Journal of NeuroVirology, 9(suppl. 1): 81–87, 2003
© 2003 Taylor & Francis ISSN 1355-0284/03 $12.00+.00
DOI: 10.1080/13550280390195379

Taylor & Francis
Taylor & Francis Group

Association of human polyomavirus JC with peripheral blood of immunoimpaired and healthy individuals

Kristina Dörries,[1] Silviu Sbiera,[1] Klaus Drews,[1] Gabriele Arendt,[2] Christian Eggers,[3] and Rüdiger Dörries[4]

[1]Institut für Virologie und Immunbiologie der Julius-Maximilians-Universität, Würzburg, Germany; [2]Neurologische Klinik, Heinrich-Heine-Universität, Düsseldorf, Germany; [3]Neurologische Klinik, Universitätskrankenhaus Eppendorf, Hamburg, Germany; and [4]Institut für Medizinische Mikrobiologie und Hygiene, Ruprecht-Karls-Universität Heidelberg, Klinikum Mannheim, Mannheim, Germany

JC virus (JCV) infection is regularly asymptomatic in healthy individuals. In contrast, in immunocompromised individuals, highly activated virus replication may lead to PML. Peripheral blood cells (PBCs) are found to habor JCV DNA in healthy and diseased individuals and it is discussed that they might be responsible for dissemination of the virus to the central nervous system (CNS) during persistence. To better understand the role of JCV DNA in PBCs for persistent infection and pathogenesis, the authors characterized the extent of JCV infection in Ficoll-gradient purified blood cells (peripheral blood mononuclear cells [PBMCs]) of healthy and human immunodeficiency virus type 1 (HIV-1)–infected individuals. Virus activation in PBMCs from healthy JCV-infected individuals was found at a rate of 0% to 38% at low polymerase chain reaction (PCR) sensitivity. In progressive multifocal leukoencephalopathy (PML) patients, a stronger signal was found, indicating increased virus activation. JCV DNA was regularly detected in T and B lymphocytes and in monocytes at low levels. However, granulocytes were shown to be the predominant reservoir of JCV DNA harboring high copy numbers. Although the overall distribution of viral genomes holds true for the population studied, in the individual, a markedly changed pattern of distribution can be found. *Journal of NeuroVirology* (2003) **9(suppl. 1),** 81–87.

Keywords: blood donor; granulocytes; HIV-1; JCV; leukocytes; persistence; PML; subpopulation

Introduction

Primary contact with the human polyomaviruses usually results in life-long persistence of viral genomic information. Virus replication seems to be closely linked to immunological competence of the infected host. Although persistent polyomavirus infection was believed to be latent in the immunocompetent healthy individual (Dörries, 2001), activated expression is seen during impairment of the immune system, as for example during pregnancy. During long-term immunodeficiency as in acquired immunodeficiency syndrome (AIDS) or lymphoproliferative disorders, asymptomatic virus infection can be followed by virus replication, resulting in cytolytic destruction of tissue and severe central nervous system (CNS) disease, namely polyomavirus-associated progressive multifocal leukoencephalopathy (PML).

Sites of persistent infection are the urogenital system and the CNS, occasionally leading to asymptomatic viruria or presence of JC virus (JCV) in the cerebrospinal fluid (CSF) of normal individuals.

Address correspondence to K. Dörries, Institut für Virologie und Immunbiologie, Versbacherstrsse 7, D-97078 Würzburg, Germany. E-mail: doerries@vim.uni-wuerzburg.de

The work was supported by a grant in the Graduate College 520 'Immunmodulation' from the Deutschen Forschungsgesellschaft and by grant 99.061.1/2 of the Wilhelm Sander-Stiftung.

Received 16 September 2002; revised 20 November 2002; accepted 3 January 2003.

Virus was observed in tonsils, bone marrow, peripheral blood cells, and plasma by polymerase chain reaction (PCR) in immunocompromised patients (Dörries, 1999; Jensen and Major, 1999) and it is feasible that JCV could be disseminated from the periphery to the CNS by infected blood cells (Dörries, 1999; Jensen and Major, 1999). In healthy individuals, full-length virus DNA was only occasionally detected by Southern blot analysis and expression of JCV genes in blood cells was not found (Andreoletti *et al*, 1999; Dolei *et al*, 2000; Koralnik *et al*, 1999a). Similar to findings in the CNS (Dörries, 2001), in human immunodeficiency virus type 1 (HIV-1)–infected patients, higher levels of JCV DNA were found in peripheral blood mononuclear cells (PBMCs) than in immunocompetent individuals. This indicates that suppression of the immune system leads to virus activation, resulting in higher copy numbers of JCV DNA in the blood.

To provide further insight into the role of hematopoietic cells as a site for JCV persistence, we examined the rate of JCV-positive blood cells in healthy German individuals and HIV-1–infected patients with and without PML by highly sensitive PCR techniques. Moreover, we investigated the distribution of JCV DNA in subpopulations of hematopoietic cells collected from immunocompetent persons.

Results

JCV DNA in PBMCs of persistently infected individuals

PBMCs of persistently infected individuals were isolated by Ficoll-Isopaque density gradients and presence of JCV DNA was analyzed by PCR. The experimental strategy involved a first round of standard PCR (sPCR) followed by nested PCR (nPCR) with internal primers, characterization of the products by gel electrophoresis, and radioactive Southern blot hybridization. Sensitivity of the primer pairs for sPCR was determined to be between 0.1 fg and 1 fg of JCV DNA or about 20 to 200 genome equivalents, although in the presence of chromosomal DNA (0.5 to 1 μg DNA), the sensitivity was reduced 10-fold. Using radioactive hybridization and nPCR increased the limits of detection between 10 and 100 times, resulting in sensitivity of 1 to 10 genome equivalents.

Analyses were performed on PBMCs collected from HIV-1–infected patients with neurological symptoms other than PML (10 cases), PML patients (6 cases), healthy blood donors (30 cases), and laboratory personel (8 cases) (Table 1, Figure 1**A**). Comparison of rates of JCV DNA between these four groups at different stages of PCR sensitivity revealed that the rate of JCV carriers after sPCR ranged from 0% to 38%. Radioactive Southern blot analysis enhanced the rate of detection to more than 60%. After PCR and radioactive hybridization, JCV DNA products were amplified in almost all samples (Table 1, Figure 1**A**).

Table 1 Prevalence of JCV in PBMCs of immunocompetent and immunoimpaired individuals

PBMCs	*No.*	*sPCR*°	*SB*°°	*nPCR*°°°
Immunocompetent				
Blood donors	30	7/30 (23.3%)	6/10 (60%)	15/16 (93.8%)
Laboratory personnel	8	3/8 (37.5%)	4/8 (50%)	8/8 (100%)
Immunodeficient				
HIV/OND§	10	0/10 (0%)	5/10 (50%)	10/10 (100%)
HIV/PML	6	2/6 (33.3%)	2/3 (67%)	5/6 (83.3%)
Total	54	12/54 (22.2%)	17/31 (54.8%)	38/40 (95%)

°Standard PCR; °°radioactive Southern blot analysis; °°°nested PCR; §OND, neurological symptoms other than PML.

Low rate of infection after sPCR confirmed that levels of viral DNA were generally very low. However, detection of JCV DNA in almost all individuals after nPCR suggested that JCV DNA is regularly associated with PBMCs of infected individuals.

Prevalence of JCV DNA in the group of immunocompromised HIV-1–infected ($n = 10$) and PML patients ($n = 6$) did not vary considerably compared to that in healthy individuals (Table 1). This was in contrast to earlier reports (Koralnik *et al*, 1999a) and prompted us to analyze JCV DNA in PBMCs of four and six serial blood specimens collected from two additional progressive PML patients. Interestingly, no JCV DNA could be amplified in any sample from one patient, whereas in the second patient, four of six samples were positive for JCV DNA after sPCR. This suggested that association of JCV DNA with PBMCs may vary considerably among PML patients and, if JCV DNA is present, virus load is not constant during the course of disease.

Semiquantitative PCR analysis of JCV target DNA by serial dilutions of PBMC DNA (10 to 500 ng) from the PML patient revealed that virus amplification was achieved at considerably lower cellular DNA concentrations (50 to 100 ng) compared to that of PBMC DNA from healthy and HIV-1–infected individuals (0.5 to 1 μg). Similarly, after *in situ* hybridization of PBMCs from a PML patient using a JCV-specific radioactive-labeled RNA probe, autoradiographic grains representing JCV DNA were more densely packed compared to PBMCs from healthy persons. In addition, the amount of labeled cells was higher than that in normal individuals (Figure 1**B**). In summary, during PML, more PBMCs carried a higher virus DNA load compared to that in healthy individuals (Dörries *et al*, 1994).

Association of JCV with subpopulations of peripheral blood cells

The role of peripheral blood cells for persistent JCV infection and possible consequences for pathogenesis

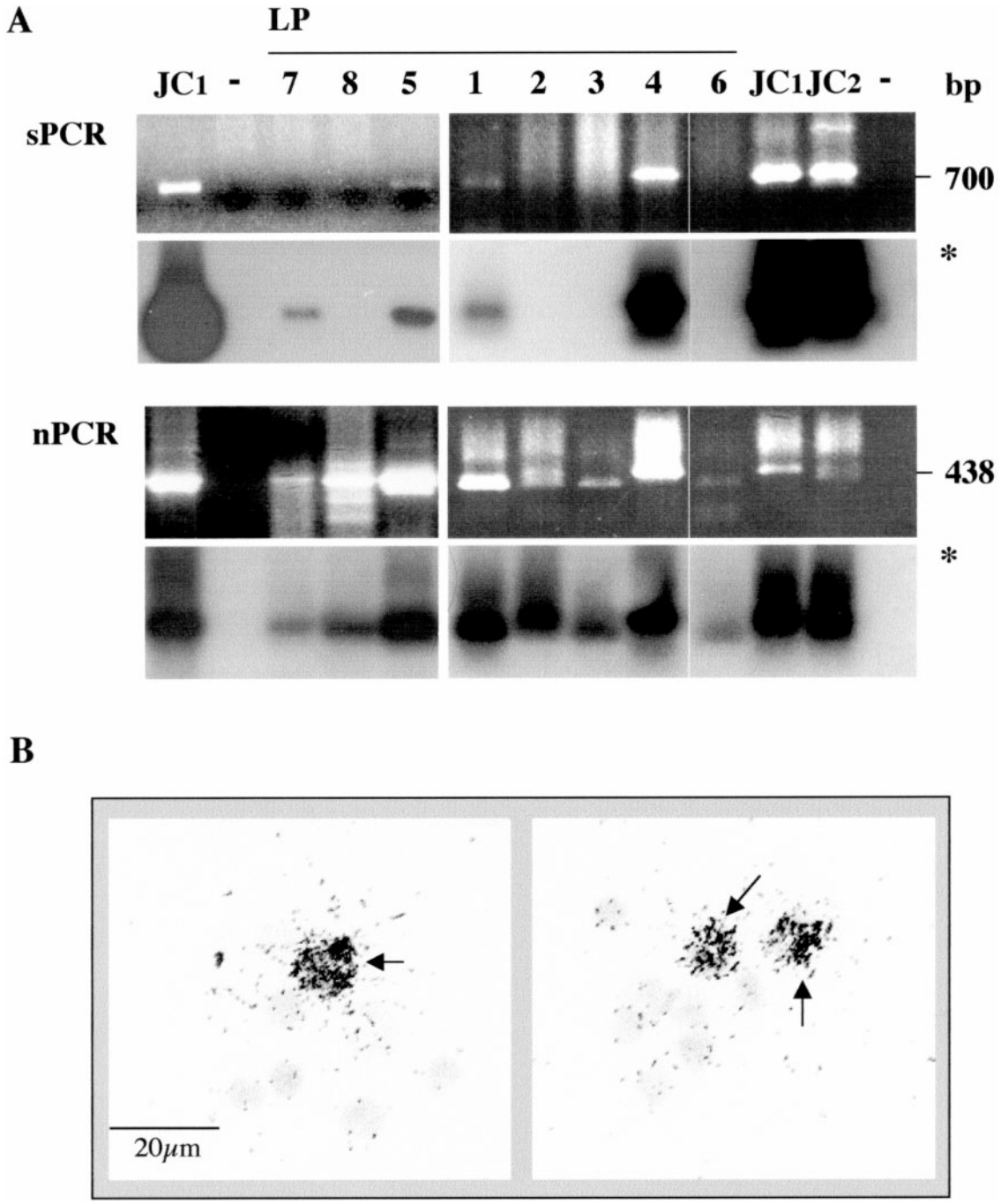
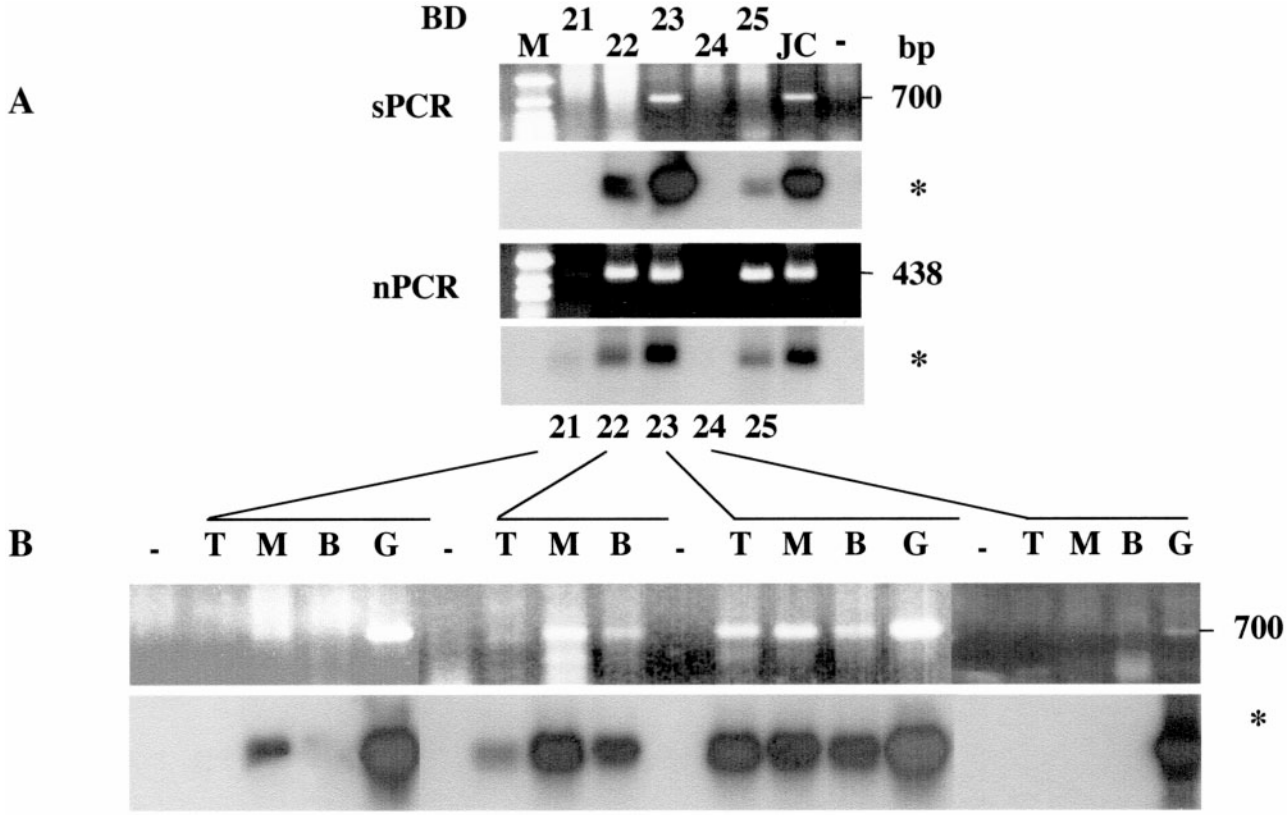


Figure 1 JCV DNA in peripheral blood cells of healthy individuals and PML patients. (**A**) JCV-specific PCR amplicons in PBMCs of healthy individuals. sPCR of JCV DNA sequences was performed on DNA from PBMCs of laboratory workers (sample LP 1–8) with primer pairs for the control region. Sensitivity was enhanced by nPCR with an internal primer pair. Electrophoretic characterization of products was followed by Southern blot hybridization using a radioactive JCV-specific ssRNA probe (◦). JC1/2: 0.1 pg of pJCVGS/B DNA served as PCR control with 1 and 2 units of Taq-polymerase, respectively; (○): contamination control without viral target DNA; bp: PCR product length. (**B**) JCV DNA associated with PBMCs of PML patients as demonstrated by *in situ* hybridization with a JCV-specific radioactive ssRNA probe followed by autoradiography. *Arrows*: Black grains represent hybridization of JCV-specific DNA above cells counterstained with Gill's hematoxilin. Size calibration line = 20 μm.

might depend on the cell type targeted by the virus in the immunocompetent individual. Therefore, we examined which subpopulations of hematopoietic cells in the blood were associated with JCV DNA by PCR analysis of buffy coat cells from healthy blood donors. Cell separation of B lymphocytes, T lymphocytes, monocytes, and granulocytes was performed by magnetic cell separation using monoclonal

antibodies (MABs) specific for CD19, CD3, CD14, and CD66b surface markers, respectively. Alternatively, granulocytes were purified from peripheral blood cells by a two-step Percoll gradient. Success of the purification procedure was controlled by tricolor fluorescence staining and flow cytometric analysis. Typically, CD19+ B lymphocytes were enriched up to 85% to 96%, CD14+ monocytes between 58% and 97%, CD3+ T lymphocytes between 94% and 96%, and CD66b+ granulocytes between 95% and 98%.

Association of JCV with hematopoietic cells was determined by analysis of DNA from PBMC of eight blood donors and the respective subpopulations of blood cells. sPCR-amplified JCV-specific products in one (NL23) out of eight blood samples. In two additional samples, (NL22, 25) JCV DNA was detected at very low concentrations in PBMCs after Southern blot analysis. After nPCR and following Southern blot analysis, JCV DNA was detected in seven of eight samples. These results were in the same range as sPCR analysis on healthy individuals (Table 1). In Figure 2, the experiments are documented for five PBMC samples. Amplification rates increased from sPCR (1/5) to hybridization (3/5) and



C

	BD 21	BD 22	BD 23	BD 24
PBMC	-"/-§	-"/+§	+"/+§	-"/-§
T CD3+	-/-	-/+	+/+	-/-
M CD14+	-/+	+/+	+/+	-/-
B CD19+	-/+	+/+	+/+	-/-
G CD66b+	+/+	nd	+/+	+/+

Figure 2 JCV DNA in PBMCs, and leukocyte subpopulations of persistently infected healthy individuals. (**A**) PBMCs of five blood donors (BD) were subjected to sPCR with primers for the control region and nPCR with an internal primer pair, each followed by Southern blotting and radioactive hybridization (°). (**B**) Subpopulations of hematopoietic cells obtained from four blood donors were subjected to PCR analysis applying the same primer pairs. sPCR and subsequent Southern blot analysis revealed amplicons in cell populations of CD3+ T lymphocytes (T) and CD19+ B lymphocytes. M sPCR: marker bands are pMALc-2/MspI DNA fragments; M nPCR: marker bands are pBluescript II KS(+)/MspI DNA fragments; JC: amplification control with pJCVGS/B DNA; (○): contamination control without viral target DNA; bp: PCR product length. (**B**), CD14+ monocytes (M) or CD66b+ granulocytes (G). (**C**) JCV amplification in PBMCs and blood cell subpopulations. +/○ : distribution of cell populations associated with JCV DNA in individual blood donors. ″/§: sPCR/Southern blot analysis.

nPCR and Southern blot analysis (4/5). Subsequent analysis of leukocyte subpopulations revealed that in cases of a low product yield in PBMCs (NL21, 22), JCV DNA was detected in CD19+ B lymphocytes, CD14+ monocytes, and CD66b+ granulocytes at varying concentrations. Similarly, in the donor with the strongest signal in unselected PBMCs (NL23) JCV DNA was amplified in each individual cell population with an important signal in the CD66b+ granulocyte fraction. Interestingly, in the unselected negative sample, NL24, exclusively the CD66b+ granulocyte population carried JCV DNA after cell separation.

In summary, our data demonstrated highly variable concentrations of JCV DNA in samples of peripheral blood cells from healthy individuals. JCV DNA can be shown to be associated with each cell population analyzed, including monocytes. However, the most prominent signal of JCV DNA was obtained by amplification of cellular DNA from CD66b+ granulocytes.

Discussion

The unresolved question for the role of JCV in peripheral blood led us to investigate the presence of JCV DNA in peripheral blood cells of healthy individuals by amplification of JCV target DNA. The strategy for PCR included standard first PCR followed by sensitivity enhancement by nPCR technique and radioactive Southern blot analysis. Apart from selection of highly sensitive primer pairs specific for JCV, each individual step from sPCR to nPCR and hybridization was controlled by samples without target DNA. Only those experiments were included in the study, in which all controls without target DNA remained negative. Stepwise analyses of PBMC samples revealed that after sPCR, between 23% and 38% of samples were found positive, whereas after Southern blot analysis of the sPCR products, 50% to 67% were found to carry JCV DNA. Only by nPCR JCV DNA was detected in nearly all samples. This confirms that the level of JCV DNA in many cells is very low, requiring highly sensitive detection techniques.

Amplification rates of JCV DNA in PBMCs of immunoimpaired patients after first PCR were comparable to those in healthy individuals. In contrast, semiquantitative PCR analysis of JCV DNA as well as *in situ* hybridization of PBMCs supported the assumption of a higher virus load in individual PBMC samples from PML patients compared to that of healthy individuals. This descrepancy was explained by the analysis of sequential blood cell samples of two PML patients. JCV DNA was detectable neither in all PBMC samples nor in each patient. From these data, the question of a possible association of JCV activation in peripheral blood and immunosuppression as induced by HIV/PML could not be decided (Andreoletti *et al*, 1999; Koralnik *et al*, 1999a, 1999b).

Although the rate of individuals carrying JCV DNA in PBMCs is controversially discussed between laboratories, there is agreement on a low virus load in peripheral blood (Andreoletti *et al*, 1999; Koralnik *et al*, 1999a, 1999b). Nevertheless, because association of JCV with blood cells might be limited to distinct subpopulations, characterization of the affected cell type could be helped by improved detection methods. With the highly sensitive amplification system, we succeeded in amplification of JCV DNA from subpopulations of blood cells where it was not possible in whole PBMCs. These findings are in line with a generally low virus load in peripheral blood cells of healthy donors and point to a preference of the virus for certain cell types.

However, the association of JCV DNA with cellular subpopulations in individual blood samples was highly heterogeneous. It ranged from all cell types analyzed to only one out of four. Similarly, the amount of JCV DNA in individual subpopulations varied from sample to sample. Although the findings were highly heterogeneous between individuals, it became clear that the granulocyte is a preferred cell type for harboring JCV DNA. Because also B lymphocytes were occasionally found positive for JCV DNA by PCR, our data are not in contradiction to earlier findings that B lymphocytes are susceptible to JCV infection. The variable amplification pattern rather suggests a complex interaction of the virus with different subpopulations of hematopoietic cells. This may include persistent virus infection (Jensen and Major, 1999) as well as attachment to cellular membranes (Wei *et al*, 2000) or phagocytosis of virus particles. The level of DNA concentration in blood donors is far below that described in the PML patients. Therefore, it remains to be determined whether the virus DNA in peripheral blood cell populations of PML patients might be a useful marker for progress of disease and thus could facilitate diagnosis and therapeutic intervention.

Material and methods

Patients material

Blood samples were collected from 6 HIV-1 patients with histopathologically and virologically diagnosed PML, from 10 HIV-1 infected patients with neurological diseases other than PML, and from a group of 8 laboratory workers. Peripheral blood cells of blood donors ($n = 30$) were prepared from buffy coats of the blood bank, Bavarian Red Cross, Würzburg.

Isolation and phenotypic characterization of blood cells

PBMCs were isolated by Isopaque Ficoll-gradient (density 1.0772 g/ml) centrifugation (Dörries *et al*, 1994). Granulocytes were purified by a two-step Percoll gradient (1.0772 g/ml and 1.090 g/ml) at 600 ∘ *g* for 30 min at 20°C. Cells were located at

the interphase of the Percoll layers. T lymphocytes, monocytes, and B lymphocytes were separated from PBMCs by positive selection with mouse MABs conjugated with magnetic beads (MACS) (Milteny, Germany). MABs were specific for human CD3 (IgG2a), CD14 (IgG2a), and CD19 (IgG1), respectively. The separation procedure was essentially as described by the manufacturer. Phenotypic characterization of enriched blood cell subpopulations was performed by direct immunofluorescence followed by flow cytometry. In addition, MABs specific for CD3 (fluorescein isothiocyanate [FITC]-conjugated, mouse IgG1, clone UCHT-1; Immunotech), CD14 (phycoerythrin-conjugated, mouse IgG2a kappa, clone RMO52; Immunotech), CD19 (phycoerythrin-cyanine dye label 5 conjugated, mouse IgG1, clone J4.119; Immunotech), or CD66b (FITC-conjugated, mouse IgG1 kappa, clone 80H3; Immunotech) were used as primary antibodies for staining of cells. Isotype-matched control antibodies (clone U7.27, mouse IgG2a; clone 679.1Mc7, mouse IgG1; Immunotech) were used as negative controls. All labeling steps were performed on ice in a total volume of 100 μl. Labelling was carried out by incubation of cells ($1 \circ 10^4$ to $2 \circ 10^5$) for 30 min with the respective MABs followed by fixation in 3% paraformaldehyde. Subsequently, cells were washed and $1 \circ 10^4$ events were assessed on a FACScan (Becton & Dickinson) using CellQuest 1.2.2 analysis software.

Characterization of JCV-specific DNA by PCR and in situ *hybridization*

DNA was purified from peripheral blood cells by the classic extraction method, including lysis of cells with 1% sodium dodecyl sulfate (SDS), proteinase K treatment, repeated phenol extraction followed by RNase treatment, and either dialysis or ethanol precipitation (Dörries *et al*, 1994). Cellular DNA was tested for inhibitory factors with beta-globin primers and JCV-specific PCR was performed using 500 ng to 1 μg cellular DNA at a reaction volume of 50 μl. The variant control region with flanking late and early coding DNA sequences of the JCV genome was amplified with primer pairs 71/72 or 71/157 (about 700-bp product length) (Dörries *et al*, 1994; Drews *et al*, 2000) and with the primer pair 160/161 for the late region (907-bp product). Sensitivity was enhanced by nested PCR with JCV-specific internal primers 53/61 for the control region (438 bp) (Drews *et al*, 2000) and 227 (–CTG CCA CAG GAT TTT CAG TAG–) and 228 (–CC G GAG CTC CAG TTA TTA CA–) for the late region (168 bp). If not stated otherwise, conditions for PCR, cycling, and quantitative competitive PCR were chosen as described previously (Drews *et al*, 2000). PCR was tightly controlled by introduction of negative controls prior and after each amplification step. Experiments were only accepted if all controls remained negative after Southern blot analysis. Amplification controls were performed with JCV plasmid DNA. Amplicons were characterized by electrophoretic separation followed by standard Southern blotting. Hybridization was performed with a JCV-specific ssRNA probe ^{32}P-radioactively labeled by *in vitro* transcription with T7 polymerase on the respective pJCV-BluescriptKS+ clone (Dörries *et al*, 1994) or by a JCV-specific internal primer 188 (–TAC TTT GGG TGT ATG AGT GGC–) labeled by T4 polynucleotide kinase (Drews *et al*, 2000; Eggers *et al*, 1999). For *in situ* hybridization (Dörries *et al*, 1994), PBMCs were air-dried onto slides, fixed with methanol–acetic acid, washed extensively in $2 \circ$ SSC, denatured, and hybridized at 45°C for 16 h in 50% formamide with a ^{35}S-radioactive labeled ssRNA of about 500 bp in length. After RNase treatment, slides were exposed to autoradiographic emulsion for 3 weeks and counter stained with Gill's hematoxilin solution.

References

Andreoletti L, Dubois V, Lescieux A, Dewilde A, Bocket L, Fleury HJ, Wattre P (1999). Human polyomavirus JC latency and reactivation status in blood of HIV-1-positive immunocompromised patients with and without progressive multifocal leukoencephalopathy. *AIDS* **13:** 1469–1475.

Dolei A, Pietropaolo V, Gomes E, Di Taranto C, Zicchedda M, Spanu MA, Lavorino C, Manca M, Degener AM (2000). Polyomavirus persistence in lymphocytes: prevalence in lymphocytes from blood donors and healthy personnel of a blood transfusion centre. *J Gen Virol* **81:** 1967–1973.

Dörries K (1999). Human polyomaviruses in target cells of hematopoietic origin and mechanisms of activation in the course of infection. *Curr Topics Virol* **1:** 84–93.

Dörries K (2001). Latent and persistent human polyomavirus infection. In: *Human polyomaviruses. Molecular and clinical perspectives*. Khalili K, Stoner GL (eds). New York: Wiley-Liss, pp 197–237.

Dörries K, Vogel E, Gunther S, Czub S (1994). Infection of human polyomaviruses JC and BK in peripheral blood leukocytes from immunocompetent individuals. *Virology* **198:** 59–70.

Drews K, Bashir T, Dörries K (2000). Quantification of human polyomavirus JC in brain tissue and cerebrospinal fluid of patients with progressive multifocal leukoencephalopathy by competitive PCR. *J Virol Methods* **84:** 23–36.

Eggers C, Stellbrink HJ, Buhk T, Dörries K (1999). Quantification of JC virus DNA in the cerebrospinal fluid of patients with human immunodeficiency virus-asociated progressive multifocal leukoencephalopathy—a longitudinal study. *J Infect Dis* **180:** 1690–1694.

Jensen PN, Major EO (1999). Viral variant nucleotide sequences help expose leukocytic positioning in the JC virus pathway to the CNS. *J Leukoc Biol* **65:** 428–438.

Koralnik IJ, Boden D, Mai VX, Lord CI, Letvin NL (1999a). JC virus DNA load in patients with and without progressive multifocal leukoencephalopathy. *Neurology* **52:** 253–260.

Koralnik IJ, Schmitz JE, Lifton MA, Forman MA, Letvin NL (1999b). Detection of JC virus DNA in peripheral blood cell subpopulations of HIV-1-infected individuals. *J NeuroVirol* **5:** 430–435.

Wei G, Liu CK, Atwood WJ (2000). JC virus binds to primary human glial cells, tonsillar stromal cells, and B-lymphocytes, but not to T lymphocytes [In Process Citation]. *J NeuroVirol* **6:** 127–136.