−	24
21	< 1 month	F	Neonatal herpes	No	2	12000	−	+	36
22	< 1 month	F	Neonatal herpes	Yes	2	130000	−	+	24
23	< 1 month	F	Neonatal herpes	No	2	180000	−	+	27
24	< 1 month	M	Neonatal herpes	No	2	240000	−	+	25
25	< 1 month	M	Neonatal herpes	No	2	690000	−	+	36
26	< 1 month	M	Neonatal herpes	No	2	3100000	−	+	30

Samples are listed in order of the number of copies of HSV DNA. Samples in bold print had discordant results in real-time PCR and LAMP

LAMP loop-mediated isothermal amplification
*Time required for the turbidity to exceed the cut-off value