CONCISE COMMUNICATIONS

Quantification of JC Virus DNA in the Cerebrospinal Fluid of Patients with Human Immunodeficiency Virus–Associated Progressive Multifocal Leukoencephalopathy—A Longitudinal Study

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In progressive multifocal leukoencephalopathy (PML) the JC virus (JCV) load in the cerebrospinal fluid (CSF) is discussed as a parameter for disease progression. To investigate the evolution of viral shedding into the CSF, the JCV DNA concentration was quantified by competitive polymerase chain reaction (PCR) in multiple CSF samples from prior to and during an unsuccessful intrathecal salvage therapy in 2 human immunodeficiency virus–infected patients with biopsy-proven PML. With continuous clinical progression the virus load varied considerably intra- and interindividually, ranging from nondetectable to \(1.2 \times 10^7\) genome equivalents/10 \(\mu\)L CSF. Whereas an overall increase during progressive disease was confirmed, the virus burden was either constant or fluctuated irregularly during the intermediate stage of disease. This shows a variability of viral shedding during active disease that must be taken into account when the JCV load is measured by quantitative PCR for both the diagnosis of PML and monitoring under investigational treatment.

Progressive multifocal leukoencephalopathy (PML) is a demyelinating disease of the central nervous system (CNS) leading to death within months after first presentation [1, 2]. Diagnosis usually relies on clinical grounds, neuroimaging, and demonstration of JC virus (JCV) DNA in the cerebrospinal fluid (CSF) by polymerase chain reaction (PCR) [3]. The diagnostic sensitivity and specificity of PCR have been documented in a large number of patients [4]. However, it was shown that, in single inhibitor-free CSF samples from patients with biopsy-proven and clinically deteriorating PML, virus may be undetectable by standard PCR [5–7] and, conversely, that low JCV DNA concentrations may be found in the CSF of human immunodeficiency virus (HIV)–infected patients without PML [6, 7]. Thus, PCR alone does not reliably differentiate activated subclinical infection from clinically overt disease, and the question remains open whether this phenomenon is limited to early-stage disease or might also happen in advanced PML stages.

In viral infections of the CNS, virus concentration in the CSF correlates with the extent of disease and the effect of antiviral therapy [8]. In PML the amount of JCV DNA in CSF was determined by semiquantitative PCR in a limited number of patients [4, 9, 10] and was reported to correlate with the clinical state. However, because of the small number of samples taken and the limitations of the semiquantitative test systems, it remained open whether the CSF JCV load correlates with the evolution of disease. Here we report on the correlation of the clinical outcome and the development of the JCV load in serial CSF samples during salvage treatment of 2 patients with PML and HIV infection.

Material and Methods

Patients. Two HIV-infected homosexual men aged 33 and 32 years (patients A and B), at CDC/WHO stages B3 and C3, respectively, presented with motor aphasia and right hemiparesis. PML was diagnosed by magnetic resonance imaging findings, JCV PCR on CSF, and brain biopsy. CD4+ lymphocytes were depleted to 40 cells/\(\mu\)L. In patient A, PML was the AIDS-defining illness, whereas patient B had had Pneumocystis carinii pneumonia. CSF findings were unremarkable, with no evidence for conditions other than PML.

The patients received Ara-C intravenously for 5 days in a 3-week cycle and intrathecally [11] received 40 mg weekly. Interferon (IFN)–\(\alpha\) was given subcutaneously, 3 \(\times 10^7\) U 3 times a week, and
by intrathecal injection, $1 \times 10^6$ U twice a week. Immunoglobulin (Ig) was given intrathecally twice weekly by application of a volume of 35 mL in total by lumbar puncture after removal of the same volume of CSF. Zidovudine was given either prior to (patient A) or concomitantly with (patient B) this regimen.

**Standard and competitive PCR techniques.** CSF was sampled by lumbar puncture prior to drug injection and stored at 70°C. HIV RNA concentration of samples 9, 11, and 13 was quantified to be 1160, 315, and 25 copies/mL, respectively, with the ultrasensitive version of the Roche Amplicor HIV-1 monitor assay (Roche Diagnostics, Mannheim, Germany).

JCV DNA was amplified by standard and nested PCR, with control-region primers JC71/72 and JC53/61 and late-coding primers JC119/121, as described elsewhere [12, 13]. Sensitivity of the primers was in the range 0.1–10 fg JCV DNA or 20–2000 genome equivalents (GE), respectively. For the analysis of inhibiting factors, each CSF sample was amplified at a 10-μL volume with 10-fold dilutions ranging from 1 to 10 fg competitor DNA. Clinical samples were subjected to competition with the primers 119/121 and an internal dilution series of competitive template DNA (pJC/Vdel-1) carrying a 201-bp deletion within the genomic target sequence. Electrophoretic separation revealed a band pattern consisting of the native JCV product (900 bp) in dilutions containing predominantly genomic DNA, the competitor DNA (700 bp) in absence of native JCV DNA, and a pattern with both product bands. This represented the zone of equivalent concentrations of genomic and competitive DNA [12, 13]. All reactions were performed in parallel with negative and positive controls, and standard techniques were applied to prevent contamination. Reproducibility of the results was assessed by at least 3 independent experiments.

**Results**

*Treatment and clinical course.* Patient A received 25 intrathecal applications in a 3.5-month period during which he worsened by developing complete motor aphasia, right hemiplegia, and left hemiparesis. When treatment was discontinued there was severe psychomotor slowing, spastic tetraparesis, pseudobulbar palsy, and oculomotor slowing. He died 8 weeks after termination of therapy (i.e., 5.5 months after diagnosis of PML). Patient B received 13 intrathecal drug applications over 6 weeks. He continuously deteriorated at a pace similar to the pretreatment period and developed right hemiplegia, dementia, and cortical blindness. Treatment was terminated, and he died from PML 4 weeks thereafter (i.e., 2.5 months after diagnosis of PML). In both patients, fever and drowsiness followed the intrathecal administration of Ara-C, whereas IFN-α and intrathecal Ig were well tolerated.

**Quantification of JCV DNA in CSF.** The JCV burden was determined by competitive PCR in 18 samples over 18 weeks in patient A (figure 1) and 9 samples over 8 weeks in patient B (figure 2). In both patients, the first samples were taken prior to the decision-making period prior to discontinuation of therapy, samples were collected twice a week. Presence of JCV DNA was examined by a qualitative standard PCR at a sensitivity of 0.1 fg DNA.

In patient A, the concentration of viral target DNA was too low to be detected by PCR in the first 4 samples (1–4) taken in the period of 8–16 weeks after first presentation (figure 1A). With the exception of sample 11, in all the subsequent samples, product bands of varying density were observed. The presence of inhibitory substances in the CSF samples was ruled out by PCR with internal competitor DNA, revealing that either no or significantly less viral DNA was present in PCR-negative samples. Quadruple analysis of sample 11 revealed the presence of JCV DNA in 1 of 4 parallel reactions, pointing to a low JCV DNA concentration. This was confirmed by the more sensitive nested PCR with internal primers, which showed the presence of JCV-specific DNA products in all CSF samples found to be negative in the standard reaction.

Quantification of the virus load in the CSF of patient A by competitive PCR revealed increasing JCV DNA concentrations within 2 weeks of therapy (figure 1B, sample 4) that reached a plateau at a range between 100 fg and 1 pg within 5 weeks (samples 5–9). Then JCV DNA concentration decreased to 1 fg (i.e., at the detection limit of the standard PCR in the inhibitor-free sample 11). During this period, clinical disease did not unequivocally stabilize or improve. The virus load increased again, reaching a steady state in the range of 1 pg ($1.2 \times 10^3$ GE).

In patient B, JCV DNA was detected in all CSF samples by standard PCR (figure 2A). Quantitative analysis revealed a JCV DNA load ranging from 1 pg ($1.2 \times 10^3$ GE) in the first samples before therapy to about 100 pg, remaining stable throughout most of the observation time (sample 1–8). After about 6 weeks of therapy, the JCV DNA concentration increased to about 1 ng ($1.2 \times 10^6$ GE; sample 9).

On routine analysis of both patients’ CSF, the only abnormality was recurring intermittent pleocytosis, up to 37 white cells/μL CSF, that was not correlated with JCV load (data not shown).

**Discussion**

The aim of this study was the longitudinal determination of the JCV concentration in the CSF of HIV-infected patients with PML during salvage treatment including Ara-C, IFN-α, and Ig. Because of intrathecal drug administration, a large series of samples from the beginnings of the disease to the end of therapeutic intervention was analyzed by quantitative PCR, thus allowing for the first time the monitoring of JCV shedding during the active disease process.

In both patients neurologic disease progressed during treatment, and survival was similar to that of untreated patients [2]. This is in agreement with a recently reported controlled trial documenting the failure of Ara-C treatment in HIV-associated
Figure 1. Presence and quantification of JC virus (JCV) DNA in serial cerebrospinal fluid samples of a progressive multifocal leukoencephalopathy (PML) patient with a highly variable virus load in the advanced progressive course of PML. A, JCV DNA was amplified in serial samples of patient A by qualitative polymerase chain reaction (PCR) with the primer pair 119/121 spanning a DNA segment within the coding region for the capsid proteins under standard cycling conditions [12, 13]. B, Competitive PCR on serial samples was performed at a volume of 10 μL with the primer pair 119/121 and internal 10-fold dilutions of competitor DNA (pJC/Vdel-1) ranging from 1 fg to 10 ng at 2.5 mM MgCl₂ at 59°C under standard cycling conditions. The competitive DNA concentration in sample 11 included the 0.1 fg dilution. PCR products were detected by electrophoretic separation on agarose gels [12]. Samples were numbered consecutively. JCV, pJCV GS/B, native target DNA (product length 900 bp); 700 bp, competitive DNA product length; black bars represent the zone of equivalence; M, marker 100 bp DNA ladder; C, control without internal target DNA; arrow, time after first symptoms.
Figure 2. Quantification of the JC virus (JCV) DNA load in a patient with a constant virus load in the intermediate stage of advanced progressive multifocal leukoencephalopathy. A, JCV DNA was amplified by qualitative polymerase chain reaction (PCR) in serial samples of patient B as described in figure 1. B, Competitive PCR was performed and products were separated as described in figure 1. Each individual sample was coamplified with 10-fold dilutions of competitor DNA (pJC/Vdel-1) ranging from 1 fg to 10 ng, respectively.
The JCV DNA load in the CSF was examined in 2 consecutive steps. With qualitative PCR the JCV DNA concentration appeared to be variable throughout the observation time. In patient A, the concentration of JCV DNA in the first specimens was low, only detectable by the highly sensitive nested-PCR technique, thus being in a range routinely found in patients at risk for PML [7]. The low virus load in samples from soon after first presentation suggests that subclinical HIV-associated JCV infection and early stages of PML cannot be reliably differentiated by PCR of the CSF [4, 6]. This overlap of the virus load in asymptomatic and symptomatic JCV infection is also found in the brain parenchyma, where JCV DNA may be detected by PCR in patients with PML as well as those at risk for the disease [14].

The quantification of JCV DNA by competitive PCR on serial CSF specimens revealed considerable differences with respect to (1) the evolution of JC viral shedding during the observation period within individual patients, and (2) to the dimension of virus load among patients. Although both patients showed continuous clinical deterioration, in the intermediate disease stage, virus load was fluctuating in patient A but was constant in patient B. An overall increase of virus load throughout the observed course was common to both individuals.

The finding of a low virus burden at 5 months after disease manifestation in 1 sample in the absence of clinical improvement was unexpected. Inhibitory factors were ruled out by the use of internal control DNA in the PCR protocol. Environmental effects were also unlikely, because the samples were handled under standardized conditions. If the disproportionately low JCV load was caused by impaired sample integrity, the HIV RNA concentration in that particular sample should be expected to be low as well. As this was not the case, we interpret the low JCV load in sample 11 to reflect a true intermittent decrease for which a short-lived increase of immunologic competence resulting in suppression of virus replication should be considered as an explanation.

In the second patient, the disease course was faster, and survival was shorter compared with patient A. After an initial steep increase in the first 3 weeks, virus burden was constant during the intermediate stages and increased at the end of observation. The difference in the development of the virus load under continuous clinical progression suggests that viral shedding might be individually influenced by at-present unknown virus-host interactions [15]. One factor governing CSF virus load might be the localization of PML lesions in relation to the CSF spaces. However, under the assumption of a continuous activation of JCV infection under severe immunosuppression and its suppression by an intact immune control, the most likely explanation to date is that the variability of JCV load reflects differences in the individual immunologic state of the host. This might even transiently reduce the virus load to below the limit of detection and may explain those 25% of patients in whom PML cannot be diagnosed by PCR on single CSF samples [4–7]. These findings suggest the analysis of serial CSF samples by PCR to avoid false-negative results.

In summary, this study on the JCV load in the CSF shows for the first time the evolution of viral shedding during the progressive course of PML. The assumption of a low virus burden early after first presentation could be confirmed. However, during progression of the disease, a constant as well as a fluctuating course of viral shedding was observed, ultimately ending in an increase at late stages of disease. In conclusion, for early diagnosis and the virologic monitoring of investigational therapy, a standardized determination of the JC virus load in serial CSF specimens is recommended.

References