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Prevalence of JC Virus viraemia in HIV-infected patients with or without neurological disorders: a prospective study

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Progressive Multifocal Leukoencephalopathy (PML) is a severe demyelinating disease, which is rapidly fatal and is due to JC virus (JCV) infection, which especially occurs in HIV-infected patients. To investigate JCV pathophysiology and to evaluate the predictive value of JCV detection in blood, we looked for JCV DNA in leukocytes and plasma of 96 patients without any neurological symptoms and 109 patients with neurological diseases, among whom 19 were suffering from PML. JCV genome was detected in about 18% of all patients, i.e. 15.6% of patients with central nervous system disorders except PML, 13.5% of patients without neurological symptoms and significantly more often in PML patients (47.6%). Both leukocytes and plasma were tested; in plasma, JCV DNA was found in 36.1% of positive patients and in cells in 80.5%. Surprisingly in seven instances only the plasma contained JCV genome. One-year follow-up of these patients showed that the absence of JCV DNA in blood was associated with a very low probability of developing PML (negative predictive value=0.99).

Keywords: JCV; leukocytes; plasma; PCR; progressive multifocal leukoencephalopathy

Introduction

The human polyomavirus JC (JCV) is responsible for a lytic infection in oligodendrocytes and causes a rare severe demyelinating disease named Progressive Multifocal Leukoencephalopathy (PML) which generally occurs in patients with marked cellular immunodeficiency. Since the onset of AIDS pandemic, the incidence of PML has considerably increased, affecting about 4% (Berger *et al*, 1987) of all AIDS patients. The disease is rapidly fatal so PML contributes significantly to mortality in HIV-infected patients. Virologic analysis of cerebrospinal fluid (CSF) allows only a late diagnosis. Serologic data suggest that exposure to JC virus is widespread in the general population since seroconversion rates reach 70% in young adults (Padgett and Walker, 1973) to 100% in the elderly (Kitamura *et al*, 1990). Primary infection with JCV occurs during childhood without any known associated clinical manifestation and the virus could persist lifelong in kidneys, with intermittent viral excretion in urine (Coleman *et al*, 1980; Kitamura *et al*, 1990, 1994; Markowitz *et al*, 1993; Sundsfjord *et al*, 1994). Some authors have reported detection of JCV DNA in the brain of neuropathologically asymptomatic patients (Elsner and Dorries, 1992; Mori *et al*, 1991, 1992; White *et al*, 1992), but others found no evidence for latency in the brain (Buckle *et al*, 1992; Henson *et al*, 1991; Rodriguez *et al*, 1992; Telenti *et al*, 1990). Moreover, JCV seems to be a lymphotropic virus: JCV has been detected in lymphoid cells of PML patients (Houff *et al*, 1988), in peripheral blood lymphocytes of non-PML-immunocompromised AIDS patients (Dubois *et al*, 1996, 1997; Tornatore *et al*, 1992), in lymphoid cell preparations after bone marrow transplantation (Schneider and Dorries, 1993) and in leukocytes of

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healthy immunocompetent individuals (Dorries *et al*, 1994). Thus, leukocytes may play a role in viral persistence or as JCV conveyors into the central nervous system (CNS).

Within the context of the French virological group for the study of polyomarivus infections, seven participating hospital laboratories looked for the presence of JCV genome in the blood of HIV-infected patients, in order to evaluate its prevalence and its predictive value. We compared JCV DNA detection in 109 patients with neurological disease (with or without PML) and 96 patients without any neurological symptoms. Moreover, the positive patients were followed up for 1 year in order to evaluate whether JCV presence in the blood could be related with PML development.

Results

JCV PCR threshold of detection

PCR threshold of detection was determined using serial dilutions of a positive control. The positive control was a full-length JCV MAD-1 strain cloned into a pBR322 vector. A JCV-negative CSF containing 10^7 plasmid copies was serially diluted from 1/100 to 1/312500. The dilutions were boiled for 15 min and used as a template. For the seven centres the PCR threshold of detection reached one to six copies for 200 μl CSF.

JCV genome detection in blood

Among the 205 patients tested with or without neurological disease, 36 (17.6%) presented JCV DNA in blood. JCV DNA was detected in 23 (21.1%) of the 109 patients with neurological disease: 9 (47.4%) of the 19 patients with PML and 14 (15.6%) of the 90 subjects without PML (Table 1). In comparison, 13 (13.5%) out of the 96 patients without neurological symptoms presented JCV genome in their peripheral blood (Table 1).

The difference between the patients with or without neurological symptoms was not significant (21.1% *versus* 13.5%, P=0.15). However, JCV DNA was detected significantly more often in the blood of PML patients (47.4% *versus* 15.6%, P=0.0031). The percentage of positive patients varied from 10.3 to 23.5% depending upon the laboratories.

We also looked for JCV genome in peripheral leukocytes and in plasma after ultracentrifugation which could contain free virions, some infected cells or cell-free DNA. To detect DNA cellular contamination, a beta globin PCR was performed for the positive JCV plasma samples. When beta globin PCR was positive plasma results were not taken into account. Thus, JCV DNA was detected in the plasma of eight patients with neurological disease (34.8% of positive results) and in five patients without neurological symptoms (38.5% of positive results) (Table 2). Moreover, JCV was found about two to three times more often in cells than in plasma, irrespective of the group (Table 2). For JCV-positive patients, cells were found to be positive in 80.5% cases and plasma in 36.1%. Surprisingly, in seven instances only plasma was found to be positive for JCV DNA, and the corresponding cells remained negative despite a repeated experiment. Such was the case for three patients with neurological symptoms, four without, and none of PML patients.

Unfortunately, CD_4 counts were available for only 145 patients at the precise time of our investigation, although all patients were known to have CD_4 counts below 200 mm^3 at some time point during the 3 previous months. Among these 145 persons, CD_4 counts ranged from 0 to 199 (mean 79); and the values for 13 PML patients ranged from 1 to 120 (mean 44). Therefore, we were unable to investigate the existence of statistical correlations between CD_4 counts and JC viremia.

Prospective study

All patients were followed up for 1 year. Among the 13 positive patients presenting no neurological symptom, two (15.4%) presented signs of PML within the year following the first JCV detection in

Table 1 Prevalence of JC Virus viraemia in HIV-infected patients with or without neurological disorders in seven French centres

	Neurological disease				*Without neurological disease*	
	Without PML		*PML*			
	Positive	n	*Positive*	n	*Positive*	n
Bordeaux	3	13	2	5	2	13
Marseille	3	16	2	3	1	9
Paul Brousse	2	21	3	6	0	8
Reims	2	5	0	0	2	15
Rennes	2	13	0	1	1	15
Rouen	1	17	2	3	4	25
Toulouse	1	4	0	2	3	11
Total (%)	14 (15.6)	90	9 (47.4)	19	13 (13.5)	96
Positive/total (%)		23/109 (21.1)			13/96 (13.5)	

n: number of patients studied per cent

Table 2 Distribution of JCV detection for different blood compartments (cells or plasma) in HIV-infected patients presenting neurological symptoms or not

	Neurological disease		*Without neurological disease*
	Without PML n=*90*	*PML* n=*19*	n=*96*
Positive cells	9 (10%)	6 (31.6%)	8 (8.3%)
Positive plasma	3 (3.3%)	0 (0%)	4 (4.2%)
Cells and plasma positive	2 (2.2%)	3 (15.8%)	1 (1%)

blood. JCV genome was detected in the cells of one patient and in the cells and plasma of the other. For the 14 positive patients with neurological disorders except PML, three (21.4%) developed PML later and the DNA was detected in the cells in all cases. Thus, five patients out of the 27 (18.5%) carrying JCV in blood at the time of the study developed PML during the following year (Table 3). On the other hand, two out of the 159 (1.3%) patients negative for JCV detection developed PML (Table 3). Therefore, JCV DNA in blood was associated with a strong negative predictive value (0.99) for the development of PML, and only a limited positive predictive value (0.19).

Moreover, the survival of the 19 PML patients was investigated in relation to JCV DNA detection in blood. Among the PML patients, 14 had a limited survival from 2 to 14 months, irrespective of JCV detection in blood. However, five PML patients are still alive 17 to 34 months after the diagnosis. Among them, three were JCV-negative in blood at the beginning of the study and two were positive. They are patients included later in the study and who received anti-retroviral treatments including protease inhibitors. This long survival might be related more to the treatment than to the detection of JCV DNA in blood. However for the seven patients who developed PML during the year of follow up, three died despite a highly active antiretroviral therapy and four are still alive.

The risk for opportunistic infection changed during the year of follow up, since the protease inhibitors were progressively introduced into anti-retroviral regimens in France in 1996. However most patients had comparable risks and treatments (associated RT-inhibitors) at the beginning of the study.

Discussion

For several years blood cells and notably B lymphocytes of PML patients (Houff *et al*, 1988; Monaco *et al*, 1996) have been suspected to play a role in the pathogenesis of human JC virus infection. In an attempt to analyse the hematogenous spread of JCV into the CNS, some authors have investigated peripheral blood cells to detect JCV DNA in immunosuppressed or immunocompetent patients (Azzi *et al*, 1996; Dorries *et al*, 1994; Dubois *et al*, 1996, 1997; Ferrante *et al*, 1996; Schneider and Dorries, 1993; Sundsfjord *et al*, 1994; Tornatore *et al*, 1992; Gallia *et al*, 1997). The results are variable and JCV genome detection in blood has varied from 0% (Sundsfjord *et al*, 1994) to 100% (Dorries *et al*, 1994). Within the context of the ANRS (Agence Nationale de Recherche sur le SIDA), seven French centres first tried to determine JCV blood prevalence for a larger population of HIV-infectd patients, and second, to monitor their clinical outcome.

Table 3 JCV presence in blood of patients who developed PML during the follow up study and those who did not

	JCV DNA in blood	
	Positive	*Negative*
PML	5 (18.5%)	2 (1.3%)
No sign of PML	22 (81.5%)	157 (98.7%)

In our study 17.6% of the 205 HIV positive patients carried JCV DNA in their peripheral blood the percentage varying from 10.3 to 23.5% depending upon the centres with an even data distribution. This result is low in comparison with most other studies (Azzi *et al*, 1996; Dorries *et al*, 1994; Dubois *et al*, 1996, 1997; Tornatore *et al*, 1992) but very similar to the data presented by Ferrante *et al* (1996) if PML patients and AIDS patients suffering from different neurological disorders were analysed together (19%). Such discrepancies could be due to the different methods used in these studies, notably the DNA extraction procedure, PCR primers, choice of amplified sequence (early, late or regulatory gene) and the PCR detection method, which may affect the sensitivity and the specificity of the reaction. For the present study, the same technique was used in all participating laboratories. It is a sensitive PCR procedure, since one to six genome copies could be detected per reaction.

Interestingly, we found no significant difference between patients with CNS diseases and subjects without neurological disease (21.1% and 13.5% respectively). However, the percentage of positive JC viraemia was increased in the group of patients with neurological symptoms because of the 19 PML patients. Indeed, JCV DNA presence in blood was significantly higher in PML patients (47.4%) than in those suffering from different CNS disorders ($P<0.005$). The question arises as to the meaning of this higher detection rate. The JC virus might have used the haematogenous route to reach the CNS; however, 55% of PML patients were negative for JCV DNA detection in blood, either because our search for JCV was conducted too late after the haematogenous spread, or because the virus was already latent in the CNS. In the latter case, JCV presence in blood might only be secondary to the onset of PML.

The follow up of positive patients showed that the presence of neurological diseases unrelated to PML did not seem to influence the subsequent development of PML. JCV detection in blood was sometimes associated with the emergence of PML. In fact, five (18.5%) of the JCV-positive patients developed PML in comparison to only two (1.3%) JCV-negative patients. In all instances, the virus was detected in leukocytes. This observation, combined

with the high rate of JCV detection in blood for PML patients, suggests that blood plays a role in PML pathogenesis. Indeed, the negative predictive value of JCV DNA detection in blood for the subsequent development of PML was particularly striking (0.99). In other words, the probability for an HIV-infected person with CD4 counts below 200×10^6 ml^{-1} to develop PML is extremely low when JCV DNA is not detectable in the blood.

By analogy with CMV or HIV plasma viral loads, JCV DNA was sought in plasma to detect free virions suggestive of an active multiplication state. The results were quite surprising. JCV DNA was found two to three times more often in cells than in plasma in the three groups of patients, and the virus genome was detected in plasma in about 36% of positive patients. For seven patients, plasma alone contained JCV DNA, suggesting that there were free virions in the plasma, but the negative result of the corresponding cells was intriguing. On the other hand, JCV DNA was never detected exclusively in the plasma of PML patients, and for those who later developed PML, JCV DNA was found in cells or in cells and plasma but never in plasma alone. These data suggest that JCV detection in blood cells could be more important for PML pathogenesis than the presence of JCV in plasma. Finally, the number of patients was small so more investigations should be conducted to confirm these observations in a larger series. However, JCV detection in blood did not seem to influence PML patients' survival.

This study by seven French laboratories points to a prevalence of about 18% for JCV viraemia in HIV-infected patients. The results also show that the existence of other neurological symptoms does not favour the development of PML. On the other hand, the absence of JCV detection in blood leukocytes should be considered as a favourable prognostic marker.

Materials and methods

Patients
Single blood samples were collected from 205 HIV-infected patients from seven hospital centres in France (Reims, Rouen, Rennes, Bordeaux, Toulouse, Marseille and Paris-Paul Brousse) between January and September 1996. Among these patients, 109 presented neurological disorders, such as CMV encephalitis (20), HIV encephalitis (13), VZV encephalitis (seven), cerebral lymphoma (seven), Toxoplasmosis (six), cryptoccal meningitis (six), encephalopathies with no etiological diagnosis (31). Nineteen had signs of PML. PML diagnosis was based on clinical, neuroimaging (Magnetic Resonance: MR) and CSF–PCR features. During the follow up of PML patients, the same criteria were used and MR was added to clinical evolution. These patients presenting neurological diseases were compared with 96 patients without any neurological symptoms. All patients had fewer than 200×10^6 CD_4 lymphocytes per ml.

Sample preparation
Peripheral blood leukocytes (PBLs) were separated from 5 to 7 ml whole blood collected in EDTA-containing tubes by sedimentation for 2 h at room temperature, centrifugation at 1500 *g*, red cell lysis (NH_4Cl 0.8%) and washing with PBS. Pellets of 10^6 leukocytes were frozen until DNA extraction which was performed using proteinase K digestion (400 μg per sample) and a classical phenol-chloroform and chloroform-isoamylalcohol procedure. The plasma was centrifuged at 10 000 *g* for 30 min, the pellet was resuspended in lysis buffer containing proteinase K and DNA was extracted with the method described above.

PCR assay
A 173 pb fragment from the large T coding early gene was amplified with the primer pair PEP1 and PEP2 (Arthur *et al*, 1989; Moret *et al*, 1993). A total of 20 μl of extracted DNA (i.e. 1 to 2 μg) was added to the 30 μl reaction mixture consisting of 10 mM Tris HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, 200 μM of each deoxynucleotide triphosphate, 2 Units Taq Polymerase (AmpliTaq Perkin Elmer) and 0.5 μM of each primer. The 173 bp DNA fragments obtained by PCR were hybridised with the 5′ biotin-labelled JCV-specific JEP-1 molecular probe (Arthur *et al*, 1989; Moret *et al*, 1993) by liquid phase hybridisation using a DNA enzyme immunoassay (DEIA, Sorin, Saluggia, Italy) (Vignoli *et al*, 1993) according to the instructions of the manufacturer. All positive results with liquid phase hybridisation were confirmed by a Southern blot procedure. For the JCV positive plasma samples, a beta globin PCR (Saiki *et al*, 1985) was performed in order to detect DNA cellular contamination.

Southern blot
For Southern analysis, PCR products (20 μl) were separated by electrophoresis on ethidium bromide stained 2% agarose gel, transferred to a nylon membrane (Hybond- N+; Amersham, Buckinghamshire, UK) and UV cross-linked. For three centres, prehybridisation conditions were $3 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% SDS, $5 \times$ Denhardt's solution and 30% formamide at 42°C for 2 h. For hybridisation, 107 c.p.m. of JEP-1 probe labelled at the 5′ end with [^{32}P]dATP and T4 polynucleotide kinase were incubated with the membrane for 18 h at 42°C. The membrane was washed for 15 min in $2 \times$ SSC–0.5% SDS at room temperature, 15 min in $1 \times$ SSC–0.5% SDS and 10 min at 50°C in $0.5 \times$ SSC–0.5% SDS. It was then dry-blotted and exposed to Kodak X-Omat film at −70°C with an intensifying screen for 1 to 3 days. For the other centres, Southern blot hybridisation was performed with digoxigenin-ddUTP end la-

belled JEP-1 probe using a 'DIG Luminescent Detection Kit' (Boehringer Mannheim, Mannheim, Germany) according to the instructions of the manufacturer.

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