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 × 106 CD4 lymphocytes per ml, 19.4% (n = 14) had between 200 and 1000 CD4 lymphocytes per ml and only 1.4% (n = 1) had more than 500 × 106 CD4 lymphocytes per ml. The mean CD4 lymphocyte count was 114.4 × 103 (range, 0 to 584 × 103) 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 ethyloxy ethanol lysis (NH4Cl, 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 CD19-coated magnetic beads (Immunotech, Lumigny, France), according to the instructions of the manufacturer. Thus, 1 × 105 to 3 × 105 Bly were purified from an additional tube of 5 to 7 ml of whole blood. PBLs, B cells, and plasma were frozen until use.
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 μg) was added to the 90-μl reaction mixture, consisting of 10 mM Tris HCl (pH 8.3), 50 mM KCl, 4 mM MgCl₂, 625 μM (each) deoxynucleotide triphosphates, 2.5 U of Taq polymerase (Boehringer), and 0.15 μM of each primer 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 molecule (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'-ATGATGACAGACCTCAAGAAGATCTAC-3') at positions 1280 to 1308, primer VM9 (5'-TCCATGCACTATAGGCTGCC-3') at positions 2399 to 2421, primer VPP13 (5'-TCCACTCCCAATTCTAATAAGG-3') at positions 1725 to 1748, and primer VMPS (5'-GGTGTGTTGACCCAGATCGAGAGC-3') at positions 2240 to 2263. Under the same conditions used for the T-gene PCR and with the primer combination VPP5 and VMP1, 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 VMPS, 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 JEP molecular probe JCAP (5'-bAAGTCAATATCTATATCAGATACA-3') at positions 1646 to 1669. Under the same conditions used for the T-gene PCR and with the primer combination JCAP and JCAFP (position 1646 to 1669) probes are also depicted.

The universal oligonucleotide dT15 primer (Boehringer) was used to synthesize single-stranded cDNA by reverse transcription (RT) before nested PCR within the T-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 MgCl₂, 0.25 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'-AATGCTGACACCTCAAGATCC-3') at positions 4043 to 4071 and primer TM5 (5'-TAAGAAGCACCCAATTTGTTGACAGAGC-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 μl (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 control PCR, an amplification control containing JCV plasmid DNA, and an RNA extraction control, that is, JCV RNA-positive cDNA 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 10⁵ 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 polyoma virus 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 and a JCV-specific hybridization with the JEP probe.
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 \times 10^6 CD4 lymphocytes per ml, 5 (17.2%) had 200 to 499 \times 10^6 CD4 lymphocytes per ml, and none had more than 500 \times 10^6 CD4 lymphocytes per ml. The mean CD4 count for these patients was 111.2 \times 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 \times 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 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. JCV RNA was detectable in 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 \times 10^5 to 3 \times 10^5 cells was obtained.

Among the 72 HIV-1-infected patients, none presented with VP1 mRNA as in blood. Large T-gene early transcripts were detected in only one person (1.4%) (Table 2), in Bly. This patient had 272 \times 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 RNase-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 plasma 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 1. JCV DNA detection in PBLs and plasma from HIV-1-infected patients without PML.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>CD4-cell count (10^6/ml)</th>
<th>T-gene DNA</th>
<th>VP1 DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PBLs</td>
<td>Plasma</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>67</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>+</td>
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<tr>
<td>5</td>
<td>1</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>33</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>120</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*–*, negative; +, positive.
TABLE 2. Detection of JCV RNA by RT-PCR in an HIV-1-infected patient without PML and two PML patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>CD4-cell count (10^6/ml)</th>
<th>Detection of JCV RNA in the indicated compartment*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T DNA Bly</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PBLs Plasma</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>67</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>272</td>
<td>+</td>
</tr>
</tbody>
</table>

* T DNA and VPI DNA designate PCR of T and VPI genes respectively. T RNA and VPI RNA indicate RT-PCR of T and VPI genes, respectively. –, negative; +, positive; ND, not determined.

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REFERENCES


