Borna Disease Virus RNA in Immunocompromised Patients in Southwestern France

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Borna disease virus (BDV) is a neurotropic RNA virus with a wide host range. Human infections, although controversial, have been described in Europe, Asia, and the United States. The present study investigated the existence of BDV infections in immunocompromised human beings, namely, 82 human immunodeficiency virus (HIV)-infected and 80 therapeutically immunosuppressed patients. BDV p40 RNAs were detected in peripheral white blood cells with reverse transcription-nested PCR and hybridization in, respectively, 11 (13.4%) and 1 (1.25%) of the two groups of patients. BDV p24 RNAs were identified in only one of those. BDV RNA was detected in the absence of any neuropsychiatric illness, suggesting that BDV infections may occur in asymptomatic carriers. The severity and particularity of cellular immunosuppression could explain the significantly increased detection of BDV RNA in HIV-infected patients.

MATERIALS AND METHODS

Patients and blood samples. Informed consent was obtained from the patients, and human experimentation guidelines of Bordeaux University Hospital (CCCPRB Bordeaux 1) were followed in this clinical research. One hundred sixty-two patients were randomly tested. The first group included 82 human immunodeficiency virus (HIV)-infected patients with leukocyte counts over 1.5 × 10^6 g/liter. The second group comprised 80 patients treated with immunosuppressive drugs, including cyclosporine or tacrolimus and corticosteroids after liver transplantation (n = 40) and cyclophosphamide or azathioprine and corticosteroids for severe lupus erythematosus (n = 40). None of the patients was recorded as presenting psychiatric symptoms. The patients were tested in a double-blind manner, independently of their belonging to one group or the other, in chronological order.

Ten milliliters of peripheral blood were collected on EDTA. Blood cells were separated by 1,200 × g (2,500 rpm) centrifugation for 5 min. Red blood cells were subsequently lysed on ice for 30 min with 8 ml of blood lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 1 mM EDTA; pH 7.4). After centrifugation at 1,200 × g for 10 min, the pellet was resuspended with 0.2 ml of sterile water and separated into two tubes. Leukocyte integrity was examined by Trypan blue exclusion. Ten leukocyte aliquots were stored at −80°C before use.

Molecular procedures. Total RNA was extracted from the 162 blood cell samples according to Chomczynski’s technique, based on guanidinium thiocyanate and phenol treatment, isopropanol precipitation, and ethanol washes (12).

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At the end of the procedure, after pooling of each patient’s extracts, the volume of an RNA extract was 60 μl. Reverse transcription (RT) was performed with RT-murine leukemia virus and the antisense primer, using 5 μl of the extract, allowing transcription of viral messenger RNAs; cDNA amplification (PCR) was obtained by a nested procedure (Genent [Paris, France] for the primers, Roche Molecular Diagnostics [Meylan, France] for all other reagents). All primers and RT-PCR techniques were as previously published (23, 35). The samples were first subjected to RT-nested PCR in the BDV p40 gene, the most abundantly transcribed in infected cells (35). Whenever the result was positive, RT-nested PCR in the BDV p24 gene was attempted (23). In 10 BDV p40-positive samples, RT-PCR for measles virus (MV) was performed (27). RT-PCR for α-actin mRNAs was applied successfully to BDV p40-negative RNA extracts.

Detection of amplified DNA was obtained by 1.5% agarose gel electrophoresis and ethidium bromide staining. Next, we carried out an enzyme-linked immunosorbent assay-format molecular hybridization (Hybridowell kit; Argene Biosoft, Vaires, France). After chemical denaturation of amplified products and their adsorption onto a microtitration plate, hybridization with an in-house-designed p40- or p24-specific biotinylated probe was carried out. Detection was performed with a streptavidin-peroxidase conjugate associated with tetramethylbenzidine substrate. Optical density was measured on a spectrophotometer at 450 nm. The cutoff value was arbitrarily determined according to the manufacturer’s recommendations, by adding 0.150 to the mean optical density of negative controls. This method was validated by using a panel of dilutions of positive controls. Primer and probe sequences are listed in Table 1.

### Table 1. Primers and probes used in this study

<table>
<thead>
<tr>
<th>Primer or probe</th>
<th>Sequence</th>
<th>Use</th>
<th>Nucleotide positions</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer A</td>
<td>5′-TTTCTACATGAAGGCTCCGACG-3′</td>
<td>First-round PCR</td>
<td>259–278 (S)</td>
<td>p40</td>
</tr>
<tr>
<td>Primer B</td>
<td>5′-GCAACTACAGGGATGTGAGGG-3′</td>
<td>First-round PCR</td>
<td>829–808 (AS)</td>
<td>p40</td>
</tr>
<tr>
<td>Primer C</td>
<td>5′-GCTCTTGTGTTTCTTATGTGG-3′</td>
<td>Second-round PCR</td>
<td>277–297 (S)</td>
<td>p40</td>
</tr>
<tr>
<td>Primer D</td>
<td>5′-GCACTCATACCTTGCGGAG-3′</td>
<td>Second-round PCR</td>
<td>805–766 (AS)</td>
<td>p40</td>
</tr>
<tr>
<td>Probe 1</td>
<td>5′-CGGGGCTCCATCGGTGAGGAC-3′</td>
<td>Hybridization</td>
<td>575–595 (S)</td>
<td>p40</td>
</tr>
<tr>
<td>Primer D2</td>
<td>5′-TGACCAAAACCCTTAGACCA-3′</td>
<td>First-round PCR</td>
<td>1387–1405 (S)</td>
<td>p24</td>
</tr>
<tr>
<td>Primer A1</td>
<td>5′-GTCCTCCTCCTCGTTGTC-3′</td>
<td>First-round PCR</td>
<td>1865–1847 (AS)</td>
<td>p24</td>
</tr>
<tr>
<td>Primer D3</td>
<td>5′-AGACACAGCCACAGCAGCCA-3′</td>
<td>Second-round PCR</td>
<td>1443–1461 (S)</td>
<td>p24</td>
</tr>
<tr>
<td>Primer A2</td>
<td>5′-ATGGGAGGATAAACAGGGGAC-3′</td>
<td>Second-round PCR</td>
<td>1834–1816 (AS)</td>
<td>p24</td>
</tr>
<tr>
<td>Probe 2</td>
<td>5′-TTCAGACAGCTACACGGCCG-3′</td>
<td>Hybridization</td>
<td>1627–1647 (S)</td>
<td>p24</td>
</tr>
</tbody>
</table>

* S, sense; AS, antisense.

### RESULTS

**Cell yields in peripheral blood samples.** An average of 3 × 10^6 white cells per ml of peripheral blood was obtained. Up to 10% of these cells did not exclude Trypan blue. The number of white blood cells present in each extraction sample was never lower than 10 × 10^6 (mean, 3 × 10^6), and each RT assay was performed with the RNA extracted from approximately 5 × 10^6 white blood cells.

**Threshold of detection.** The sensitivity of the RT-PCR technique for BDV p40 detection was first investigated with RNA extracted from serial dilutions of BDV-infected C6 cells. Viral RNA was detected in reaction tubes containing as little as 0.075 infected cell. When we attempted to test the sensitivity with the RNA transcribed from p40 INS, we consistently detected a signal using 1,000 copies of it (the detection of 100 copies was inconsistent).

**Detection of BDV RNA and controls.** BDV p40 RNA was detected in the peripheral blood leukocytes of 11 of the 82 HIV-infected patients (13.1%). Their CD4 counts ranged from 1 to 724 (mean = 279; median = 199), and 6 of them had CD4 counts equal to or greater than 200/mm^3. Four had recently experienced encephalitis, either opportunistic (Toxoplasma gondii and Cryptococcus neoformans infections, progressive multifocal leukoencephalopathy) or HIV-induced; these had the lowest CD4 counts (1 to 18 CD4/mm^3). Figure 1 illustrates the results of agarose gel electrophoresis for various HIV-infected patients.

Only one of the 80 patients under therapeutic immunosuppression was positive (1.25%); he was a liver graft recipient treated with tacrolimus and corticosteroids who presented no biological feature of immunodepression at the time of blood sampling.

The difference between HIV-infected and HIV-negative immunosuppressed patients for the detection of BDV RNA was significant (χ^2 = 8.73, P = 0.003).

BDV p24 RNA was detected in only one of the twelve p40-positive patients; he belonged to the HIV-infected group.

Negative controls showed the absence of DNA contamination in RNA extracts and of cross-amplification for either MV or VSV.

**BDV sequence analysis.** Nucleotide sequencing showed complete identity between the BDV p40 DNA fragments amplified from 11 clinical samples and BDV-infected C6 cells.
The sequence obtained from the twelfth (HIV-infected) patient demonstrated a single 120-bp deletion, as shown in Fig. 2. All sequences differed from the p40 INS-derived amplified products.

DISCUSSION

BDV infections, well studied in various animal species, remain controversial in humans (25, 31, 38, 40), although the wide host range of this virus rather suggests such a possibility. The present study indicated that “BDV-like” RNA could be detected by RT-PCR in the peripheral white blood cells of a significant number of immunocompromised patients. To our knowledge, such results were not yet available in France, although similar data were previously published elsewhere (9, 22, 25).

Because scientific doubts were repeatedly expressed on this topic, this study was conducted with constant attention to any source of contamination; this went as far as performing the BDV-infected C6 cell cultures in a level 3 containment laboratory otherwise dedicated to HIV culture diagnostic procedures and located in a building distant from the laboratory where patients samples were analyzed. Negative controls included “RT-minus,” RT-PCR MV and VSV because of the structural similarities among Mononegavirales, and none were positive. In addition, sequencing of our PCR products never indicated any contamination with the plasmid used as a positive control. On the other hand, the RT-nested PCR technique used in this study was reasonably sensitive, since it allowed the detection of 0.075 in vitro-infected cell (such cells were shown to contain approximately 0.0008 infectious BDV each [11]) or 1,000 copies of the RNA-transcribed plasmid. mRNAs of the first gene to be transcribed, p40, represent most of the viral RNA species present in infected cells and were therefore chosen as the main RT-nested PCR target. Finally, hybridization of the amplified DNA fragments enhanced the specificity of the detection because a specific probe was used.

The BDV p40 sequence detected in one patient presented an unusual deletion. Unfortunately, p24 RNA could not be amplified from this sample, which precluded any phylogenetic comparison.

Although this study was not designed to investigate the prevalence of BDV infection, the percentage of positive results in HIV-infected patients (13.41%) was comparable to previously published serological results (3, 7, 8). Another study did not detect any BDV RNA in 27 HIV-infected patients (4); the limited number of patients and the use of a single PCR could account for this discrepancy. Of note, BDV RNA was detected...
in only one of the 80 patients subjected to immunosuppressive treatments (1.25%), a result similar to those obtained with control patients (7, 8, 23). HIV-associated and drug-induced immunosuppressive conditions present important qualitative differences with regard to the immune system, which could account for the significant difference observed between the two groups of patients in the detection of the BDV RNA. In comparison, human cytomegalovirus infection in these two populations of patients differs in both frequency and clinical expression. There is not yet a definitive explanation for this, but it could depend on subtle differences in the host immune capacity of defense against cytomegalovirus. Other viruses have a preferential expression in particular immunocompromised subjects too, like JC virus in HIV-infected patients or Epstein-Barr virus in some but not all patients with cellular immunodeficiencies. BDV infection could depend on similar mechanisms. On one hand, HIV-infected patients may be altogether more susceptible to BDV infection than other immunocompromised patients, and on the other hand, HIV-specific functional abnormalities in T-cell-mediated immunity could favor BDV reactivation in latently infected individuals, thus improving its detection.

Interestingly, none of the patients investigated in this study showed acute psychiatric symptoms or was treated for a chronic psychiatric disease. Indeed, it was recently suggested that most BDV infections could occur asymptptomatically (31, 38). In animals, symptomatic Borna disease seems to result predominantly from immunopathologic mechanisms; BDV-induced neuronal destruction results from a strong host cellular immune response to the virus, including the recruitment of CD8+ and CD4+ T cells (39). Therefore, the relative impairment of cellular immune responses in HIV-infected patients could diminish the risk of clinical expression and bring about an asymptomatic carriage of the virus. Moreover, in the present study, there was no clinical indication that BDV could act as an opportunistic pathogenic agent.

BDV detection in humans has raised a sustained controversy, possibly because of its alleged implication in the physiopathology of psychiatric disorders. BDV load in circulating blood could be very low (one infected cell out of 5 × 106 mononucleated cells in rats) (17). Therefore, the cell yields tested in molecular assays could represent a critical factor: an insufficient cellular input could lead to chance-driven loss of the virus and subsequent false-negative results in certain sample aliquots. Since mononucleated cells may not be the exclusive viral carriers (28), the present study focused on total circulating blood cells, after exclusion of erythrocytes. The analysis of large cell pellets presented technical drawbacks: most commercially available extraction devices were not designed to analyze large amounts of cells and RNA. A classical extraction method was therefore chosen. Molecular techniques including an internal standard should certainly be preferred in future investigations (24, 41). Questions about the best available method for achieving optimal detection of BDV RNA in blood and definitively eliminating false-negative results are still unanswered.

This work, performed in two initially BDV-naive medical laboratories trained for routine molecular diagnostic procedures, under strict experimental conditions, confirmed the presence of BDV or BDV-like sequences in asymptomatic immunocompromised human beings. Defining the prevalence of BDV infection and confirming the putative link with a particular cellular immunosuppression should now be attempted.

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