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Short communication

Failure of mefloquine therapy in progressive multifocal leukoencephalopathy: Report of two Japanese patients without human immunodeficiency virus infection

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ABSTRACT

Although progressive multifocal leukoencephalopathy (PML) cases showing responses to mefloquine therapy have been reported, the efficacy of mefloquine for PML remains unclear. We report on the failure of mefloquine therapy in two Japanese patients with PML unrelated to human immunodeficiency virus. One of the patients was a 47-year-old male who had been treated with chemotherapy for Waldenström macroglobulinemia, and the other was an 81-year-old male with idiopathic $CD4^+$ lymphocytopenia. Diagnosis of PML was established based on MRI findings and increased JC virus DNA in the cerebrospinal fluid in both patients. Mefloquine was initiated about 5 months and 2 months after the onset of PML, respectively. During mefloquine therapy, clinical and radiological progression was observed, and JC virus DNA in the cerebrospinal fluid was increased in both patients. Both patients died about 4 months and 2 months after initiation of mefloquine, respectively. Further studies are necessary to clarify the differences between mefloquine responders and non-responders in PML.

1. Introduction

Progressive multifocal leukoencephalopathy (PML) is a brain disorder caused by JC polyomavirus, which causes death in one-half of patients within 1 year [1]. Primary infection usually occurs during childhood and is often asymptomatic. The initial site of JC virus (JCV) infection is thought to be the tonsils, and it is then carried by lymphocytes to the kidneys and bone marrow. Reactivation of JCV occurs due to severe cellular immunodeficiency, and the virus crosses the blood–brain barrier (BBB) and infects oligodendrocytes, causing widespread demyelinating lesions. A recent study revealed promyelocytic leukemia nuclear bodies as an intranuclear target of JCV [2].

A study of 9675 cases of PML between 1998 and 2005 showed that 82% of patients had human immunodeficiency virus (HIV), 8.4% hematologic malignancies, 2.83% solid organ cancers, and 0.44% rheumatologic diseases [3]. Recently, a new category of PML patients has emerged among patients treated with immunomodulatory medications including natalizumab, rituximab, and efalizumab. PML may also occur in patients with minimal or occult immunosuppression including idiopathic $CD4^+$ lymphocytopenia [4]. In Japan, the proportion of hematological malignancies or rheumatologic diseases as underlying diseases is relatively high, whereas that of HIV infection is low [5,6].

The estimated probability of survival at 1 year is reported to be 52% in HIV related PML [1] and variable in PML unrelated to HIV among reports. Some patients with PML do survive for extended periods of time after diagnosis [7,8]. Survival in PML is influenced by the presence of JCV-specific cytotoxic T-lymphocytes, $CD4^+$ cell counts, or JCV DNA levels [1,9]. One study reported that estimated 1-year survival was 48% in patients with HIV related PML with $CD4^+$ cell counts $<200/\mu l$ at PML diagnosis compared to 67% in those with $CD4^+$ cell counts $>200/\mu l$ [1]. Another study showed that JCV DNA levels >4365 copies/ml of cerebrospinal fluid (CSF) correlated significantly with shorter survival in patients with HIV related PML not receiving highly active antiretroviral therapy (HAART) [9].

To date, although antiviral drugs such as cytarabine and cidofovir show activity against JCV *in vitro* [10,11], large clinical studies have failed to establish the efficacies of these drugs in the treatment of PML [12–14]. The reason for this may be that these drugs are not able to cross the BBB and accumulate throughout the entire brain parenchyma at a dose sufficient to suppress JCV proliferation [15].

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In 2008, mefloquine, an anti-malarial drug, was reported to show activity against JCV *in vitro* [15]. Since then, there have been at least 5 reported cases of PML in which mefloquine was effective [16–20]. In contrast, a recent mefloquine trial of 24 patients with PML (21 HIV-positive and 3 HIV-negative) reported failure in reducing JCV DNA levels in the CSF [21], although it is pending publication. Because there have been no reports describing the details of PML patients demonstrating mefloquine treatment failure, we report two HIV-negative patients with PML in whom mefloquine was not effective.

2. Case reports

2.1. Case 1

A 47-year-old male presented with progressive left hemiparesis. The patient had been treated with chemotherapy including rituximab for Waldenström macroglobulinemia for six years in our hospital. The interval between the last administration of rituximab and occurrence of hemiparesis was about 1 month. Diffusion weighted images (DWI) of brain MRI about 3 months after the onset of hemiparesis demonstrated high intensity areas with internal low intensity areas in the white matter of the right frontal lobe. The apparent diffusion coefficient (ADC) values of the lesion were increased. Because serum IgM had been prominently elevated (around 5000 mg/dl) in association with Waldenström macroglobulinemia, we presumed that the hyperviscosity syndrome resulted in brain infarction.

About 4 months after the onset of hemiparesis, the patient was admitted to our hospital because a convulsion occurred in the left upper and lower limbs. At that time, the patient did not receive any immunosuppressive therapy. On admission, neurological examination revealed upper limb-dominant left hemiparesis, and Babinski's sign and Chaddock's reflex on the left. MRI on admission demonstrated lesion expansion and extension to the right parietal and insular white matter, right putamen, right internal capsule, right thalamus, corpus callosum, left frontal white matter, and midbrain. There was no edema or gadolinium-enhanced lesions. Peripheral blood tests showed white blood cell count (WBC): 3790/μl (normal range: 4500–9000), hemoglobin: 10.4 g/dl (normal range: 13–16), and platelet count: 3.7×10^4/μl (normal range $15–30\times10^4$), indicating pancytopenia. C-reactive protein (CRP) was below 0.1 mg/dl. Testing for HIV was negative. On the next day of admission, a nasogastric feeding tube was inserted because of dysphagia. Four days after admission, CSF examination demonstrated cell count: 1 cell per 3 μl, total protein: 97 mg/dl, and glucose: 67 mg/dl. PCR was positive for JCV DNA in the CSF and detected 1200 copies/ml of DNA. A diagnosis of PML was established based on MRI findings and increased JCV DNA in the CSF.

After diagnosis, the patient developed right hemiparesis and apraxia of speech. Brain MRI 18 days after admission demonstrated lesion expansion and extension to the left insular white matter and left putamen (Fig. 1A). The JCV DNA copy number in the CSF was increased to 4300 copies/ml. $CD4^+$ cell count of the peripheral blood was 219/μl (normal range: 500–1300). Nineteen days after admission, about 5 months after the onset of PML, mefloquine was initiated at a dose of 275 mg/day orally for 3 days, followed by 275 mg once a week [17]. We used Mephaquin Hisamitsu tablets (Hisamitsu Pharmaceutical, Tosu, Japan), which show maximum concentration (C_{max}) of 3.1 μM, time at which C_{max} is observed (T_{max}) of 5.2 h, and terminal half-life (T1/2) of 400.1 h when 1100 mg of drug is once administered. Treatment with mefloquine was approved by the Ethics Committee in our hospital. We obtained written, informed consent from the patient's family. We also used 1 mg/day of risperidone, a 5HT2A receptor blocker at the same time. After initiation of mefloquine, we observed no symptoms suggestive of mefloquine neurotoxicity such as nausea, dizziness, sleep disturbances, anxiety, and psychosis [22]. Eight days after initiation of mefloquine, the JCV DNA copy number in the CSF was increased to 150,000 copies/ml, and the dose of mefloquine was returned to 275 mg/day for 3 days per week (Fig. 2).

However, the JCV DNA copy number in the CSF 22 days after initiation of mefloquine was increased to 850,000 copies/ml. Because of severe aspiration pneumonia, tracheotomy was performed 37 days after initiation of mefloquine. Brain MRI 38 days after initiation of mefloquine demonstrated lesion expansion and extension to the right temporal and occipital white matter and pons (Fig. 1B). The JCV DNA copy number in the CSF 50 days after initiation of mefloquine increased to 3,700,000 copies/ml. Changes in the JCV DNA load are shown in Fig. 2. Brain MRI about 3 months after initiation of mefloquine demonstrated lesion expansion and extension to the left temporal and parietal white matter, left internal capsule, left thalamus, and medulla oblongata (Fig. 1C). The patient died of respiratory failure about 4 months after initiation of mefloquine. The total clinical course of PML was about 9 months. Autopsy could not be performed.

2.2. Case 2

An 81-year-old male with a three-week history of gait disturbance presented with muscle cramp in the bilateral upper limbs and was taken to another hospital by ambulance. Past medical history included hypertension, hyperuricemia, chronic heart failure, and chronic renal failure due to renal sclerosis. A diagnosis of brain infarction of the

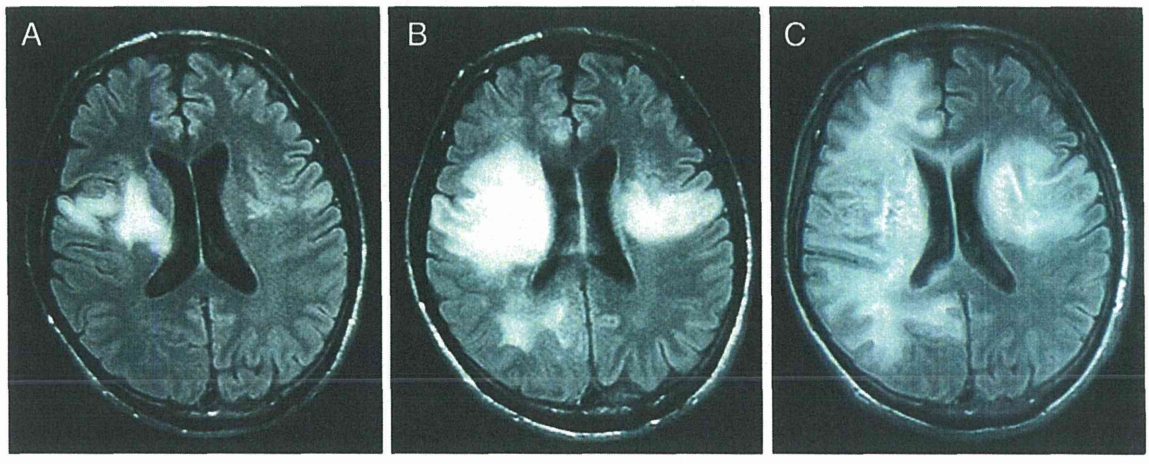

Fig. 1. A. Fluid-attenuated inversion recovery (FLAIR) sequence of brain MRI before initiation of mefloquine demonstrated high intensity areas in the white matter of the bilateral frontal lobes. B, C. FLAIR sequence of brain MRI 38 days (B) and about 3 months (C) after the initiation of mefloquine showed lesion expansion.

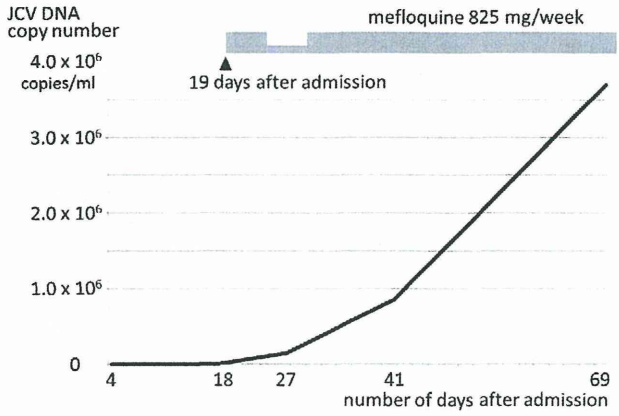

Fig. 2. Changes in the JCV DNA load of case 1 are shown. The JCV DNA copy number in the CSF was increased even after initiation of mefloquine.

subacute phase and the worsening of renal failure was made in the emergency room and the patient was transferred to our hospital.

Physical examination on admission demonstrated muscle cramp in the bilateral upper limbs and face, and right hemiparesis. Consciousness was slightly disturbed, but the orientation to time and place was preserved. Peripheral blood tests showed WBC: 5240/μl, hemoglobin: 7.8 g/dl, platelet count: 17.6×10^4/μl, albumin: 2.7 g/dl (normal range: 3.9–4.9), blood urea nitrogen (BUN): 147 mg/dl (normal range: 8–20), creatinine: 7.83 mg/dl (normal range: 0.6–1.1), creatinine kinase (CK): 445 IU/l (normal range 50–200), CRP: 0.3 mg/dl (normal range <0.1), and glucose: 103 mg/dl. Testing for HIV was negative. Hemodialysis was started on the next day of admission.

DWI of brain MRI 3 days after admission demonstrated high intensity areas in the white matter of the left frontal and parietal lobes and right parietal lobe. ADC values of the lesions were increased. Right hemiparesis progressed after admission, and 18 days after admission, the left hemiparesis emerged. Because of dysphagia, a nasogastric feeding tube was inserted 19 days after admission. CSF examination 20 days after admission demonstrated cell count: 6 cells per 3 μl, total protein: 35 mg/dl, and glucose: 60 mg/dl. PCR was positive for JCV DNA in the CSF, and detected 2223 copies/ml of DNA. A diagnosis of PML was established based on MRI findings and increased JCV DNA in the CSF.

Brain MRI 32 days after admission demonstrated lesion expansion and extension to the corpus callosum and right frontal white matter. Thirty six days after admission, the patient manifested akinetic mutism. Thirty eight days after admission, about 2 months after the onset of PML, mefloquine was initiated at a dose of 275 mg/day orally for 3 days per week. Treatment with mefloquine was approved by the Ethics Committee in our hospital. We obtained written, informed consent from the patient's family. At that time, the JCV DNA copy number in the CSF was increased to 2,790,000 copies/ml. The $CD4^+$ cell count of the peripheral blood was 294/μl. Because whole body CT demonstrated no mass lesions or abnormal lymph node swelling, underlying diseases causing immunodeficiency remained unclear in this patient.

After initiation of mefloquine, we observed no acute neurological deterioration suggesting mefloquine neurotoxicity. Brain MRI 15 days after initiation of mefloquine demonstrated lesion expansion and extension to the bilateral temporal and occipital white matter (Fig. 3A). Twenty nine days after initiation of mefloquine, the JCV DNA copy number in the CSF was increased to 24,075,000 copies/ml. Changes in the JCV DNA load are shown in Fig. 4. Brain MRI 31 days after initiation of mefloquine demonstrated lesion expansion (Fig. 3B). Thirty three days after initiation of mefloquine, hemodialysis was discontinued because of hypotension. The patient died 19 days later. The total clinical course of PML was about 4 months. Autopsy could not be performed.

3. Discussion

Because there is no known specific antiviral agent against JCV, we treated PML in the two HIV-negative patients with mefloquine based on case reports describing the efficacy of mefloquine for PML [16–20]. However, during mefloquine therapy, clinical and radiological progression was observed, and JCV DNA in the CSF was increased in both patients.

Our case 1 had been treated with chemotherapy including rituximab for Waldenström macroglobulinemia. The interval between the last administration of rituximab and diagnosis of PML was about 6 months. Although it is difficult to exclude the possibility that the immunodeficiency due to Waldenström macroglobulinemia itself was related to the occurrence of PML [23], rituximab is well known to cause PML [7]. Rituximab is an anti-CD20 monoclonal antibody that targets human B cells. The pathogenesis of rituximab in PML is considered to decrease B cells in the cerebral perivascular spaces, resulting in decreased antigen presentation to T cells and subsequent alterations in the cellular immune response [7]. One study reported that a median $CD4^+$ cell count was 216/μl in 25 patients who received rituximab [24]. The interval between the last administration of rituximab and

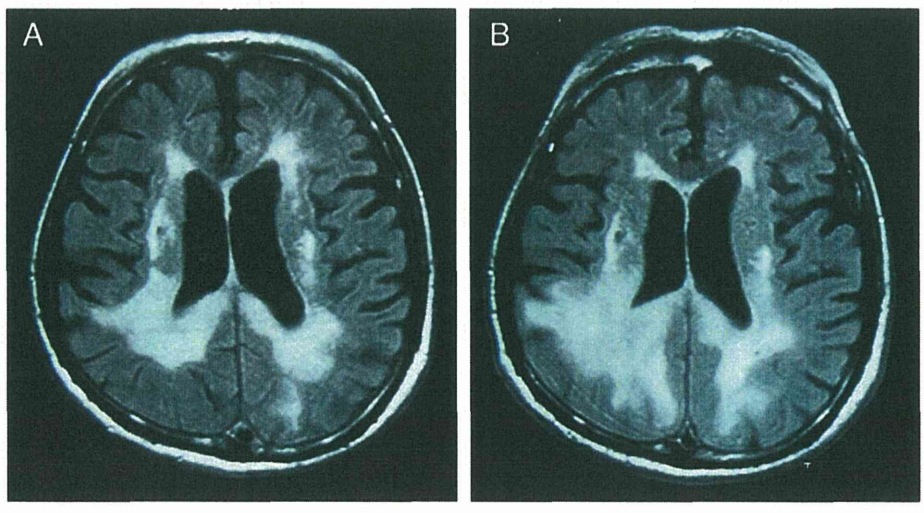

Fig. 3. A. FLAIR sequence of brain MRI 15 days after initiation of mefloquine demonstrated abnormal high intensity areas in the bilateral temporal and occipital white matter. B. FLAIR sequence of brain MRI 31 days after the initiation of mefloquine showed lesion expansion.

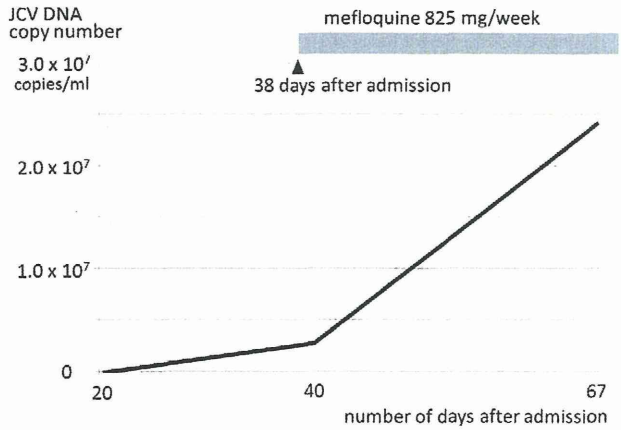

Fig. 4. Changes in the JCV DNA load of case 2 are shown. The JCV DNA copy number in the CSF was increased even after initiation of mefloquine.

diagnosis of PML has been reported to be 5.5 months [25]. Considering that 90% of patients with PML after rituximab therapy die [25], the unfavorable clinical course of our case 1 may be associated with the use of rituximab. In case 2, while $CD4^+$ lymphocytopenia was documented, there were no underlying diseases causing immunodeficiency. However, as PML may occur in patients with minimal or occult immunosuppression [4], idiopathic $CD4^+$ lymphocytopenia may be associated with the occurrence of PML in this patient.

Mefloquine is an anti-malarial drug used both for prophylaxis and treatment of chloroquine resistant *Plasmodium falciparum*. Because mefloquine is highly lipophilic and has a long terminal half-life of more than 1 week [26], a single dose of 15–25 mg/kg is used for treatment and 250 mg/week for prophylaxis. Among subjects administered 250 mg weekly, blood concentrations vary between 1 μM to 5 μM [27]. Mefloquine readily crosses the BBB, where active efflux by the P-glycoprotein membrane transporter prevents its accumulation in the brain [27].

In 2008, mefloquine was reported to show activity against JCV *in vitro* [15]. Brickelmaier et al. showed that mefloquine inhibits viral DNA replication, using quantitative PCR to quantify the number of viral copies in cultured cells. In this study, mefloquine reduced the number of infected cells by 50% or more at a concentration of 3.9 μM [15]. Brickelmaier et al. presumed that efficacious concentrations of mefloquine for PML are achieved in the brains of patients receiving approved doses of the drug [15].

Since the publication by Brickelmaier et al. [15], there have been at least 5 reported cases of PML in which mefloquine was effective [16–20]. The underlying diseases or conditions included sarcoidosis [16], umbilical cord blood transplant [17], HIV infection [18], and systemic lupus erythematosus [19]. $CD4^+$ cell counts in the peripheral blood of patients were described in 3 reports, and were 187/μl [18], 419/μl [17], and 420/μl [16], respectively. JCV DNA loads in the CSF before mefloquine therapy were available in these reports, and were 33,700 copies/ml [16], 535,500 copies/ml [18], and 911,175 copies/ml [17], respectively. The intervals between symptom onset and initiation of mefloquine therapy were about 3 months [17,19], 5 months [18], and 6 months [16,20], respectively. In 4 reports [16–19], the authors stated that PCR for JCV in the CSF became negative after mefloquine therapy. At present, the patients' background or laboratory data common among these cases showing responses to mefloquine therapy is unclear.

In contrast to these cases, a recent mefloquine trial of 24 patients with PML (21 HIV-positive and 3 HIV-negative) reported failure in reducing JCV DNA levels in the CSF [21]. Participants took 250 mg of mefloquine 4 times daily, followed by 250 mg weekly. The failure of this trial and the poor outcome of our patients raise the possibility that the improvement observed in mefloquine therapy in reported PML patients [16–20] may actually reflect the natural favorable course of those patients.

At present, we cannot tell the difference in patient backgrounds or laboratory data between patients showing responses to mefloquine [16–20] and our patients. Regarding the presence of both mefloquine responders and non-responders in PML, Nevin stated that responses to mefloquine may correlate with polymorphisms in the *MDR1* gene coding for P-glycoprotein that affect drug efflux across the BBB [28]. In cases of unsuccessful treatment of PML, active efflux as a result of drug induced upregulation of P-glycoprotein expression in the BBB may be preventing therapeutic concentrations of mefloquine [28]. From this point of view, co-administration of P-glycoprotein inhibitors or substrates such as risperidone may be recommended in the treatment of PML [27]. On the other hand, considering the failure of the mefloquine trial and the poor outcome of our patients, re-evaluation of the anti-JCV activity of mefloquine may be required. If the anti-JCV activity of mefloquine is verified again, further studies are necessary to clarify whether the response to mefloquine in PML is influenced by the presence of HIV infection, $CD4^+$ cell counts, JCV DNA levels in the CSF, blood concentration of mefloquine, interval between disease onset and initiation of therapy, or *MDR1* polymorphism.

Conflict of interest statement

The authors have no conflicts of interest.

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Case Report

Does anti-JCV therapy improve the prognosis of AIDS-related PML?

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1. Introduction

Progressive multifocal leukoencephalopathy (PML) is a subacute, fatal demyelinating disease of the brain that occurs in immunosuppressed patients. The causative agent is the JC virus (JCV). Approximately 5% of patients with AIDS contract PML. The only known intervention for these patients is combination antiretroviral therapy (cART): survival at 1 year is reported to be between 38.6% and 56%, and those patients who survive are usually left with severe neurological sequelae. Although several medications with in vitro activity against JCV have been employed during cART for PML patients, they have proven largely ineffective. However, the anti-malarial drug mefloquine, which is known to have anti-JCV activity in vitro, has produced successful outcomes in some PML patients [1]. We report here the use of mefloquine alongside cART in a case of AIDS-related PML in which the JCV level in cerebrospinal fluid (CSF) was relatively high compared to previously reported cases.

2. Case report

In May 2011, a 61-year-old man presented with weakness in all limbs. He had been well until 2 months earlier. He was diagnosed with HIV-1 infection at his first visit. Viral load was 99,000 copies/mL and the CD4 count was 58 cells/μL. Brain MRI showed asymmetric, patchy foci in the cerebral hemispheres, with perifocal edema. Real-time PCR testing for JCV genomic DNA in CSF revealed 315,000,000 (8.50 log) copies/mL. On this basis, we diagnosed him with AIDS-related progressive multifocal leukoencephalopathy (PML). He was immediately started on a cART regimen of abacavir, lamivudine, and efavirenz according to national protocol, but he lost consciousness 14 days after admission. Because the JCV viral load in CSF was extremely high, concomitant administration of mefloquine hydrochloride tablets was started at this time (initially 275 mg daily for 3 days, then 275 mg once weekly).

Immunological variables showed rapid improvement, with CD4 increased up to 234 μL^{-1} and HIV-1 viral load decreased to 41 copies/mL at 3 months after the initiation of cART. At this time the patient's Karnofsky Performance Scale Index score for functional impairment had progressively worsened from 70/100 on admission to 20/100. At 4 months after starting cART and with concomitant mefloquine therapy, the JCV viral load in CSF was markedly suppressed, below the detection sensitivity limit and comparable to previous reports. Follow-up MRI showed extension

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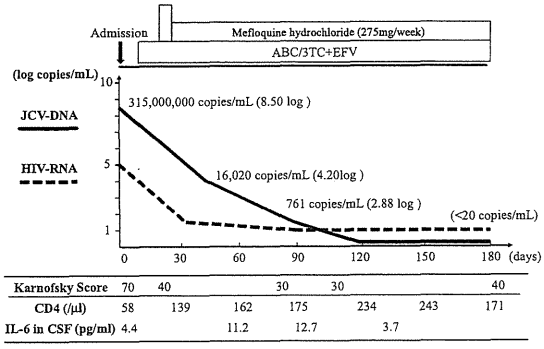

Fig. 1. Clinical course after administration of the mefloquine.

of an area of T2 hyperintensity within the white matter, but no further extension occurred after disappearance of JCV in the cerebrospinal fluid. As of December 2011, he had recovered the ability to make eye contact with medical staff and his clinical appearance had improved, with a Karnofsky Performance Scale Index score of 40/100. Unfortunately, he died in hospital from pneumonia (Fig. 1).

3. Discussion

The use of highly active cART, which is the only known intervention for AIDS-related PML, can leave many patients who survive with severe neurological sequelae. In the present case, the JC viral load was high at 8.50 log copies/mL and the eradiation rate with cART and mefloquine was fast at 7.0×10^{-2} log copies per month, compared with the highest JC viral load in CSF of 7.30 log copies/mL reported previously and a median eradiation rate with cART of 3.0×10^{-3}–1.4×10^{-2} log copies per month [2–4]. García de Viedma et al. previously reported a significant difference in survival between patients with a JCV viral load >4.68 log copies/mL in CSF and those with a JCV viral load below this [5]. Another report highlighted that early eradication or suppression of JCV in CSF was associated with the stabilization of symptoms caused by PML. It appears that our patient's neurological condition improved because of the rapid eradication of JCV.

Immune reconstitution inflammatory syndrome (IRIS) may develop secondary to restoration of immunity in HIV-positive patients with PML receiving cART and may cause paradoxical clinical deterioration despite recovery of the immune system. Risk factors for IRIS include a decrease in CD4 T cells at the initiation of cART, with a count <50 μL^{-1} posing a high risk, and a decrease in HIV-1 RNA levels in AIDS patients of >2 log copies/mL within 90 days of starting cART. Our patient was therefore at high risk for IRIS, with a CD4 count of 58 μL^{-1} at the initiation of cART and his HIV-1 RNA level falling >3 log copies/mL within 90 days of starting cART. It must be noted that the severity of PML-IRIS is varied however: some cases are mild and resolve with continued cART, while others may lead to significant morbidity and even mortality because of a severe inflammatory response characterized histopathologically by a marked influx of CD8 T cells and macrophages in areas of demyelination and inflammatory reaction. In the present case, we cannot determine whether the additional contribution of mefloquine improved the patient's clinical status because of an antiviral effect against JCV or an anti-inflammatory effect against IRIS. Accordingly, our observations here should be considered as preliminary findings only and require confirmation with a larger number of patients.

Although the clinical effectiveness of mefloquine combined with cART is controversial and remains to be proven, our case demonstrates its potential in eradicating JCV from the CSF and slowing clinical exacerbation.

4. Conclusion

The potent management of HIV infection is crucial because HIV-induced immune suppression can lead to rapid and widespread dissemination of JCV in the brain and subsequent demyelination. At that point, co-therapy with mefloquine may help with the early eradication of JCV from the CSF and contribute to neurological improvement.

Conflict of interest

The authors of this manuscript report no disclosures or conflicts of interest.

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Ethical approval

This study was approved by the National Defense Medical College Research Ethics Committee (reference Biyou-4).

Contributors

All authors were involved in discussion about diagnosis, care of the patient, and preparation of the report. Written consent to publish was obtained.

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RESEARCH ARTICLE Open Access

Detection of human herpesviruses in the cerebrospinal fluid from patients diagnosed with or suspected of having progressive multifocal leukoencephalopathy

Kazuo Nakamichi[1*], Naoki Inoue[1], Toshio Shimokawa[2], Ichiro Kurane[1], Chang-Kweng Lim[1] and Masayuki Saijo[1]

Abstract

Background: Progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease caused by JC virus (JCV), occurs mainly in immunocompromised patients. While JCV DNA is detected in the cerebrospinal fluid (CSF) from a certain proportion of patients suspected of having PML, JCV-negative patients may also develop brain lesions due to other infectious agents. This study assessed the prevalence of six herpesviruses in the CSF from patients diagnosed with or suspected of PML.

Methods: Two hundred and ninety-nine CSF specimens and clinical data were collected from 255 patients, including 31 confirmed PML cases. Quantitative PCR assays were carried out to detect the genomic DNA of JCV, herpes simplex virus (HSV), varicella-zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), and human herpesvirus 6 (HHV-6).

Results: Herpesvirus DNAs were detected in the CSF specimens from 29 of 255 patients (11.4%). HSV-1 and CMV were detected in JCV-negative patients, whereas VZV and EBV were detected in both CSF JCV-positive and -negative individuals. The herpesvirus-positive patients had underlying disorders that caused immunosuppression, such as HIV infection, congenital immunodeficiencies, and hematologic malignancies, and presented with neurologic symptoms and MRI lesions, mainly in the cerebral white matter. The median values of CSF cell counts and protein levels in the herpesvirus-positive patients were slightly higher than those in the PML patients.

Conclusions: The results demonstrate that herpesviruses are occasionally detected in the CSF from PML patients and immunocompromised individuals suspected of having PML. Thus, this study provides a significant basis for the diagnosis and treatment of neurological disorders in immunocompromised patients.

Keywords: Cerebrospinal fluid, Human herpesvirus, JC virus, Progressive multifocal leukoencephalopathy, Quantitative PCR testing

Background

Progressive multifocal leukoencephalopathy (PML) is a rare but fatal demyelinating disease of the central nervous system (CNS) caused by JC virus (JCV), a small DNA virus belonging to the family *Polyomaviridae*, genus *Polyomavirus* [1-3]. Humans are infected with JCV asymptomatically during childhood and are persistently infected with it throughout life. From 50–90% of adults have been reported to be serologically positive for JCV [1-4]. In some severely immunocompromised patients, JCV activates and causes a lytic infection in the oligodendrocytes, leading to PML [1-4]. Although PML is mainly diagnosed in patients with HIV-infection, it is also observed in patients with immunodeficiency due to a hematological malignancy, chemotherapy, transplantation, lymphocyte depletion, or autoimmune disorders, such as systemic lupus erythematosus, and in those under treatment with immunosuppressive agents [1,3,5]. In addition, PML has recently been identified in patients receiving immunomodulatory therapies with monoclonal

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antibodies, such as natalizumab, rituximab, and efalizumab [1-3,6].

The detection of JCV DNA in the cerebrospinal fluid (CSF) by PCR is a reliable and less-invasive diagnostic marker of PML, particularly when combined with typical magnetic resonance imaging (MRI) patterns [1,7]. CSF testing for JCV DNA using a quantitative PCR technique has become the current diagnostic standard [6]. In Japan, real-time PCR testing for JCV DNA in CSF specimens has been partly supported by the Laboratory of Neurovirology, Department of Virology 1, National Institute of Infectious Diseases (NIID), Tokyo, Japan, since 2007. The CSF from approximately 11% of patients (48 of 419) was found to be positive for JCV DNA and these patients were diagnosed with PML [8]. However, no JCV could be detected in the CSF samples from the remaining approximately 89% of patients, implying that a large proportion of these subjects might have developed brain disorders due to other infectious or non-infectious causes.

Herpesviruses, in particular, herpes simplex virus (HSV), varicella-zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), or human herpesvirus 6 (HHV-6), are major etiological agents of encephalitis and other CNS infections in immunocompromised persons [9-13]. This study sought to assess whether these herpesviruses contribute to the CNS involvement in patients diagnosed with or suspected of having PML.

Methods

Collection of CSF specimens and clinical data

The study was conducted under the approval from the Ethical Committee for Biomedical Science in the NIID (approval number 339). Informed consent from patients or their family members was also obtained. Patients suspected of having PML on the basis of neurological symptoms and/or MRI patterns were enrolled in this study. Upon request to the patients' physicians, CSF testing for JCVDNA was routinely performed regardless of patient age, gender, underlying disease, or medical history. Two hundred and ninety-nine CSF specimens were collected by lumbar puncture from 255 patients from April 2007 to the end of January 2010, immediately frozen, and then transferred to the NIID for PCR testing. For 44 of the patients, CSF testing was repeated during their follow-up period. Clinical data were collected from the patients' physicians through standardized questionnaires. The following data were analyzed: age, gender, underlying diseases, manifestations of neurologic symptoms, pattern of brain MRI lesions, and CSF leukocyte counts and total protein levels.

Real-time PCR testing for viral DNA in CSF specimens

Total DNAs were extracted from the CSF specimens using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) and then used as PCR templates as described previously [8,14,15]. Since most of the CSF specimens had been frozen at the hospitals, they also contained cellular components. Quantitative real-time PCR assays targeting the DNAs of JCV [8,14,15], VZV [16], CMV [17], and HHV-6 [17] were carried out as described in the earlier reports. HSV-1/2 and EBV DNAs were quantified using an artus HSV-1/2 LC PCR Kit (Qiagen) and LightCycler EBV Quantification Kit (Roche, Penzberg, Germany), respectively, according to the protocols supplied by the manufacturers.

Statistical analysis

The detection rates of herpesvirus DNAs in the CSF specimens and the sex ratios of patients in each group were statistically compared by means of a two-tailed Fisher's exact test. For multiple testing, the resulting *P*-value was corrected using the Benjamini-Hochberg method. Differences in the ages and CSF cell counts and protein contents between patient groups were compared by nonparametric analyses using the Mann–Whitney *U* test. All *P*-values less than 0.05 were judged to be statistically significant.

Results

Detection of JCV in CSF from patients suspected of having PML

The study population comprised 166 males and 89 females. The mean age of the subjects was 56.3 years (median 59.0 years, range 4–89 years, SD = 18.0), excluding 1 male patient whose age was not definitely stated. The underlying diseases of 255 subjects were as follows: HIV infection ($n = 52$, 50 males, 2 females), hematologic disorders ($n = 51$, 39 males, 12 females), autoimmune disorders ($n = 33$, 10 males, 23 females), other diseases ($n = 46$, 29 males, 17 females), and unknown ($n = 73$, 38 males, 35 females). A total of 299 CSF specimens from 255 patients were subjected to the real-time PCR assay for JCV DNA, and 42 samples (14%) were found to be positive for JCV DNA. The median JCV load in these specimens was 3.2×10^4 copies/mL (range $1.5 \times 10^2 - 4.8 \times 10^8$ copies/mL, SD = 7.8×10^7). The prevalence of JCV DNA and underlying diseases in the patient population are shown in Table 1. Thirty-one of 255 patients (12%) were diagnosed with PML based on clinical findings and JCV DNA-positive CSF. These PML patients had HIV infection (10 patients, 19%), hematologic disorders (13 patients, 26%), autoimmune disorders (3 patients, 9%), or other diseases (5 patients, 11%). No JCV DNA was detected in the CSF specimens from 73 patients who had no clinically apparent underlying disorders.

Detection of herpesvirus DNA in CSF from patients diagnosed with or suspected of having PML

The next series of analyses were conducted to clarify the etiological contribution of herpesviruses to CNS disease

Table 1 Prevalence of CSF JCV DNA and underlying diseases in the patient population

	No. (%) of patients		
	Total	JCV-positive	
Underlying disease	(*n* = 255)	(*n* = 31)	
HIV infection	52	10	(19.2)
Hematologic disease	51	13	(25.5)
Autoimmune disease	33	3	(9.1)
Other disease	46	5	(10.9)
Unknown	73	0	(0)

by the detection of herpesvirus DNA in 299 CSF specimens from 255 patients diagnosed with or suspected of having PML. Among the 299 CSF samples, 31 were positive for herpesvirus DNA (Table 2). HSV-1, VZV, CMV, and EBV were detected in 1 (0.3%), 8 (2.7%), 5 (1.7%), and 19 (6.4%) specimens, respectively. Two specimens were positive for CMV and either HSV-1 or EBV. No amplification signal was observed for HSV-2 and HHV-6 in any sample. HSV-1 and CMV were detected only in JCV-negative CSF specimens. In contrast, VZV and EBV were detected in both JCV-positive and -negative samples. The viral DNA level of HSV-1 in 1 specimen was 1.3×10^3 copies/mL. The median viral loads of VZV, CMV, and EBV were 3.3×10^2, 1.1×10^3, and 1.5×10^3 copies/mL, respectively (Figure 1). Although the DNA levels of these viruses in most specimens ranged from 10^2 to 10^4 copies/mL, more than 10^4 copies/mL of VZV, CMV, and EBV DNAs were found in some samples.

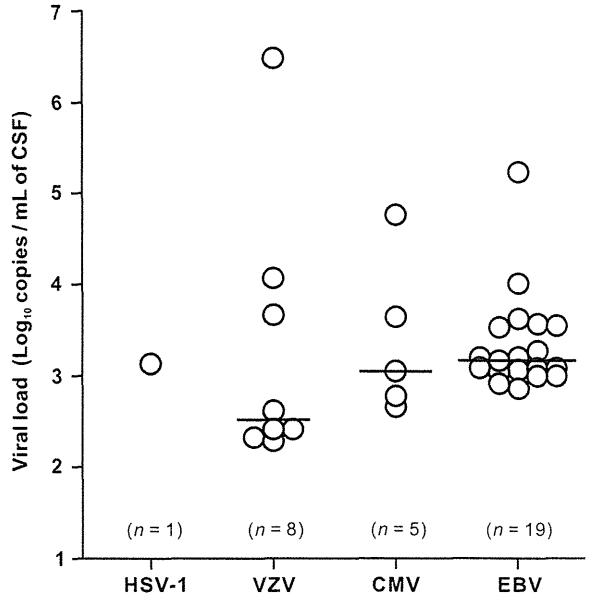

Figure 1 Viral loads of herpesvirus DNAs in the CSF specimens from patients. The CSF viral loads of HSV-1, VZV, CMV, and EBV are shown (left to right). Each open circle indicates the copy number of viral DNA in each sample, and the horizontal lines represent the medians.

Proportion of patients for whom CSF specimens were herpesvirus DNA positive

Table 3 shows the numbers and proportion of patients for whom the CSF specimens were positive for herpesvirus DNA. Among the 255 subjects, CSF herpesvirus DNA was detected in 29 (11%). HSV-1 and CMV were detected in CSF JCV-negative patients, while VZV and EBV were detected in JCV-negative and positive-patients. The detection rate of herpesvirus DNAs in JCV-positive patients (19%) was higher than that in JCV-negative individuals (10%), but this difference was not statistically significant. VZV was detected in 8 patients, from one of whom EBV-positive CSF specimens were obtained during the follow-up. Five patients were found to be positive for CMV, and HSV-1 and EBV were concomitantly detected in CMV-positive samples from 1 patient each. Sixteen individuals were found to be positive for EBV but not for other herpesviruses. The EBV-positive rate appeared to be higher than those of other herpesviruses. In addition, the detection rate of EBV in JCV-positive patients was statistically higher than that in JCV-negative patients ($P = 0.032$).

Table 2 Number of herpesvirus DNA-positive and -negative CSF specimens

	No. (%) of CSF specimens		
Herpesvirus	Total[a]	JCV-positive	JCV-negative
DNA	(*n* = 299)	(*n* = 42)	(*n* = 257)
HSV-1	1 (0.3)	0 (0)	1 (0.4)
HSV-2	0 (0)	0 (0)	0 (0)
VZV	8 (2.7)	1 (2.4)	7 (2.7)
CMV	5 (1.7)	0 (0)	5 (1.9)
HHV-6	0 (0)	0 (0)	0 (0)
EBV	19 (6.4)	5 (11.9)	14 (5.4)

[a]Two specimens were positive for CMV and either HSV-1 or EBV DNA.

Table 3 Number of patients in whom herpesvirus DNA was detected in the CSF

	No. (%) of patients		
Herpesvirus	Total	CSF JCV-positive	CSF JCV-negative
DNA in CSF	(*n* = 255)	(*n* = 31)	(*n* = 224)
Total	29 (11.4)	6 (19.4)	23 (10.3)
HSV-1 and CMV	1 (0.4)	0 (0)	1 (0.4)
VZV	7 (2.7)	1 (3.2)	6 (2.7)
VZV or EBV[a]	1 (0.4)	0 (0)	1 (0.4)
CMV	3 (1.2)	0 (0)	3 (1.3)
CMV and EBV	1 (0.4)	0 (0)	1 (0.4)
EBV	16 (6.3)	5 (16.1)	11 (4.9)

[a]VZV and EBV DNA was detected in different CSF specimens collected from the same patient during the follow-up period.

Underlying diseases of herpesvirus DNA-positive patients

The clinical data of 29 herpesvirus DNA-positive patients were analyzed. The patients comprised 23 males and 6 females. The mean age of all except 1 patient was 52.5 years (median 53.5 years, range 30–84 years, SD = 14.7). There were no statistically significant differences in the age and sex ratios between the herpesvirus DNA-positive and -negative patients. The underlying diseases of the patients who provided herpesvirus DNA-positive CSF specimens are summarized in Table 4. Of the 29 patients, 27 (93%) were found to have underlying disorders that may cause immunosuppression. Sixteen patients (55%) had HIV infection, and the severe loss of peripheral blood CD4-positive T cells was observed in most cases. VZV, CMV, and/or EBV were detected in CSF from the HIV-positive patients. Eight patients (28%) suffered from hematologic diseases, such as non-Hodgkin's lymphoma and aplastic anemia, and had been treated with hematopoietic stem cell transplantation, combination chemotherapy, or other immunosuppressive drugs. Three patients (10%) had other underlying diseases, such as lupus nephritis, chronic renal failure, or primary angiitis of the CNS, and received immunosuppressive therapy. Among the subjects in each group, the proportion of the herpesvirus DNA-positive patients with HIV infection (31%) and that with hematologic diseases (16%) were statistically higher than that with other underlying diseases (4%), as compared by multiple statistical testing ($P = 0.000$ and 0.048, respectively). In addition, both JCV and herpesvirus DNAs were detected in the CSF specimens from 6 patients with either HIV infection or hematologic diseases.

Clinical features of herpesvirus DNA-positive patients

In the final set of analyses, the clinical features of the patients with herpesvirus-positive CSF were compared to those of PML patients. Table 5 shows the appearance patterns of neurologic symptoms and brain lesions. In the study population, 25 patients were positive for JCV but provided herpesvirus-negative CSF specimens. These PML patients presented with diverse neurologic symptoms, such as paralysis, dementia, dysarthria, dysphagia, and/or visual impairment (data not shown). MRI lesions were found mainly in the cerebral white matter (CWM) (84%), and a smaller proportion of patients showed lesions in other sites, such as the cerebellum (16%) and brain stem (28%). Among the 24 patients in whom herpesviruses were detected in the CSF, 20 individuals (83%) had neurologic manifestations. MRI lesions were identified in the CWM (75%), cerebellum (29%), brain stem (8%), and other sites (13%). There were no statistically significant differences in the proportion of individuals with lesions at each site between the PML and herpesvirus-positive patients. The VZV- or EBV-positive patients displayed lesions not only in the CWM, but also in other areas of the brain. In contrast, the lesions were localized in the CWM in the HSV-1- and/or CMV-positive patients. Figure 2 shows the results of CSF cell counts and total protein contents of the PML and CSF herpesvirus-positive patients. Since the CSF cell counts and/or total protein contents were not defined in the questionnaires in some cases, the numbers of patients are not identical in Figure 2A and 2B. In both patient groups, the median values of cell counts for both mono- and polynuclear cells (Figure 2A) and protein contents (Figure 2B) were at normal or near-normal levels. However, a considerable proportion of herpesvirus-positive patients exhibited higher cell numbers and protein levels when compared to those of PML patients, and these differences were statistically significant ($P = 0.049$ and 0.004, respectively).

Discussion

The present study aimed to comprehensively assess the prevalence of six human herpesviruses in the CSF specimens from patients diagnosed with or suspected of having PML. Since the aim of this study was the detection of herpesviruses DNA using PCR from the CSF samples of the patients diagnosed with or suspected PML associated with several immuno-suppressive underlying diseases, the

Table 4 Underlying diseases of patients in whom herpesvirus DNA was detected in the CSF

Herpesvirus DNA in CSF	No. of patients	Underlying disease (%)							
		HIV infection		Hematologic disease		Other disease[a]		Unknown	
Total	29	16	(55.2)	8	(27.6)	3	(10.3)	2	(6.9)
HSV-1 and CMV	1	0	(0)	1	(100)	0	(0)	0	(0)
VZV	7	2[b]	(28.6)	3	(42.9)	2	(28.6)	0	(0)
VZV or EBV	1	1	(100)	0	(0)	0	(0)	0	(0)
CMV	3	3	(100)	0	(0)	0	(0)	0	(0)
CMV and EBV	1	0	(0)	0	(0)	1	(100)	0	(0)
EBV	16	10[c]	(62.5)	4[d]	(25.0)	0	(0)	2	(12.5)

[a]The data include patients with autoimmune disorders.
[b]One patient was positive for JCV.
[c,d]The results include 2 and 3 JCV-positive patients, respectively.

Table 5 Neurologic symptoms and brain MRI patterns in the PML and herpesvirus-positive patient groups

Detected Viral DNA in CSF	No. of patients	Neurologic symptom (%)	MRI(T2/FLAIR) lesion (%)[a]				
			Cerebral white matter	Cerebellum	Brain stem	Other	Unknown
JCV[b]	25	25 (100)	21 (84.0)	4 (16.0)	7 (28.0)	2 (8.0)	2 (8.0)
Herpesviruses[c]	24	20 (83.3)	18 (75.0)	7 (29.2)	2 (8.3)	3 (12.5)	1 (4.2)
HSV-1 and CMV	1	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
VZV	6	6 (100)	5 (83.3)	1 (16.7)	1 (16.7)	0 (0)	1 (16.7)
VZV or EBV	1	1 (100)	1 (100)	1 (100)	0 (0)	1 (100)	0 (0)
CMV	3	2 (66.7)	3 (100)	0 (0)	0 (0)	0 (0)	0 (0)
CMV and EBV	1	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
EBV	12	9 (75.0)	7 (58.3)	5 (41.7)	1 (8.3)	2 (16.7)	0 (0)

[a]The data include patients with multiple lesion sites.
[b]The patients were negative for herpesvirus DNA in their CSF.
[c]The patients were negative for JCV DNA in their CSF.

detection of virus DNA indicates the existence of these viruses, but the data do not always indicate the main contribution to brain damage.

One of the most important findings is that EBV was present in the CSF of approximately 16% of the confirmed PML patients. Although the detection of EBV as well as JCV in the CSF has been reported previously [18-20], the prevalence of EBV in the CSF specimens from the relatively large number of patients suspected of having PML has not been determined previously. This data suggest that it is not rare to concomitantly detect JCV and EBV in PML cases. Although the EBV-positive patients had not been diagnosed with EBV-related diseases when the specimens were collected, it would be interesting to see whether these patients later developed EBV-associated neurological disorders. It would be attractive to hypothesize that EBV infection is involved in the progression of PML progression as the detection rate of EBV in the CSF from JCV-positive patients was higher than that in JCV-negative patients. However, this result might be due to differences in the proportion of immunocompromised individuals between the JCV-negative and -positive populations. VZV was detected in the CSF from one PML patient with AIDS (CD4 cell count, 8 cells/μl). The detection rate of VZV appeared to be lower than that of EBV, and the co-detection of JCV and VZV in CSF was reported in one previous report [20]. It is possible that this patient developed both PML and VZV encephalitis.

It is of interest to note that HSV-1/2, CMV, and HHV-6 were not detected in the CSF specimens from any PML patient. Two previous reports demonstrated that JCV was concomitantly detected with CMV or HSV-1 in the CSF [20,21]. The data obtained in this study indicate that the CSF from some PML patients was HSV-1 and/or CMV DNA-positive, although the prevalence was low. Another important finding in this study is that

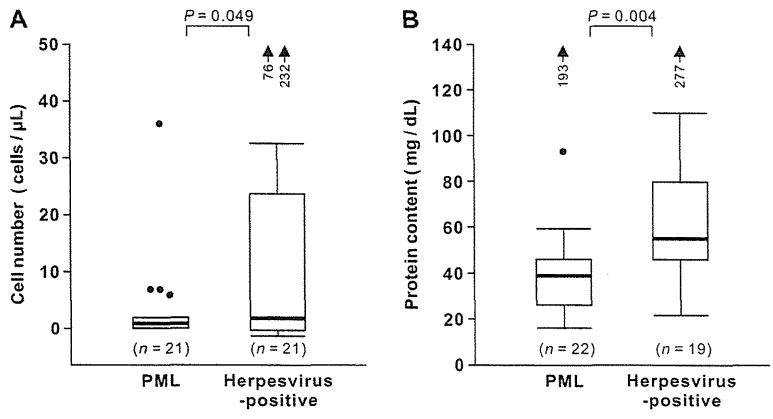

Figure 2 CSF cell counts (A) and total protein contents (B) in PML and herpesvirus-positive patients. Since the CSF cell counts and/or total protein contents were not defined in the questionnaires in some cases, the numbers of patients are not identical in panels **A** and **B**. The data of patients positive for both CSF JCV and herpesvirus DNAs are not included in each panel. In box-and-whisker plots, the thick horizontal line within each box is the median; the lower and upper boundaries are the 25th and 75th percentiles, respectively; vertical whiskers extend over the range; and dots and arrows show outliers.

HSV-1, VZV, CMV, and EBV were detected in more than 10% of the CSF specimens from patients suspected of having but not diagnosed with PML. Based on neurological symptoms, MRI lesion patterns, and underlying disease, it seems reasonable that these patients were suspected of having PML. A large proportion of the patients that were found to be positive for herpesviruses had HIV infection or hematologic disorders, suggesting that there is a significant relationship between the presence of herpesviruses in the CSF and severe immunosuppression due to AIDS, chemotherapy, or hematopoietic stem cell transplantation. As the distribution of CSF cell numbers and protein contents partially overlapped in the PML and herpesvirus-positive patients, these parameters may not directly contribute to a diagnosis of PML. It is likely that the inflammatory response was inhibited under the immunosuppressive conditions in both patient groups. However, it is worth focusing on the significant proportion of the herpesvirus-positive patients showing high CSF cell counts. In these patients, it can be postulated that the brain inflammation was induced by the lytic herpesvirus infection. In such a situation, the amount of herpesvirus DNA might be increased by the migration of the infected cells into the CSF, as the PCR assays were performed using total DNA extracted from frozen CSF.

In some herpesvirus-positive cases, a combination of two herpesviruses, such as CMV and HSV-1, CMV and EBV, and VZV and EBV, were detected in the CSF, which is consistent with previous reports describing the co-detection of herpesviruses in CSF [18,20,22,23]. It was also observed that VZV and EBV were detected in different CSF specimens from one patient during the follow-up period. This patient presented with lesions in the CWM and basal ganglia, at which time VZV was detected. At repeat CSF testing 3 months later, EBV, but not VZV, was detected in the CSF, and lesions were identified in the CWM and cerebellum. This observation indicates that although VZV propagation in the CNS was reduced, EBV infection or reactivation occurred during the follow-up period in this patient.

Currently, no specific treatment has been established for PML. Restoration of the immune system, either by combination antiretroviral therapy for patients with AIDS or by moderating the immunosuppressive therapies for non-AIDS patients, is the only treatment option for the management of PML, although several experimental treatments are being investigated [1]. In contrast, acyclovir is effective in the treatment of encephalitis caused by HSV or VZV [24]. It is also known that ganciclovir and foscarnet are beneficial for patients with CMV-related encephalitis [24]. Thus, the present data suggest that comprehensive testing for these herpesviruses as well as JCV is important for early diagnosis and proper management of patients suspected of having PML.

Conclusions

In summary, as herpesviruses can contribute to CNS disorders in a significant proportion of patients suspected of having PML, comprehensive testing for the herpesviruses as well as for JCV is required for the accurate diagnosis and treatment of CNS diseases in patients diagnosed with or suspected of having PML.

Abbreviations

PML: Progressive multifocal leukoencephalopathy; CNS: Central nervous system; JCV: JC virus; CSF: Cerebrospinal fluid; HSV: Herpes simplex virus; VZV: Varicella-zoster virus; CMV: Cytomegalovirus; EBV: Epstein-Barr virus; HHV-6: Human herpesvirus 6; MRI: Magnetic resonance imaging; NIID: National Institute of Infectious Diseases; CWM: Cerebral white matter.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KN and MS conceived of the study. KN and NI carried out real-time PCR testing, and KN created the database of patients. KN analyzed the clinical data and drafted the manuscript. TS performed the statistical analyses. MS, NI, TS, IK, and CKL participated in the study design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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TNF-α Stimulates Efficient JC Virus Replication in Neuroblastoma Cells

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JC polyomavirus (JCV) causes progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system (CNS) in immunocompromised patients, and particularly in the severe immunosuppression associated with acquired immunodeficiency syndrome (AIDS). HIV-1 can lead to the production of tumor necrosis factor-alpha (TNF-α) in the CNS. Our aim was to examine the effects of TNF-α on JCV gene expression and replication using a human neuroblastoma cell line, IMR-32, transfected with JCV DNA, M1-IMRb. Quantitative RT-PCR analysis of JCV large T antigen and VP1 mRNA, the viral DNA replication assay, and the DNase protection assay were carried out. TNF-α treatment of IMR-32 cells transfected with JCV DNA induced large T antigen mRNA and JCV DNA replication, while other effects on VP1 mRNA expression and virus production were marginal. In addition, ELISA analysis of the nuclear p65 subunit of nuclear factor κB (NF-κB), which is a hallmark of NF-κB pathway activation, of IMR-32 cells upon TNF-α treatment showed that TNF-α treatment activated the NF-κB pathway in IMR-32 cells. Taken together, our results suggest that TNF-α stimulation could induce JCV replication associated with the induction of JCV large T antigen mRNA through the NF-κB pathway in IMR-32 cells transfected with JCV DNA. Our findings may contribute to further understanding of the pathogenesis of AIDS-related PML. ***J. Med. Virol. 86:2026–2032, 2014.*** © 2014 Wiley Periodicals, Inc.

KEY WORDS: TNF-α; T antigen expression; IMR-32; NF-κB; DNA replication

INTRODUCTION

JC polyomavirus (JCV) causes progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system (CNS) in immunocompromised patients [Major et al., 1992]. Demyelination in the brain of PML patients is caused by the destruction of oligodendrocytes, the myelin-producing cells of the CNS [Major et al., 1992], which are preferentially infected by JCV in the human brain. The pathological features of PML are the presence of cytolytic oligodendrocytes with giant nuclei, and unusual astrocytes with hyperchromatic nuclei. However, a previous report also demonstrated the ability of JCV to infect neurons and macrophages in the CNS [Major et al., 1992]. JCV propagates in the CNS, in particular, leading to PML in patients with severe immunosuppression, such as that associated with acquired immunodeficiency syndrome (AIDS).

In general, JCV persists in a latent state, in which viral protein expression and replication are not detectable after primary infection [Hou and Major,

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2000; Khalili et al., 2006]. However, HIV-1 can lead to the production of tumor necrosis factor-alpha (TNF-α) in the CNS [Benveniste, 1994; Yeung et al., 1995; Kaul et al., 2005]. It has been indicated that TNF-α stimulates JCV transcription in both the early and late phases of infection via nuclear factor κB (NF-κB) in a human oligodendroglioma cell line [Wollebo et al., 2011]. NF-κB, a transcriptional factor, is induced by TNF-α. However, it has also been reported that TNF-α did not stimulate JCV transcription or multiplication in human fetal glial cells [Atwood et al., 1995]. Thus, it remains unclear whether TNF-α stimulates JCV transcription and replication. A few cell lines are susceptible to JCV propagation. Previous studies with the human neuroblastoma cell line IMR-32 revealed that JCV was produced by serial passage, because three JCV (designated M1-IMRa, M1-IMRb, and M1-IMRc) had altered regulatory regions that were involved in adaptation. After transfection, M1-IMRb showed the highest virus replication in IMR-32 cells among the three adapted clones [Yogo et al., 1993]. Thus, IMR-32 cells transfected with M1-IMRb are a useful tool for studying the role of TNF-α in JCV replication.

In this study, the effects of TNF-α on the expression of JCV large T and VP1 genes in neuroblastoma IMR-32 cells were analyzed using a real-time RT-PCR assay, and viral genome replication was analyzed using a DNA replication assay. Moreover, JCV production in IMR-32 cells transfected with M1-IMRb was determined using real-time PCR analysis combined with DNase treatment. In addition, the effect of TNF-α on nuclear translocation of the p65 subunit of NF-κB in IMR-32 cells was determined.

MATERIALS AND METHODS

Cell Lines, Plasmids, and Cytokines

IMR-32 cells were obtained from DS-Pharma Biomedical (Osaka, Japan). IMR-32 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin [Nukuzuma et al., 1995]. The plasmid M1-IMRb, derived from IMR-32-adapted JCV, was kindly provided by Dr. Yoshiaki Yogo. TNF-α was obtained from Sigma–Aldrich (St. Louis, MO), and dissolved in a small volume of distilled water.

Quantitation of Cell Proliferation

Cytotoxicity was measured using a Cell Proliferation Kit I (MTT; Roche, Indianapolis, IN) as described in previous studies [Nukuzuma et al., 2012, 2013]. IMR-32 cells in 100 μl medium were plated at a concentration of 5.4×10^4 cells/well into 96-well microtiter plates and incubated in the absence or presence of TNF-α at a final concentration of 25 ng/ml for 3 days at 37°C in a CO_2 incubator, following by the addition of 10 μl (final concentration 0.5 mg/ml) of the MTT labeling reagent to each well. The microtiter plates were incubated for 4 hr. A solubilization solution (100 μl) was added to each well and left to stand overnight in the incubator. The solubilized formazan product was spectrophotometrically quantified using an ELISA reader (Bio-Rad, Hercules, CA) at a wavelength of 550 nm. The reference wavelength was 650 nm.

JCV DNA Transfection

JCV DNA transfection was carried out essentially as described previously [Nukuzuma et al., 2012, 2013]. IMR-32 cells were cultured in 35-mm dishes containing 2 ml complete growth medium (DMEM-10% FBS) until they reached 70–80% confluency. An M1-IMRb clone was used for the DNA replication assay as M1-IMRb showed the highest HA activity in IMR-32 cells [Yogo et al., 1993]. For transfection, 1.0 μg viral DNA, excised from the recombinant plasmid with *Eco*RI, was introduced into the cells using FuGENE 6 transfection reagent (Roche). Endonuclease-cleaved linear viral DNA can be re-circularized after transfection [Yogo et al., 1993].

Real-Time RT-PCR for Expression of the JCV Large T or VP1 Antigen

In order to examine the stimulation of large T or VP1 gene expression in TNF-α-treated IMR-32 cells, a real-time RT-PCR assay was conducted. IMR-32 cells transfected with JCV genome DNA were incubated in culture medium in the absence or presence TNF-α at a final concentration of 25 ng/ml. At 48 hr after TNF-α treatment, the cells were harvested, and total RNA was extracted from three cell cultures using an RNeasy Mini Kit (Qiagen, Hilden, Germany). One microgram of RNA was treated with DNase I and then introduced into the RT reaction as follows. DNase I-treated RNA was mixed with 40 μl of a reaction mixture containing RT buffer (Toyobo, Osaka, Japan), 1 mM dNTPs, 40 U RNase inhibitor (Toyobo), 200 U ReverTra Ace, as a reverse transcriptase (Toyobo), and 10 pmol oligonucleotide dT_{15} (Roche). The RT mixture was incubated for 10 min at 30°C, 60 min at 42°C, heated to 99°C for 5 min, and then added to the real-time PCR mixture.

For real-time RT-PCR analysis of large T and VP1 gene expression, two sets of PCR primers and TaqMan probes were used according to previous reports [McNees et al., 2005; Nukuzuma et al., 2009]. Real-time PCR analyses were performed in a total volume of 25 μl of 2× TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA), 300 nM of each primer, 200 nM TaqMan probe and 5 μl RT product. For the absolute quantification of T and VP1 antigen copies, plasmids containing target sequences were serially diluted from 10^2 to 10^6 copies per reaction and then used as standard DNA templates. Real-time PCR amplification was carried out using an ABI PRISM 7900HI system (Applied Biosystems).

The amplification conditions were as follows: 2 min at 60°C, 10 min at 95°C, 40 cycles of 95°C for 15 s, and 60°C for 15 s. As an endogenous reference, the copy number of the β-actin gene, a housekeeping gene, in each RNA extract was determined by real-time RT-PCR using TaqMan β-actin Control Reagents (Applied Biosystems). The copy number of the T antigen and VP1 mRNA in each sample was normalized against β-actin mRNA copies.

DNA Replication Assay

The DNA replication assay was carried out essentially as described previously [Nukuzuma et al., 2012, 2013]. At 48 hr after TNF-α treatment, the cells were harvested, and low-molecular-weight DNA was extracted from cells according to the Hirt procedure [Hirt, 1967]. The DNA (1 μg) derived from the transfected cells was digested with *Dpn* I and *Bam* HI. The resulting fragments were separated by electrophoresis on a 1.0% agarose gel, transferred onto a nylon membrane (Roche), and hybridized at 65°C in an incubator to digoxigenin-labeled JCV DNA using a DIG DNA Labeling Kit (Roche). Digoxigenin-labeled DNA Molecular Weight Marker was used as the size marker on the gel. Replicated DNA was detected using a DIG Luminescent Detection Kit (Roche), and exposed to film overnight. The exposed X-ray films were then scanned and the intensities of the bands were quantified using ImageJ (National Institutes of Health, Bethesda, MD, http://rsbweb.nih.gov/nih-image/). Relative band intensities were normalized against the intensities of the input DNA and background. The relative intensities were presented with reference to the lowest detectable band intensities by assigning them a value of 1.

DNase Protection Assay of JCV Replication in Transfected IMR-32 Cells

For the DNase protection assay, IMR-32 cells were untreated or treated TNF-α in 25 cm^2 flasks and were resuspended in 250 μl phosphate-buffered saline (pH 7.15) containing 0.2% bovine serum albumin (BSA) and subjected to freeze-thawing three times. The cell lysates were centrifuged at 1,500 rpm for 10 min at 4°C. The resultant supernatants were applied to real-time PCR assay combined with DNase treatment. To eliminate transfected JCV DNA from the cell extracts, 4 μl sample was mixed with 16 μl reaction mixture containing 2U Baseline-Zero DNase and Baseline-Zero buffer (AR Brown, Madison, WI) and incubated for 60 min at 37°C. Next, 15 μl reaction sample was mixed with an equal volume of DNAzol Direct reagent (Molecular Research Center, Cincinnati, OH) and incubated for 10 min at 80°C to inactivate DNase. Then, samples were directly subjected to real-time PCR analysis of the large T antigen [McNees et al., 2005] without any DNA extraction protocol, essentially as described in our previous report [Nakamichi et al., 2011].

Analysis of NF-κB Activation

The activation of the NF-κB pathway was assessed using a NF-κB/p65 ActivELISA Kit (Imgenex, San Diego, CA) according to the manufacturer's instructions. This kit measures the amount of p65 subunit of NF-κB translocated into the nuclei during activation of the NF-κB pathway. IMR-32 cells in 2 ml medium were plated into six-wells plates at a concentration of 8.0×10^5 cells/well and incubated in the absence or presence of TNF-α at a final concentration of 25 ng/ml for 48 hr. The nuclear fraction was extracted according to the lysate preparation manual, and 100 μl (50 μg protein) of each nuclear fraction were then transferred to the wells of an anti-p65 antibody-coated microtiter plate. Next, an anti-p65 antibody, alkaline phosphate-conjugated secondary antibody, and NPP substrate were added to generate a colorimetric signal. The absorbance of the colorimetric signal was measured at a wavelength of 415 nm using a BIO-RAD Model 3550 Microplate Reader (Bio-Rad). For the absolute quantification of p65, the recombinant p65 protein was diluted serially from 15.6 to 1,000 ng/ml per reaction and used as the standard curve.

Statistical Analysis

The significance of intergroup differences was determined using a statistical program and Student's *t*-test.

RESULTS

TNF-α Does Not Induce Any Cytotoxic Effect on IMR-32 Cells

The relative number of live cells was determined by measuring mitochondrial succinate dehydrogenase activity using an MTT assay. The absorbance of formazan products from TNF-α-untreated and -treated cells was 0.711 ± 0.003 and 0.697 ± 0.004, respectively. The enzyme activity of TNF-α-treated IMR-32 cells was slightly lower than that of TNF-α-untreated control cells, but this difference was not statistically significant. This result indicates that TNF-α does not induce a cytotoxic effect on IMR-32 cells under the assay conditions.

TNF-α Stimulates JCV T Antigen Gene Expression in IMR-32 Cells

IMR-32 cells were transfected with JCV genome DNA and incubated in the absence or presence of TNF-α. The expression levels of the JCV large T antigen and VP1 genes were determined by real-time RT-PCR, as described in section Materials and Methods. The mRNA of the large T and VP1 genes from three cultures was quantitatively amplified by real-time RT-PCR. The expression level of the large T antigen gene in TNF-α-treated IMR-32 cells was 6.2-fold higher than that in TNF-α-untreated control cells (Fig. 1), and this difference was statistically

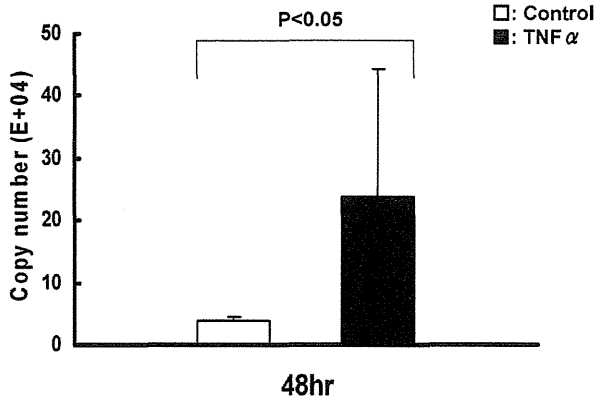

Fig. 1. Stimulation of JCV large T antigen gene expression in IMR-32 cells by TNF-α. The expression levels of the large T antigen gene from three cultures were measured by real-time RT-PCR after incubation for 48 hr in the absence or presence of TNF-α. The linear standard curve was generated from serial dilution with the plasmid M1-IMRb. The amount of large T antigen mRNA in each sample was normalized with reference to the copy numbers of human β-actin mRNA. Data are shown as the mean ± standard deviation of the means. $^*P < 0.05$ (Student's *t*-test).

significant ($P < 0.05$). On the other hand, the expression of the VP1 antigen in TNF-α treated IMR-32 cells was only 2.0-fold greater than that in the control cells (Fig. 2), and this difference was not statistically significant. These results indicate that TNF-α preferentially induces the expression of the large T antigen gene in IMR-32 cells.

TNF-α Stimulates JCV Replication in IMR-32 Cells

IMR-32 cells were harvested at 48-hr post-transfection with JCV genome DNA, and the replicated JCV

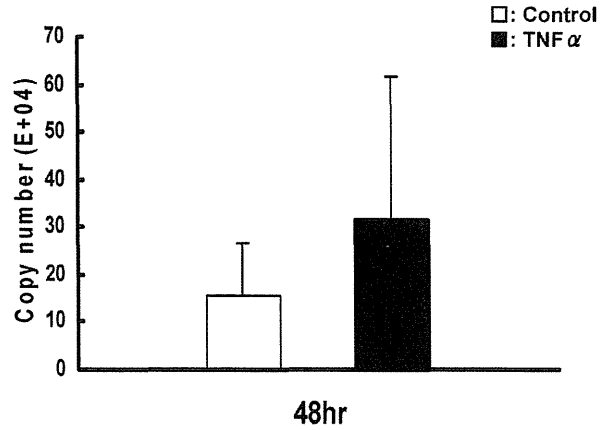

Fig. 2. TNF-α-induced expression of JCV VP1 antigen in IMR-32 cells. The expression levels of the JCV VP1 gene from three cultures were measured by real-time PCR after incubation for 48 hr in the absence or presence of NF-α. The linear standard curve was generated from serial dilution with the plasmid containing the VP1 gene. The amount of VP1 mRNA in each sample was normalized with reference to copy numbers of human β-actin mRNA. Data are shown as the mean ± standard deviation of the means.

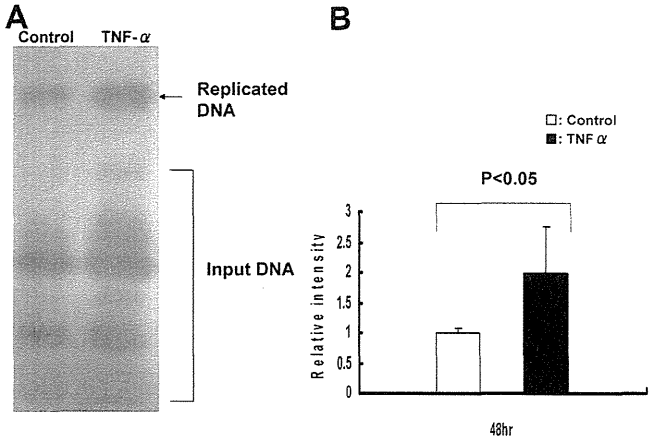

Fig. 3. Stimulation of JCV replication by TNF-α in IMR-32 cells. **A**: IMR-32 cells were transfected with 1.0 μg M1-IMRb and incubated in culture medium in the absence (Control) or presence (TNF-α) of TNF-α. Position of bands representing replicated (5.1 kb, *Dpn* I-resistant) and input (various fragments, *Dpn* I-sensitive) DNA are shown. **B**: The intensities of *Dpn* I-resistant bands were determined as described in Methods section. The columns of the histogram were derived from an analysis of the intensities of the replicated DNAs (*Dpn* I-resistant bands) from three independent experiments using ImageJ. Relative band intensities were normalized against the intensities of the input DNA and background. The relative intensities were presented with reference to the lowest detectable band intensities (Control, 48 hr) by assigning them a value of 1. Data are shown as the mean ± standard deviation of the means. $^*P < 0.05$ (Student's *t*-test).

DNA was detected as described in the Methods section. In TNF-α-treated IMR-32 cells, replicated viral DNA was clearly detected 48-hr post-transfection (Fig. 3A). The columns of the histogram were derived from the densitometry of *Dpn* I-resistant bands from *Bam* HI-digested low-molecular-weight DNA extracted from IMR-32 cells transfected with JCV genome DNA (Fig. 3B). The relative intensities are presented with reference to the lowest detectable band intensities (Control, 48 hr) by assigning the lowest intensities a volume of 1. It can be seen that the relative intensities of JVC genome DNA in TNF-α-treated cells were 2.0-fold greater than those in control cells (Fig. 3B), and this difference was statistically significant ($P < 0.05$). These results indicate that TNF-α stimulates JCV replication in IMR-32 cells.

TNF-α Stimulates JCV Production in IMR-32 Cells

JCV production in TNF-α-treated or -untreated IMR-32 cells was compared using real-time PCR analysis combined with DNase treatment, designated here as a DNase protection assay. Transfected IMR-32 cells were harvested on day 21 after treatment with TNF-α. Extravirion DNAs derived from transfected JCV genome were eliminated with a potent DNase, and the amount of JCV DNA from DNase-protected particles was determined. Under the