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Latency and Reactivation of JC Virus in Peripheral Blood of Human Immunodeficiency Virus Type 1-Infected Patients

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JC virus (JCV) acts as an opportunistic virus in immunocompromised human immunodeficiency virus type 1 (HIV-1)-infected patients. The role of peripheral blood cells in central nervous system invasion, before the onset of progressive multifocal leukoencephalopathy (PML), remains controversial. In order to clarify JCV latency or reactivation status in peripheral blood, 72 HIV-1-infected patients were studied, together with 7 HIV-1-positive PML patients and 50 blood donors. Blood leukocytes, plasma, and B lymphocytes were investigated by two complementary DNA amplification procedures within the early T and late VP1 JCV genes and two reverse transcription techniques for the detection of corresponding early transcripts and mRNAs. JCV DNA was detected in 40.3% of the HIV-1-infected patients but only 8% of the blood donors ($P < 0.001$). Leukocytes represented 82.7% of the positive samples, but plasma from 12 patients (41.4%) contained JCV DNA. B lymphocytes seemed to be involved in the natural history of JCV but did not represent the unique cell target. JCV DNA was intermittently found in blood, and JCV mRNAs for VP1 capsid protein were detected exclusively in one PML patient. Such observations demonstrate that JCV, when detected in blood, does not undergo active multiplication. They support the JCV hematogenous spread hypothesis, but do not indicate any direct link between peripheral virus and dissemination in the central nervous system at the time of immunodepression.

Progressive multifocal leukoencephalopathy (PML) is a rare, severe demyelinating disease caused by the human polyomavirus JC virus (JCV), which induces lytic infection of myelin-producing oligodendrocytes. Primary infection with JCV occurs during childhood, and seroconversion rates reach 70% in young adults (17) to 100% in elderly people (11). This primary infection is not associated with any known clinical manifestation, and the virus could persist lifelong in humans, with intermittent viral production in kidneys (4, 10, 11, 13, 21). Under marked immune system impairment due to severe primary immunodeficiency, malignant diseases, organ transplantation, or human immunodeficiency virus (HIV) infection, the virus can be reactivated and induce PML. Three to 4% (2) of all AIDS patients develop PML. This disease is rapidly fatal and consequently contributes significantly to mortality in HIV-infected patients.

The organs which harbour the virus during latency remain controversial: the kidneys, brain (16) and leukocytes seem to be involved. JCV was detected in lymphoid cells of PML patients (9), in peripheral blood lymphocytes of non-PML-immunocompromised AIDS patients (6, 22), in lymphoid cell preparations after bone marrow transplantation (20) and in leukocytes of healthy immunocompetent individuals (5). JC virus seems therefore to be a lymphotropic virus, B lymphocytes (Bly) being usually considered as JCV specific targets (9, 14). Leukocytes may play a role in viral persistence or as JCV conveyors into the central nervous system.

We looked for JCV DNA in peripheral blood in 72 non-PML-AIDS patients, 7 PML-AIDS patients and 50 healthy blood donors. Our aim was to determine whether the opportunistic JC virus productively infects peripheral blood leukocytes (PBLs) in HIV_1-infected patients, long before the onset of PML. Active JCV multiplication was ascertained in JCV-DNA-positive PBLs and plasma samples by the detection of mRNAs for a capsid-coding gene (VP1 gene). In addition, whenever JCV DNA was present in PBLs or plasma, the presence of JCV mRNAs was investigated in Bly, when available, in order to evaluate the role of these cells in the development of PML.

MATERIALS AND METHODS

Samples. Blood specimens were collected from 72 HIV-1-infected patients attending the Pellegrin and Haut-Lévêque Hospitals in Bordeaux, France, between February and September 1995. Blood was collected after most clinical consultations, i.e., only once for 43 patients, twice for 21 patients, and three times for 8 patients. Thus, sequential samples were obtained at 5-month intervals for 2 patients, 4-month intervals for 1 patient, 3-month intervals for 2 patients, 2-month intervals for 25 patients, and 1-month intervals for 7 patients. These patients presented no signs of PML, and all stages of HIV disease were represented among the patients. Among them 79.1% ($n = 57$) had less than 200×10^6 CD4 lymphocytes per ml, 19.4% ($n = 14$) had 200 to 499×10^6 CD4 lymphocytes per ml and only 1.4% ($n = 1$) had more than 500×10^6 CD4 lymphocytes per ml. The mean CD4 lymphocyte count was 114.4×10^6 (range, 0 to 584×10^6 CD4 lymphocytes per ml). The mean age in this group of patients was 39.4 years (range, 25 to 72 years).

One blood sample was collected from 50 healthy blood donors, whose mean age was 41.1 years (range, 22 to 51 years).

For seven patients who presented with clinical and radiological signs of PML, it was possible to obtain one blood sample, cerebrospinal fluid (CSF), and urine.

Sample preparation. PBLs and plasma were separated by sedimentation, centrifugation, and erythrocyte lysis (NH_4Cl, 0.8%) of 5 to 7 ml of whole blood collected in EDTA-containing tubes. A total of 109 samples were studied, and peripheral blood Bly were obtained from 86 patients by using monoclonal antibody CD_{19}-coated magnetic beads (Immunotech, Lumigny, France), according to the instructions of the manufacturer. Thus, 1×10^5 to 3×10^5 Bly were purified from an additional tube of 5 to 7 ml of whole blood. PBLs, B cells, and plasma were frozen until use.

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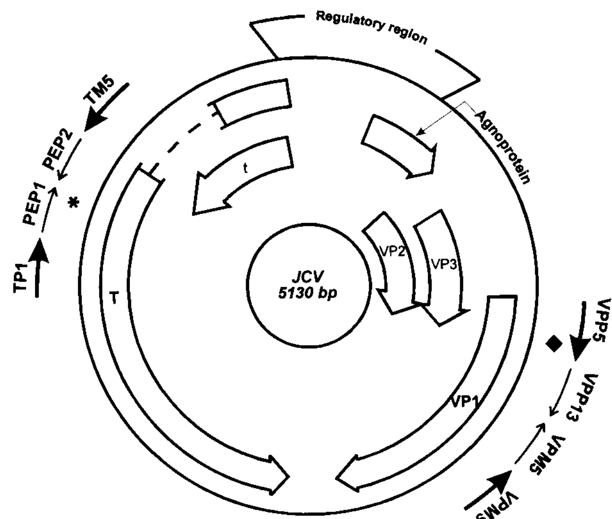

FIG. 1. JCV genome showing the regions of PCR primers TP1 (positions 4043 to 4071), TM5 (positions 4611 to 4637), PEP1 (positions 4255 to 4274), and PEP2 (positions 4408 to 4427) in the T gene and primers VPP5 (positions 1280 to 1308), VPM9 (positions 2399 to 2421), VPP13 (positions 1725 to 1748), and VPM5 (positions 2240 to 2263) in the VP1 gene (adapted from Major et al. [12]). JEP (*; positions 4283 to 4322) and JCAP (◆; position 1646 to 1669) probes are also depicted.

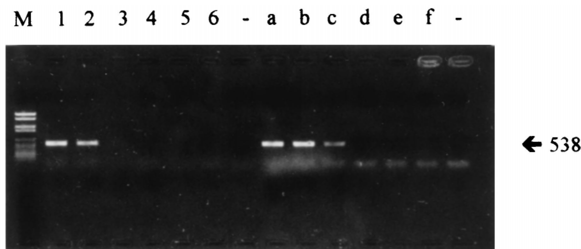

FIG. 2. Sensitivity of VP1 nested PCR with various $MgCl_2$ concentrations and primer annealing temperatures. Lanes 1 to 6, dilutions of 1:3,200 (31.25 copies), 1:6,400, 1:12,800, 1:25,600, 1:51,200, and 1:102,400 (0.97 copies), respectively, with 1.5 mM $MgCl_2$ and hybridization at 65°C during the first round of amplification and 60°C during the nested PCR; lanes a to f, the same dilutions as for lanes 1 to 6, respectively, after a modification of the nested PCR conditions (2.5 mM $MgCl_2$); lane −, negative control. The last positive dilution contained 7.8 plasmid copies/10 μl introduced in the PCR mixture. The number on the right is in base pairs.

DNA extraction and PCR assay. DNA was extracted from 200 μl of plasma, one pellet of PBLs, 200 μl of urine, and 200 μl of CSF by proteinase K digestion (400 μg per sample) (Boehringer GmbH, Mannheim, Germany) and a classical phenol-chloroform and chloroform-isoamyl alcohol procedure. DNA was quantified by spectrophotometry (Pharmacia, Cambridge, United Kingdom), and for each sample, the DNA concentration was adjusted to 1 to 2 μg of DNA/10 μl.

Two regions of the JCV genome were amplified (Fig. 1). The first pair of primers recognized a nucleotide sequence located in BK virus and JCV large T early gene. A total of 10 μl of extracted DNA (i.e., 1 to 2 μg) was added to the 90-μl reaction mixture, consisting of 10 mM Tris HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 250 μM (each) deoxynucleotide triphosphates, 2.5 U of *Taq* polymerase (Boehringer), and 0.15 μM (each) primers PEP1 and PEP2 (1, 15). The 173-bp DNA fragments obtained by PCR were hybridized with the 5′ biotin-labeled JCV-specific JEP molecular probe (1, 15) by liquid-phase hybridization by a DNA enzyme immunoassay (DEIA; Sorin, Saluggia, Italy) (23), according to the instructions of the manufacturer.

The second amplified region was located in the VP1-encoding late gene. Two pairs of primers were designed for a nested PCR by using a computer program (PC/gene): primer VPP5 (5′-ATGATGCAGACAGCATTCAAGAAGTTACC-3′) at positions 1280 to 1308, primer VPM9 (5′-TCCATGCCATACATAGGC TGCCC-3′) at positions 2399 to 2421, primer VPP13 (5′-TTCCACTACCCAAT CTAAATGAGG-3′) at positions 1725 to 1748, and primer VPM5 (5′-GTTTGT AAACATGCCACAGACATC-3′) at positions 2240 to 2263. Under the same conditions used for the T-gene PCR and with the primer combination VPP5 and VPM9, the first round of amplification resulted in a 1,141-bp fragment after 35 amplification cycles (1 min of denaturation at 94°C, 1 min of annealing at 65°C, and 1 min of extension at 72°C). With the primer combination VPP13 and VPM5, a 538-bp fragment was obtained during the nested PCR at an annealing temperature of 60°C. Each PCR was extended with a first denaturation cycle for 5 min and with a last extension cycle for 5 min. The amplification products were observed by exposure to UV light after 2% agarose gel electrophoresis in the presence of ethidium bromide.

Unfortunately, no JCV-specific probe could be identified within the VP1 nested PCR product. In order to check the amplification specificity, a liquid-phase hybridization (DEIA; Sorin) with the 5′ biotin-labeled JCV-specific molecular probe JCAP (5′-bAAGTCAATATCTATATCAGATACA-3′) was performed after the first round of the VP1 PCR. This probe was applied to VP1-positive samples when the T-gene PCR and hybridization remained negative.

Primers and labeled probes were synthesized by Genset (Paris, France).

RNA extraction and RT-PCR assay. RNA was extracted from the DNA-positive samples (100 μl of plasma, PBLs, Bly when available, 100 μl of CSF, and 100 μl of urine) by using guanidium thiocyanate (Interchim, Montlucon, France), 2-mercaptoethanol (7%), phenol, and chloroform-isoamyl alcohol as described by Chomczynski and Sacchi (3). RNA was precipitated from the aqueous phase in isopropanol, washed in 70% ethanol, air dried, and dissolved in 50 μl of distilled water.

The universal oligonucleotide dT15 primer (Boehringer) was used to synthesize single-stranded cDNA by reverse transcription (RT) before nested PCR within the VP1 gene. Extracted RNA (5 μl) was added to 5 μl of a reaction mixture containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 625 μM (each) deoxynucleotide triphosphates, 20 U of RNase inhibitor (Boehringer), 10 U of Moloney murine leukemia virus reverse transcriptase (Boehringer), and 40 pmol of oligonucleotide dT15. The RT mixture was incubated for 1 h at 37°C, heated to 95°C for 5 min, and then introduced into the nested VP1 PCR mixture.

The RT-PCR conditions for the detection of the large T-gene early transcript were slightly different. Primers TP1 and TM5, external to PEP1 and PEP2 (Fig. 1), were designed to perform a nested PCR in order to increase the sensitivity of RNA detection: primer TP1 (5′-AATTGCTGACACTCTATGTCTATGTG GTG-3′) at positions 4043 to 4071 and primer TM5 (5′-TAAGAAGCAGCCCA CTTGTGTGGATAG-3′) at positions 4611 to 4637. Unfortunately, primer TM5 hybridizes with a sequence removed during the differential splicing of T and t mRNAs (Fig. 1). These specific primers therefore had to be used instead of the oligonucleotide dT15 primer during the RT step, because dT15 could not allow for the subsequent nested PCR within the large T gene. This procedure enabled the detection of large T-gene early transcripts but not mRNAs. The RT conditions in the T-gene PCR were identical to those in the VP1 RT-PCR procedure, with 1 μM (each) specific primer. The nested PCR was realized first with the TP1-TM5 primer combination and next with primers PEP1 and PEP2. The 173-bp products were visualized on ethidium bromide-stained 2% agarose gels.

All experiments included a negative buffer control, an amplification control containing JCV plasmid DNA, and an RNA extraction control, that is, JCV RNA-positive CSF from a PML patient. Beta-globin DNA was amplified by PCR (19) to check the quality of the cellular DNA present in the DNA extraction samples and the efficiency of RNA extraction. Indeed, the absence of beta-globin DNA in RNA extracts before RT argues against the possibility of JCV DNA parasite amplification. Moreover, to prove the absence of JCV DNA in the RNA extracts, RT-PCR was performed with or without 0.5 mM DNase-free RNase (Boehringer) (1 h at 37°C).

Statistics. The results were analyzed by the chi-square test.

RESULTS

Sensitivity of the PCR assay for JCV. The sensitivity of the PCR was determined by successful amplification of serial dilutions of a positive control. The positive control was a full-length JCV MAD-1 strain cloned into a pBR322 vector (kindly given by H. Moret and D. Ingrand, Reims, France). Serial dilutions starting with 10^5 plasmid copies and ending with 0.97 copies were used as a template. As demonstrated in Fig. 2, VP1 primers amplified as little as 7.8 copies of the pMAD-1 template (nested PCR). Similar results were obtained with primer pair PEP1 and PEP2, combined with the subsequent hybridization procedure.

Detection of JCV DNA in blood. (i) HIV-1-infected patients. We compared plasma and PBLs for the presence of polyomavirus DNA. Two different PCR procedures were used: the first method combined a PCR with one set of primers within the T gene and a JCV-specific hybridization with the JEP probe. The second technique consisted of a nested PCR within the VP1

gene. The specificity of VP1 amplification was confirmed by liquid hybridization with the JCAP molecular probe when the samples were negative by the T-gene PCR.

Plasma and PBLs were available for DNA detection from all HIV-1-infected patients, the 50 healthy blood donors, and the 7 PML patients. For several HIV-1-infected patients, multiple samples were studied: two samples from 21 patients and three samples from 8 other patients. A total of 218 samples (109 PBLs and 109 plasma samples) were tested for the 72 HIV-1-infected patients.

Taking multiple samples into account, for 29 (40.3%) of the 72 HIV-1-infected patients, JCV DNA was detectable in their plasma and/or PBLs. For 10 of the 29 JCV-positive patients, both the T and the VP1 genes could be amplified, whereas for 12 patients the T-gene PCR alone was positive and for 7 patients the VP1 PCR alone was positive. JCV DNA was detected in PBLs from 17 patients, plasma from 5 patients, and both PBLs and plasma from 7 patients. Thus, 82.7% (24 patients) of PBL samples were positive for JCV DNA, whereas 41.4% (12 patients) of plasma samples and 24.1% (7 patients) of PBLs and plasma samples were positive for JCV DNA.

Whenever multiple samples were studied (29 patients), JCV DNA detection was reproducible for samples from 7 patients (24.1%). Ten patients remained systematically negative (34.5%). The JCV genome was intermittently amplified from samples from 12 patients (41.4%). Certain patients would have been considered negative if only one sample had been studied. However, if the results obtained with the first sample alone were considered, JCV DNA would be detected in 25 (34%) instead of 29 (40%) HIV-1-infected patients.

Among the 29 HIV-1-infected patients in whose blood JCV DNA was detectable, 24 (82.7%) had less than 200×10^6 CD4 lymphocytes per ml, 5 (17.2%) had 200 to 499×10^6 CD4 lymphocytes per ml, and none had more than 500×10^6 CD4 lymphocytes per ml. The mean CD4 count for these patients was 111.2×10^6 CD4 lymphocytes per ml. The numbers of HIV-infected patients in the clinical and biological stages and the mean CD4 count (114.4×10^6 CD4 lymphocytes per ml) were similar in the whole group of HIV-1-infected patients.

(ii) Healthy blood donors. Plasma and PBLs from each healthy blood donor were tested, and JCV DNA was detected in 4 of them (8%): twice in plasma and PBLs and twice in PBLs only. For two healthy donors a positive signal was found within the VP1 gene only, and for two healthy donors positive signals were found by both PCR methods. JCV DNA was detected significantly less often in this group than in the HIV-1-infected group ($P < 0.001$; chi-square test), even if only the first sample was considered for HIV-1-infected patients.

(iii) PML patients. Plasma and PBLs from seven PML patients were tested. Five of them had JCV DNA in peripheral blood, one had JCV DNA in PBLs, three had JCV DNA in plasma, and one had JCV DNA in PBLs and plasma (Table 1). Three samples were found to be positive by both amplification methods: one by T-gene PCR only and one with VP1 primers alone.

JCV DNA was detected in the CSF of all 7 PML patients by the T-gene PCR and hybridization.

Detection of JCV RNA in blood. (i) HIV-1-infected patients. In order to assess whether the virus detected in blood was latent or reactivated, we looked for the presence of RNAs including VP1 gene mRNAs and large T-gene early transcripts. DNA-positive PBLs or plasma samples were tested for RNAs, and the corresponding Bly were also analyzed, whenever they were available (26 of the 29 patients). The presence of JCV DNA was not investigated in Bly since only one pellet of 1×10^5 to 3×10^5 cells was obtained.

TABLE 1. JCV DNA detection in PBLs and plasma from HIV-1-infected patients without PML

Patient no.	CD4-cell count (10^6/ml)	Detection of DNA in the indicated compartments[a]			
		T-gene DNA		VP1 DNA	
		PBLs	Plasma	PBLs	Plasma
1	1	−	+	−	+
2		−	−	−	−
3	67	+	−	−	−
4		−	+	−	+
5	1	+	−	−	+
6	33	−	−	−	+
7	120	−	−	−	−

[a] −, negative; +, positive.

Among the 72 HIV-1-infected patients, none presented with VP1 mRNAs in blood. Large T-gene early transcripts were detected in only one person (1.4%) (Table 2), in Bly. This patient had 272×10^6 CD4 lymphocytes per ml.

To check the efficiency and specificity of RNA extraction, a beta-globin DNA PCR (19) was performed directly after the RNA extraction but before RT; the beta-globin DNA PCR was negative, thus proving the absence of contaminating DNA in the RNA extracts. Moreover, the RT-PCR procedure was applied simultaneously in the presence or in the absence of DNase-free RNase: the JCV amplification signal from RNA extracts was removed with RNase (Fig. 3).

(ii) PML patients. Among the five PML patients who presented with JCV DNA in blood, for two JCV RNA was detectable in PBLs or plasma (Table 2). For one patient, JCV RNA was found both in PBLs and in plasma, and for the other patient, JCV RNA was found in PBLs alone. All three samples contained the large T-gene early transcript, whereas one had only VP1 gene mRNAs. They were detected in plasma but not in blood cells.

DISCUSSION

The presence of JCV DNA in kidneys, lymph nodes, spleen, bone marrow, and liver (8) suggests a widespread hematogenous distribution of the virus in PML patients. The multifocal and perivascular distribution of white matter lesions further supports the putative role of blood cells in the spread of JCV into the central nervous system (12).

In our HIV-1-infected patients, whether or not they were affected by PML, JCV DNA was frequently present in peripheral blood. It was also found in healthy blood donors, although significantly less frequently. Moreover, JCV DNA was detectable only intermittently, thus supporting the hypothesis of viral reservoirs, such as lymphoid organs. Finally, JCV mRNAs were seldom associated with DNA, suggesting that JCV reactivation does not take place in peripheral blood. These observations agree with the theory of a hematogenous distribution of JCV. However, they do not indicate whether the dissemination of JCV occurs during primary infection or later, just before the onset of PML in the immunocompromised patient.

Among the 72 HIV-1-infected patients who showed no sign of PML, 29 exhibited JCV DNA in peripheral blood (40.3%). These results corroborate those initially published by others (18, 22) and our own previous observations (6, 7). In the present study, JCV DNA was detected in the blood of five of the seven PML patients. Does its absence from the two others exclude any intervention of leukocytes in PML pathophysiol-

TABLE 2. Detection of JCV RNA by RT-PCR in an HIV-1-infected patient without PML and two PML patients

Patient no.	CD4-cell count (10^6/ml)	Detection of JCV RNA in the indicated compartment[a]											
		T DNA			VP1 DNA			T RNA			VP1 RNA		
		PBLs	Plasma	Bly	PBLs	Plasma	Bly	PBLs	Plasma	Bly	PBLs	Plasma	Bly
1	1	−	+	ND	−	+	ND	+	+	ND	−	+	ND
2	67	+	−	ND	−	−	ND	+	−	ND	−	−	ND
3	272	+	−	ND	−	−	ND	−	ND	+	−	ND	−

[a] T DNA and VP1 DNA designate PCR of T and VP1 genes respectively. T RNA and VP1 RNA indicate RT-PCR of T and VP1 genes, respectively. −, negative; +, positive; ND, not determined.

ogy? This group was too small for us to be able to draw any conclusion, and several factors may explain our incapacity to detect JCV in blood: leukocytes might have transported JCV into the brain long before our study started, molecular techniques may lack sensitivity, and the presence of JCV in peripheral blood was clearly intermittent.

JCV DNA was present significantly less frequently in HIV-negative blood donors ($P < 0.001$). In a previous study, we compared HIV-1-infected patients with HIV-negative immunocompromised individuals (6). The difference between the two groups, although notable, did not reach significance. However, these HIV-negative patients were, on average, 20 years older than the HIV-1-positive patients. In the present work, we eliminated this bias by choosing a control population perfectly matched in age to the group of HIV-1-infected patients.

Altogether, if JCV remains latent in peripheral organs and is intermittently released into the blood circulation, immunosuppression might enable JCV reactivation in peripheral reservoirs and thus facilitate its detection in blood. However, circulating virus might have no link with the emergence of PML.

JCV DNA was frequently detected in the leukocyte compartment, but its amplification from plasma was possible in a few instances (12 patients), suggesting the recent production and release of viral particles. On the other hand, the amount of JCV DNA in blood was probably low. Sensitive methods were needed to identify the presence of the JCV genome, and intermittent positivity in certain patients might have been explained by the presence of DNA amounts close to the cutoff value. These observations suggested that JCV was mainly latent in blood cells. They were further confirmed by results concerning VP1 mRNAs, the presence of which demonstrates JCV replication. Indeed, mRNAs for a capsid protein such as VP1 are considered specific for JCV multiplication (12). In contrast, early gene RNAs, such as T-gene pre-mRNAs, may be present in the absence of viral particle production, since the T protein serves as a regulatory factor in early and late transcription and in DNA replication. Interestingly, JCV mRNAs were detectable in a single patient. He was infected with HIV-1 and suffered from PML. Early RNAs for the T gene were found in three patients, including the VP1 mRNA-positive one. The two other patients were also HIV-1 positive, but one only had PML. In order to confirm the good sensitivity of our procedure, we tested CSF and several urine samples from PML patients (data not shown). DNA-positive CSF contained JCV VP1 mRNAs in all instances, and DNA-positive urine contained JCV VP1 mRNAs in 38% of the patients.

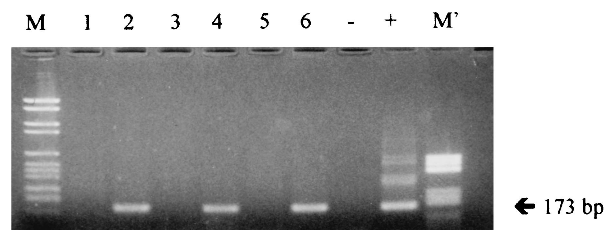

FIG. 3. T-gene RT-PCR performed in the presence (lanes 1, 3, and 5) and absence (lanes 2, 4, and 6) of DNase-free RNase for patients presenting with JCV RNA in their blood. Lane +, positive control; lane −, negative control.

For several years, Bly have been depicted as JCV-infected leukocytes (9). Recently, JCV infection of Bly was confirmed with peripheral blood from one patient (14). The present observations are in agreement with previous studies, since JCV RNAs were detected in the Bly subpopulation. However, preliminary results indicate that non-Bly cells in peripheral blood may carry JCV DNA (data not shown).

Altogether, leukocytes seem to harbor latent JCV. If they do convey JCV into the central nervous system, they might deliver various amounts of JCV DNA, in the absence of active viral production. The neurological outcome of JCV infection might be determined by a DNA threshold in blood. Our results do not eliminate the possible link between peripheral transport of JCV and the onset of PML. However, they suggest the existence of a subtle balance and indirect interactions between the different compartments of the infected organism.

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