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New JC virus infection patterns by *in situ* polymerase chain reaction in brains of acquired immunodeficiency syndrome patients with progressive multifocal leukoencephalopathy

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Progressive multifocal leukoencephalopathy (PML), caused by the human polyomavirus JC (JCV), is an opportunistic infection of the central nervous system (CNS), the histopathological diagnosis of which can be made by routine staining. Very low copy numbers of JCV nucleic acid can be detected in paraffin sections by the specific and highly sensitive *in situ* polymerase chain reaction (*in situ* PCR). The authors evaluated JCV infection in 12 acquired immunodeficiency syndrome (AIDS) patients with PML by comparison of hematoxylin and eosin (H&E) staining, *in situ* hybridization (ISH), and *in situ* PCR. Phenotype of infected cells was determined by immunohistochemistry with antibodies against glial fibrillary acidic protein (GFAP) or cluster of differentiation 68 (CD68), focusing on cells containing low JC viral copy numbers, and on cell types that are normally not associated with papovavirus infection. The number of detectable JCV-positive oligodendrocytes increased markedly upon PCR amplification and hitherto unknown oligodendrocytic staining patterns were discernible: JCV DNA was detectable in both nucleus and cytoplasm, in cytoplasm only, and as ghost-cell silhouettes appearing as a membranous "rim" of staining product in some cells. The authors suggest that the staining patterns correspond to different stages of the viral replication cycle. Some human immunodeficiency virus (HIV)-type giant cells (HIV-GCs) were shown to contain JCV DNA, thus probably revealing a double infection. Macrophages and HIV-GCs showed staining in the cytoplasm and the nuclei, indicating that they not only may phagocytize JCV particles but may also be actively infected. CD68-positive GCs were occasionally noted to contain a complete JCV DNA–positive nucleus in their center, and were accordingly called JCV-type giant cells (JCV-GCs). Rarely, JCV DNA signals were noted in vascular endothelium. No JCV infection was detectable in lymphocytes, neurons, or in brain tissue of

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JCV-negative age-matched controls. The authors report new findings concerning inter- and intracellular JCV infection patterns in PML, possibly shedding new light on JCV susceptibility of different cell types in the brain of AIDS patients with PML. *Journal of NeuroVirology* (2004) **10,** 1–11.

Keywords: AIDS; CD68; double-staining methods; GFAP; HIV-GC; HIV; ISH; PCR; JCV-GC; JC virus; PML

Introduction

Progressive multifocal leukoencephalopathy (PML), caused by the human polyomavirus JC (JCV), is an opportunistic infection which can occur in patients with impaired cellular immunity. In the time span of about 15 years between the initial description of the acquired immunodeficiency syndrome (AIDS) and the availability of combined antiretroviral medication, AIDS accounted for the majority of all PML cases.

The histopathological diagnosis of PML can be made by conventional hematoxylin and eosin (H&E) staining. When abundant virus copies are present, the pathognomonic feature of nuclear viral inclusions within oligodendrocytes is easily recognizable. Further typical findings are bizarre or enlarged astrocytes and multifocal areas of demyelination (Aström *et al*, 1958; Richardson, 1961). JCV nucleic acid can be detected specifically by conventional *in situ* hybridization (ISH). However, this technique requires at least 200 to 1000 copies of viral genome per cell (Houff *et al*, 1988). A highly sensitive technique to detect smaller viral copy numbers in tissue sections is *in situ* polymerase chain reaction (*in situ* PCR) (Samorei *et al*, 2000). The specific aim of this postmortem study is the detection of JCV-infected cells containing low copy numbers and their further phenotypic characterization.

Results

Patterns of PML lesions in glial cells and myelin
A histopathological diagnosis of PML could be made by all three techniques used. Cellular JCV staining, number of JCV-infected cells, and intracellular distribution of viral deoxyribonucleic acid (DNA) were judged. Therefore, H&E-stained brain tissue sections revealing absent, mild, or severe involvement of PML were compared with corresponding JCV DNA distribution in individual cells by *in situ* PCR and ISH on parallel sections. As expected, sensitivity of detecting PML was highest using *in situ* PCR, because the number of stained oligodendrocytes increased two- to threefold upon PCR amplification of JCV sequences (Samorei *et al*, 2000), revealing an enhanced picture of JCV infection over that appreciated in ISH. The highly sensitive *in situ* PCR suggests a five-tiered staging of JCV infection: uninfected, sparse, mild, moderate, and severe (Table 1) (Samorei *et al*, 2000).

JCV-infected oligodendrocytes in sparse and mild PML
By *in situ* PCR, JCV DNA could be visualized in oligodendrocytes that appeared uninfected by H&E and even by ISH (degree 1). These oligodendrocytes revealed JCV DNA in their nuclei whereas the cells mostly still showed normal size and shape, and they were present either as single cells or in small clusters (degree 1 or 2). The underlying white matter appeared to be intact (degree 1), or showed pallor and rarely vacuolization (degree 2) (Table 1) (Samorei *et al*, 2000).

Intracellular oligodendroglial infection patterns in moderate and severe degrees of PML
Regardless of the degree of PML, the typical picture of oligodendrocytic infection was dominated by intranuclear JCV DNA. In rare cases, and only in moderate and severe degrees of infection, three additional hitherto novel staining patterns of individual oligodendrocytes were discernible using *in situ* PCR. Oligodendrocytes were seen to harbor JCV DNA in both nucleus and cytoplasm. Surprisingly, in some cells, JCV DNA also occurred as a peripheral linear (Figure 1) or less linear (Figure 2) membrane staining in the cytoplasm only. An unexpected finding was that of ghost-cell silhouettes with oligodendrocyte-like morphology. The latter were detectable within very necrotic regions, and appeared as a "rim" of staining product surrounding a nucleus-shaped cavity, and seemingly reflecting a stage of apoptosis (Figures 1 and 3, Tables 1 and 2) (Samorei *et al*, 2000). These structures, which tend to have ramified processes, resembling to be remnants of oligonucleoglial cells, were almost exclusively observed in *in situ* PCR sections and very rarely by ISH.

Cerebral white matter
Whereas in sparse or mild JCV infection the white matter appeared to be largely intact, in more advanced stages, the characteristic white matter destruction of different degrees became manifest either as pallor, demyelination, or frank necrosis. In regions of active infection, prominent neuropil staining was observed within areas harboring JCV-positive oligodendrocytes (Figure 3), as has previously been shown using ISH (von Einsiedel *et al*, 1993; Samorei *et al*, 2000). This staining, possibly representing cell-free DNA, was more prominent on *in situ* PCR sections than on parallel ISH-stained slides (Samorei *et al*,

Table 1 Histopathological staging of PML and corresponding JCV DNA distribution in individual cells by *in situ* PCR

	Degree of infection				
Degree of JCV infection	*0 (uninfected)*	*1 (sparse)*	*2 (mild)*	*3 (moderate)*	*4 (severe)*
Architecture of lesions					
JCV DNA-positive oligodendrocytes	All cells JCV negative	Single	Small clusters	3 components of lesions*: Necrotic centers: very few Margins: abundant Periphery: few	Few
JCV DNA-positive astrocytes	None	None	None	Rarely	Rarely
Myelin and neuropil	Intact	Intact	Pallor Rarely vacuolization	Necrotic centers: Frank necrosis No residual myelin Abundant macrophages	Disseminated macrophages Confluent lesions Extensive necrosis and marked disruption of tissue architecture
				Margins and periphery of PML lesions: Pallor Vacuoles and/or demyelination Cell-free viral DNA in vicinity of active JCV infection	Rarely: cell free JC viral DNA
JCV DNA distribution in individual oligodendrocytes					
	Normal size and configuration No JCV DNA staining	Normal size and configuration JCV DNA–positive nuclei	Normal and enlarged size JCV DNA–positive nuclei	Necrotic centers: Very few JCV DNA-positive oligodendrocytes Ghost cells Margins: Abundant enlarged JCV DNA–positive nuclei close to centers JCV DNA in cytoplasm JCV DNA in cytoplasm and nucleus Periphery: Enlarged JCV DNA–positive nuclei	Enlarged JCV DNA–positive nuclei JCV DNA in cytoplasm JCV DNA in cytoplasm and nucleus Ghost cells

*JCV infection proceeds in a centrifugal manner; lytic lesions start from the center.

Table 2 Novel JCV infection patterns by *in situ* PCR and surmised correlation to JCV replication cycle

Surmised stags of JCV replication cycle	*Localization of JCV DNA in oligodendrocytes and mylein*	*Comments*
Host cell entry	No oligodendrogilal staining	Not discernible because of low viral particles present
Active viral nuclear replication	Nuclear oligodendrogilal staining	Common intranuclear staining pattern
a) Virus replication and assembly as well as b) Transport of the progeny virions out of the cell	Both nucleus and cytoplasm of oligidendrocytes: a) Nuclear staining b) Cytoplasmic staining	Novel staining patterns
End stage in the course of viral cell infection	Cytoplasmic staining of oligodendrocytes only Nuclei are uninfected and intact	Novel staining patterns
Late stage after cytolysis	Ghost cell silhouettes of former oligodendrocytes in necrotic regions	Novel staining patterns
JCV debris in “burnt out” regions inside centers of necrosis	Cell-free virus	Novel staining patterns

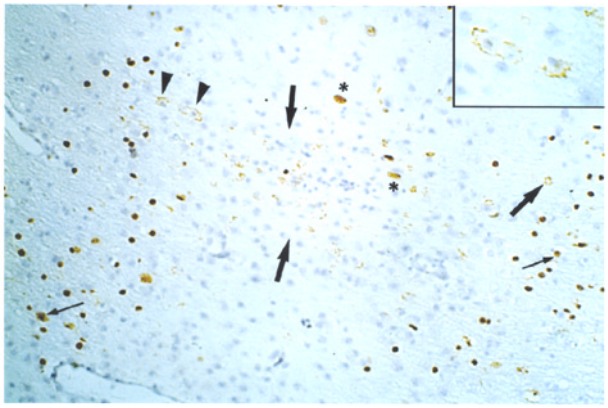

Figure 1 White matter with a necrotic center in a "moderate" stage JCV infection. Two small vessels are visible in the vicinity of the necrotic lesion. White matter surrounding this lesion appears to be intact. Enlarged oligodendrocytic nuclei with brown JCV DNA staining product dominate the picture, and surround the lesion. Some oligodrocytes show JCV DNA in both nuclei and cytoplasm (*small arrows*). Few cells harboring JCV DNA in the cytoplasm only, are present, which are evident as a linear outline at the peripheral cell membrane (*arrowheads* and *inset*). Ghost cell sillhouettes are detectable within the necrotic region (*thick arrows*). Traces of cell-free virus, depicted as granular stains, appear in the "burnt out" region of the necrotic center. The two JCV-infected oval-shaped cells (*) might be astrocytes. (*In situ* PCR for JCV DNA with brown DAB staining product, hematoxylin counterstain. Magnification ×125.)

2000). Unexpectedly, we also found cell-free DNA in the centers of necrosis, but only when using *in situ* PCR (Figures 1 and 3, Table 1).

Astrocytes with bizarre or enlarged nuclei, as well as glial fibrillary acidic protein (GFAP)-positive astrocytes of normal shape, were rarely detectable. In two cases however, we found both JCV and GFAP staining products in some of these glial cells, indicative of active JCV infection.

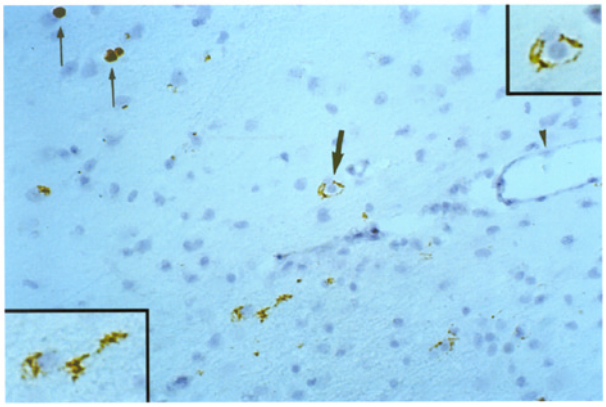

Figure 2 A rim of JCV DNA around an uninfected nucleus, which is presumably an oligodendrocyte. Although the cytoplasmic staining is similar to the appearance in Figure 1, it is less linear (*thick arrow*). A vessel (*arrowhead*) and some enlarged JCV-infected oligodendrocytes are notable (*thin arrows*). (*In situ* PCR for JCV DNA with brown DAB staining product, hematoxylin counterstain. Magnification ×250.)

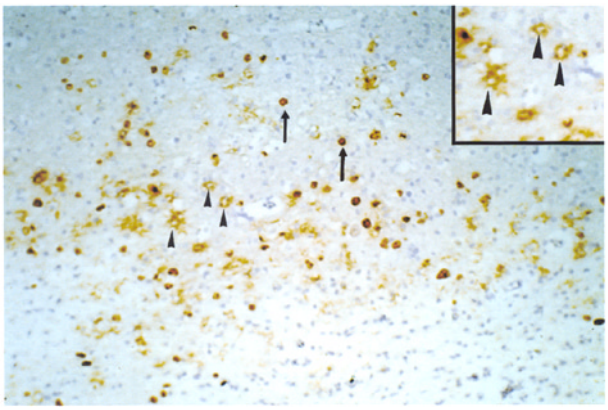

Figure 3 Severe JCV infection with frank necrosis (at the bottom of the micrograph). Enlarged oligodendrocytic nuclei (*arrows*), and abundant ghost cells without apparent nuclei outline the PML lesion. These structures appear as a "rim" of staining product surrounding a nucleus-shaped cavity, with ramified processes, resembling remnants of oligodendrocytes, apparently reflecting a stage of apoptosis (*arrowheads*). Prominent neuropil staining is present (cell-free JCV DNA). (*In situ* PCR for JCV DNA with brown DAB staining product, hematoxylin counterstain. Magnification ×125.)

JCV infection patterns by in situ *PCR in cells of nonglial origin*

Macrophages accumulated in severely necrotic regions were infrequently found to contain JCV DNA. JCV staining in these lipid-laden foamy macrophages was always very pale, and viral DNA was either located in the cytoplasm or concurrently also within the nucleus of these histiocytes (Figures 4 and 5). Double labeling of *in situ* PCR or ISH in combination with cluster of differentiation 68 (CD68) was performed on some cerebral and cerebellar sections of brain. Technically, *in situ* PCR was performed prior to immunohistochemistry on the same sections, whereas others have accomplished immunohistochemistry first (Bagasra *et al*, 1996). In areas of virus spill into the neuropil, we could differentiate single CD68-positive macrophages harboring traces of cytoplasmic JCV DNA. In a cerebellar tissue section with severe necrosis of white matter, CD68-positive multinucleated cells infrequently demonstrated single phagocytized JCV-positive oligodendrocytes (Figure 6, Table 3). The cytoplasm of the cell shown is largely negative for CD68, and it cannot be completely ruled out that the structure might be an extracellular vacuole.

Human immunodeficiency virus (HIV)-type giant cells (HIV-GCs), which are cells in brain tissue strongly indicative of HIV infection (Sharer *et al*, 1985, 1986; Budka, 1986; Navia *et al*, 1986a, 1986b; Vinters and Anders, 1990), were evaluated for the presence of JCV DNA. We could only detect these syncytial cells very rarely, either in the proximity of or within necrotic PML lesions. Two different JCV infection patterns were present in HIV-GCs. JCV DNA could be found in the cytoplasm only (Figure 7).

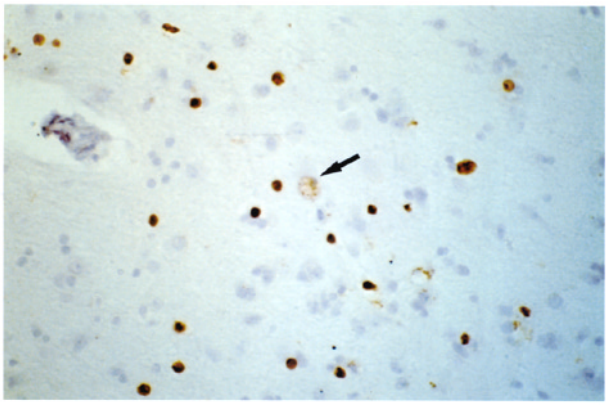

Figure 4 A macrophage within a necrotic PML region harboring JCV-staining product in the cytoplasm in small clusters (*arrow*), surrounded by an JCV-infected oligodendroglial nuclei of different sizes. (*In situ* PCR for JCV DNA with brown DAB staining product, hematoxylin counterstain. Magnification ×250.)

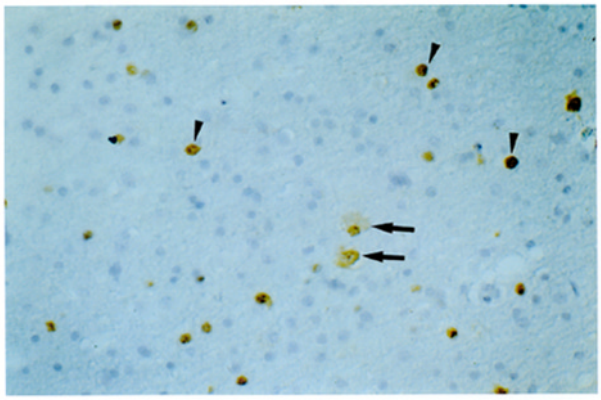

Figure 5 Two JCV-infected macrophages (*arrows*) with light staining product in the excentric nuclei and the cytoplasm. Infected oligodendroglial nuclei are indicated by arrowheads. The white matter is moderately preserved. (*In situ* PCR for JCV DNA with brown DAB staining product, hematoxylin counterstain. Magnification ×250.)

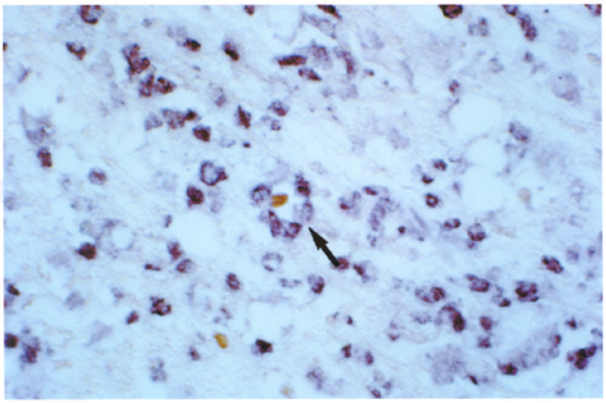

Figure 6 CD68-positive single-cell macrophages (*blue stain*) in a region of severe necrosis. One multinucleated JCV giant cell (JCV-GC) in the center (*arrow*), composed of CD68-positive macrophages (*blue staining product*) enclosing one JCV DNA-positive nucleus (*brown staining product*). The cytoplasm of the cell shown is largely negative for CD68, and it cannot be completely ruled out that this structure might be an extracellular vacuole. (Double labeling: *In situ* PCR for JCV DNA showing brown DAB staining product, and CD68-positive cells using NBT/BCIP to reveal a blue staining product. Magnification ×250.)

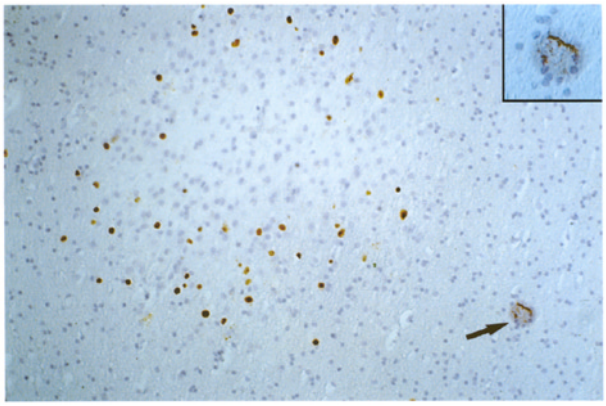

Figure 7 Presumed JCV-infected HIV-GC (*arrow* and *inset*) close to a region of moderate JCV infection. The HIV-GC harbors JCV DNA in the cytoplasm. (*In situ* PCR for JCV DNA with brown DAB staining product, hematoxylin counterstain. Magnification ×125).

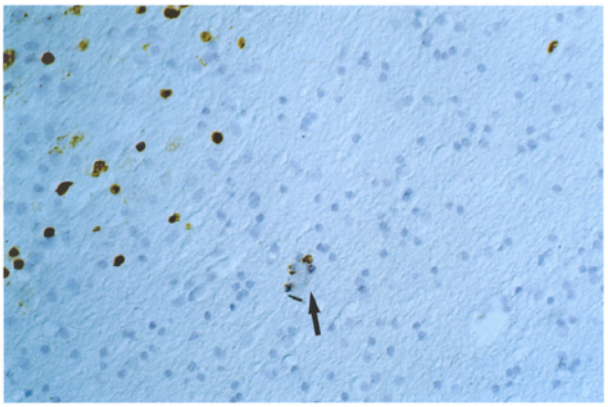

Figure 8 Presumed HIV-GC (*arrow*) with five nuclei, of which three are JCV infected whereas the cytoplasm is almost JCV free, apart from a few small granules in the cytoplasm. The nuclei of this cell are not as large as the JCV-positive oligodendrocyte nuclei in the nearby PML lesion; they reveal a paler stain and are not as uniformly stained as the oligodendrocytes. Artificial blue bar below the cell. The HIV-GC is located next to a lytic region of JCV infection. The edge of the necrotic lesion reveals the typical enlarged infected oligodendrocytic nuclei. (*In situ* PCR for JCV DNA with brown DAB staining product, hematoxylin counterstain. Magnification ×250.)

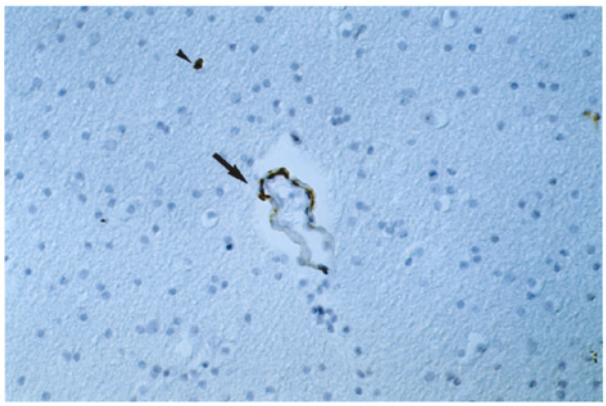

Figure 9 JCV-infected vascular endothelial cells (*arrows*) in a region with only one JCV-infected oligodendrocyte (*arrowhead*). (ISH for JCV DNA with brown DAB staining product, hematoxylin counterstain. Magnification ×250.)

Table 3 Novel JCV infection patterns by *in situ* PCR in cells of nonglial origin.

Cells of nonglial origin	*Localization of JCV DNA*	*Comments*
Macrophages	Cytoplasmic staining	JCV debris phagocytized
	JCV giant cell	Complete cells phagocytized
	Both nuclear and cytoplasmic staining	Both active infection and phagocytosis
HIV-GCs	JCV DNA in the nucleus	Double infection of HIV and JCV = active JCV infection
	JCV DNA in cytoplasm	Double infection of HIV and JCV = phagocytosis
Vessels	Vascular endothelial cells*	Active infection

*Only perfomed by ISH.

Interestingly, few multinucleated HIV-GCs also contained JCV staining product, mainly within several of their nuclei, and diminutive granules in the cytoplasm. The nuclei of these cells were not as large as the positive oligodendrocyte nuclei in the nearby PML lesion, nor were they darkly or uniformly stained. These JCV DNA-positive HIV-GCs were located in close proximity to PML lesions, and apart from necrotic areas (Figure 8, Table 3).

Vascular endothelial cells usually remained free of JCV DNA even when located within necrotic regions, or in vicinity of infected oligodendroyctes. In one brain specimen, however, these cells seemed to be stained positive for JCV in several capillaries using ISH (Figure 9). Unfortunately, the experiment could not be repeated with *in situ* PCR, as tissue from directly adjacent sections was not available.

Cell types without JCV infection. In numerous brain tissue sections of one patient, perivascular cuffing (PVC) was unusually severe and suggested an atypical immune response against JCV. In all regions investigated by *in situ* PCR and ISH, none of these mononuclear inflammatory cells—which were mainly lymphocytes—revealed JCV DNA. Neurons were never shown to harbor JCV by *in situ* PCR, even when JCV-positive satellite oligodendrocytes were in direct contact with them. Finally, the small number of brain samples serving as negative controls did not show JCV DNA.

Discussion

The primary aim of this *in situ* PCR study was the detection of cells containing low JCV copy numbers. Special emphasis was placed on the interpretation of different JCV oligodendrocytic staining patterns as well as on the investigation of potentially JCV-infected cells of non-glial origin.

Patterns of PML lesions in glial cells and myelin

Oligodendrocytic JCV DNA infection patterns by in situ *PCR correlated with JCV replication cycle:* JCV particles are directly detectable using electron microscopy in PML tissue. Different stages of the JCV replication cycle can be surmised from the intracellular localization of viral particles. Upon host-cell entry, JCV is located in the cytoplasm and transported to the nucleus, where replication takes place. Abundant virus copies are generated in the nucleus of the lytically infected oligodendrocytes. Subsequently, progeny virions are released into the cytoplasm and JCV particles are then present in both cell compartments. Cell lysis begins by disintegration of the cytoplasmic components followed by the nuclear membrane, with consequent virus spill into the neuropil (Mazlo and Tariska, 1980; Cole, 1996). Although nuclear integrity may be impaired in lytically infected oligodendrocytes, cell morphology could be maintained at least transitorily as has been demonstrated for the polyomavirus simian virus 40 (SV40)-infected epithelial cells releasing virions through cytoplasmic smooth membrane vesicles before cell lysis (Clayson *et al*, 1989).

Indirect visualization of viruses is possible using light microscopic methods; the most sensitive technique available for the detection of nucleic acids in tissue sections is *in situ* PCR. We hypothesize that different intracellular oligodendrocytic JCV infection patterns can be correlated with JCV replication stages on a light microscopic level.

At host-cell entry, the number of viral particles might be too low to be visualized, even when utilizing *in situ* PCR, which is the most sensitive indirect light microscopic detection method so far. Typical nuclear staining probably corresponds to the stage of active viral replication. The novel finding of intranuclear and cytoplasmic oligodendrocytic staining (Figure 1, Table 2) most likely mirrors the stage of virus replication and assembly (nuclear staining) and transport of the progeny virions out of the cell (cytoplasmic staining). In a former ultrastructural study using some of the same tissue samples, we could show membrane-bound viral particles within the cell cytoplasm (von Einsiedel *et al*, 1993), suggesting that our present finding is not an *in situ* PCR artifact due to overdigestion of the nuclear membrane. Another electron microscopy study has also localized JCV virions in both oligodendrocytic cell compartments thus supporting our assumption (Boldorini *et al*, 1993a, 1993b).

In regions of advanced PML, cells with cytoplasmic staining, but an uninfected and intact nucleus, were seen, either appearing as a linear peripheral cell membrane staining (Figure 2) or as a less linear outline (Figure 2). Based on its exclusive occurrence in moderate and severe lesions, this staining pattern possibly represents an end stage in the course of

viral cell infection. However, we cannot exclude that cells with cytoplasmic JCV staining product are possibly macrophages, because for technical reasons, we did not perform double staining on every slide investigated.

In necrotic regions in which tissue architecture was no longer recognizable, ghost cells became visible after amplification, detected as a rim of cytoplasmic membrane–bound staining product with ramified processes, surrounding a nucleus-shaped cavity, resembling remnants of oligodendrocytes. We do not consider these structures to mirror astrocytes, although they present starlike processes, as it would be very unusual to find abundant astrocytes in areas of JCV-induced necrosis. Generally, we rarely found GFAP-positive astrocytes, and they were not located in regions of advanced regions of PML (Figures 1 and 3) (Samorei *et al*, 2000). The hazy outline consisted of JCV DNA staining product, which was no longer cell bound but still located close to the former host cell. Yet again, the distinction of these cells from macrophages cannot be made conclusively. With respect to the JCV replication cycle, these cell-like structures might represent a late stage after cytolysis, which may result in clearing the nucleus from viral DNA, as has been shown by SV40 (Clayson *et al*, 1989), speculatively secondary to apoptosis.

Neuropil
Cell-free JCV DNA is detectable in the neuropil by ISH (von Einsiedel *et al*, 1993) and *in situ* PCR. The myelin of severely infected oligodendrocytes revealed reaction product when using ISH, however, the amount of extracellular viral DNA was markedly enhanced after amplification, as has been shown previously (Samorei *et al*, 2000). By *in situ* PCR, we observed JCV DNA, appearing as granular stains not only in the myelin but also large amounts of cell-free DNA in regions of necrosis, so called "burnt out" regions (Figures 1 and 3). We do not consider the myelin-associated extracellular staining signal to represent diffusion artifact, because we monitored optimal cytoplasmic and nuclear pore size by fluorescein isothiocyanate (FITC)-labeled molecules (Samorei *et al*, 2000). Furthermore, several ultrastructural studies (ZuRhein and Chou, 1965; Papadimitriou *et al*, 1966; Kepes *et al*, 1975; Mazlo and Tariska, 1980,1982; Orenstein and Jannotta, 1988), including our own, have revealed extracellular papovavirus particles either between myelin lamellae or free in the interstitium (von Einsiedel *et al*, 1993).

JCV infection patterns by in situ *PCR in cells of non-glial origin*

Macrophages: In regions with central necrosis, large numbers of macrophages were present (Figures 1, 3, 4, 6, 7, Table 3). These phagocytes may contain JCV in their cytoplasm, as has been demonstrated ultrastructurally (Boldorini *et al*, 1993a, 1993b) as well as by ISH (Wiley *et al*, 1988; Vazeux *et al*, 1990). In a previous ISH study (von Einsiedel *et al*, 1993), we failed to demonstrate JCV in macrophages, in accord with the results of others (Ironside *et al*, 1989; Aksamit *et al*, 1990). When using single labeling by *in situ* PCR, we could confirm that macrophages reveal very pale staining product, suggesting low amounts of DNA (Figures 4 and 5). After double labeling with *in situ* PCR or ISH in combination with CD68 in some sections of cerebral or cerebellar tissue, we observed different forms of macrophages phagocytizing JCV DNA. In areas of virus spill into the neuropil, we detected single macrophages containing cytoplasmic JCV DNA. In addition, CD68-positive multinucleated cells had also appeared to phagocytize complete JCV-positive oligodendrocytes. These cells may be called JCV-type giant cells (JCV-GCs), in analogy to HIV-GCs (Figure 6). Some of the macrophages additionally showed nuclear staining, suggesting that they can also be actively infected (Figure 5), as has been shown by Mesquita *et al* (1992, 1996). We do not believe these cells to be HIV-GCs, because they are usually discovered in microscopically preserved tissues and in some distance to PML lesions, whereas JCV-GCs were found exclusively in centers of severe PML (degree 4).

Cells with double infection by JCV and HIV
In a previous study on some of the same tissue, we identified concurrent infection in three of the nine autopsy brains. We showed HIV-GCs and mononuclear cells to be HIV positive by immunohistochemistry for p24 antigen (von Einsiedel *et al*, 1993). Using *in situ* PCR, we have now investigated HIV-GCs for the presence of JCV DNA, and chose these HIV-pathognomonic syncytial cells by their light microscopic features. We admit not to have performed double labeling of HIV and JCV, because we used up to 20 parallel sections for validation of the *in situ* PCR method and had not always adjacent section available for further double-staining methods. Likewise, we had no non–HIV-infected PML cases obtainable for investigation of the presence of multinucleated, macrophage-derived cells. We are aware that there are many pathways leading to syncitial cell formations, and chose the rare multinucleated giant cells present in regions apart form PML lesions and without other opportunistic infections than PML. We consider that we investigated HIV-GCs, and found JCV DNA in these presumed HIV-GCs, either as cytoplasmic or as both cytoplasmic and nuclear staining (Figures 7 and 8, Table 3). It is conceivable that JCV cytoplasmic staining reflects phagocytosis of JCV-positive nuclei. The nuclear infection of HIV-GCs, however, cannot be explained as easily. These cells, accepted markers for HIV infection of the CNS, are thought to originate from histiocytes or microglia,

and either stem from cell-to-cell fusion or from nuclear division. HIV is either captured within these cells or they are primarily infected by HIV (Kato *et al*, 1987; Vinters and Anders, 1990). Assuming these cells to originate from histiocytes, they can potentially be infected by JCV as has been shown for individual macrophages (see above). The mechanism of how this occurs remains enigmatic, because the infected syncytial cells are often seen at some distance from PML lesions (Figures 6 and 7). The JCV-GCs showed CD68-positive macrophages enclosing a central JCV DNA oligodendrocytic nucleus in a region of advanced necrosis with abundant single magcrophages or JCV-negative multinucleated cell formations (Figure 6).

Vasular endothelium
Papovavirus particles have never been observed in vascular endothelial cells by electron microscopy or immunohistochemistry (Aksamit *et al*, 1990; Boldorini *et al*, 1993a; von Einsiedel *et al*, 1993). However, JCV-infected endothelial cells could be detected by ISH (Dörries *et al*, 1979; Shapshak *et al*, 1986) (Figure 9). We believe the infection of endothelial cells to be a very rare event, possibly explained by mutations occurring in the course of active replication in the region of the JCV genome encoding the VP1 protein, which is known to mediate cell attachment. It is unlikely that rare infection of endothelial cells has any substantial pathogenetic role in the intracerebral distribution of JCV. It might be conceivable that this rare finding might represent JCV passing through endothelial cells from blood stream, as has been shown for HIV (Kim *et al*, 2003), or that we observed perivascular microglia immediately adjacent to vascular endothelial cells.

Cells uninfected by JCV using in situ PCR
In our investigation on postmortem brain tissue, we examined all cell types present by *in situ* PCR and never observed JCV DNA in neurons, not even when JCV-infected oligodendrocytes were located as satellite cells surrounding them. Some groups, however, have shown that neurons can occasionally harbor JCV DNA, but this rare finding was only observed when neurons were located within affected regions of gray matter. Ultrastructurally, viral particles were more often present within the cytoplasm and only very rarely in nuclei of neurons (Dörries *et al*, 1979). Boldorini *et al* (1993a) concluded that neurons are probably not effectively infected by JCV. Perivascular and inflammatory cells always remained free of JCV DNA, even after PCR amplification, which supports previous observations (Aksamit *et al*, 1985, 1986, 1987, 1990; Vazeux *et al*, 1990; Boldorini *et al*, 1993a; von Einsiedel *et al*, 1993), although there have been reports of occasional infected mononuclear cells in and around blood vessels in PML brain tissue (Shapshak *et al*, 1986; Houff *et al*, 1988; Ironside *et al*, 1989). The usual absence of JCV DNA in vascular endothelial cells does not rule out a hematogenous dissemination of JCV to the brain. It is possible that *in situ* PCR merely fails to detect JCV DNA in these cells for reasons of technique sensitivity. A hematogenous route to the brain is strongly supported by multifocal lesions of the white matter occurring in the vicinity of blood vessels and by the severe involvement of the corticomedullary junction demonstrable in almost all cases of PML investigated. As already mentioned in our previous investigation using the combined JCV DNA detection method and conventional ISH, we were unable to find JCV-positive cells in our non-PML cases, which served as negative controls (von Einsiedel *et al*, 1993; Samorei *et al*, 2000). In summary, the four patients whose brain tissue we used as negative-control cases were in their 30s, and PML patients' ages ranged from 28 to 63. Our and the findings of others (Chesters *et al*, 1983; Dörries *et al*, 1979; Telenti *et al*, 1990; Ferrante *et al*, 1995) are controversial to some investigators reporting the brain a sanctuary site for JCV, and that this phenomenon is increasing with age (Mori *et al*, 1991; Elsner and Dörries, 1992; White *et al*, 1992). Since *in situ* PCR only allows to investigate a section measuring 2.4 cm^2, it is theoretically possible that we missed JCV-positive cells, because we did not investigate different brain regions systematically in the small number of control brains. So far, our findings rules out the brain to be a potential site of latency for JCV infection.

Summary and conclusion

This investigation of archival brain tissue using *in situ* PCR for JCV DNA revealed new intra- and intercellular infection patterns in glial cells as well as in cells of nonglial origin. New aspects of the understanding of JCV infection emerged from the examination of cells containing low JCV copy numbers. The intracellular distribution of JCV in oligodendoglial cells probably permits the indirect visualization of different stages of the JCV replication cycle at a light microscopic level.

We have shown that after *in situ* amplification, which realistically reflects the actual extent of JCV infection, PML can be very advanced even without extensive necrosis. Our data led us to propose that cells of non-glial origin, e.g., HIV-GCs and macrophages, can be actively infected by JCV. Their infection patterns show parallels to astrocytes, which may reveal abortive infection, and therefore remain in an early stage of infection indefinitely. Furthermore, double infection of cells with HIV and JCV are possible. Negative controls did not reveal JCV DNA. In summary, we report significant new findings concerning inter- and intracelluar JCV infection patterns that may shed new light on JCV infectivity of different cell types in the brain of AIDS patients with PML. *In situ* PCR is indicated in the investigation of diseases for which a

rather detailed picture of infection has not been obtained with conventional techniques.

Materials and methods

Brain tissue specimens were obtained from 12 AIDS patients with PML, autopsies were carried out between 1983 and 2000, at University of California Los Angeles Medical Center. The clinical, radiographic, and laboratory data of affected patients as well as processing of brain material and its pathologic features have been extensively described elsewhere (von Einsiedel *et al*, 1993; Samorei *et al*, 2000). Patient ages ranged from 28 to 63 years, PML dominated the clinical and pathological findings in each case and was the proximate cause of death in all cases. Brain tissue used as negative controls was obtained from three age-matched patients with no underlying immune deficiency and from one AIDS patient without PML.

For neuropathologic investigation, brain tissue was formalin-fixed and paraffin-embedded. All brains examined showed evidence of PML. Light microscopic areas with absent, mild, or severe involvement of PML in H&E sections von Einsiedel *et al*, 1993; Samorei *et al*, 2000) were selected for further examination. Validation of *in situ* PCR technique, ISH, and routine evaluation of have been described (Samorei *et al*, 2000). In brief, for all detection methods, brain tissue blocks were cut with a separate microtome blade for each block, tissues were directly mounted on silane-coated slides (Perkin Elmer Applied Biosystems, Foster City, USA), with omission of a water bath. Sections were heat-fixed thereafter.

In situ *PCR and ISH for JCV DNA*

A highly sensitive and specific molecular biological method for the detection of JCV DNA was applied, performing *in situ* PCR (GeneAmp *in situ* PCR system 1000, Perkin Elmer Applied Biosystems, Foster City, USA), consecutively followed by ISH. For *in situ* PCR, pretreatment of brain tissue samples comprises deparaffinization, followed by cell permeabilization, which has been monitored for each section to reach exact pore membrane sizes of cells and *nuclei* for efficient reagent diffusion to the target nucleic acid, using the Situs control kit (Situs, Düsseldorf, Germany). For *in situ* amplification, a 'semi-hot start' was introduced for which the PCR master mix at room temperature were applied to the preheated (70°C) slide on the assembly tool (Perkin Elmer Applied Biosystems). Fifty microliter of the master mix containing 1 μM of each JCV primer (5′ACTGAGGAATGCATGCAGATCTAC3′ nucleotides 4225 to 4249; 5′TAGGTGCCAACCTATGGAACA3′, nucleotides 4409 to 4429) (Stoner *et al*, 1988), 3 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.2 mM of each desoxyribonucleoside triphosphate (dNTP), and 10 units of AmpliTaq DNA polymerase IS (Perkin Elmer Applied Biosystems) were added to each slide. AmpliCover rubber discs and metal clips (Perkin Elmer Applied Biosystems) were assembled onto the slides, thus producing an air-tight reaction chamber. Initial denaturation step of 94°C for 3 min was accomplished, and DNA was amplified by 20 cycles of denaturation at 94°C for 2 min, annealing at 48°C for 2 min, and elongation at 72°C for 2 min. On completion of a final extension step at 72°C for 7 min, the AmpliCover discs and clips were dismantled and the slides rinsed in PBS. After following JCV DNA amplification by *in situ* PCR, ISH was performed. ISH was also carried out alone as a conventional instrument for JCV DNA detection in brain tissue sections (von Einsiedel *et al*, 1993; Samorei *et al*, 2000). The tissue sections were subsequently counterstained with hematoxylin.

We attached great importance to controls for *in situ* PCR to disclose false-positive or false negative results, and included up to 13 controls (Samorei *et al*, 2000). Inter-alia, formalin-fixed brain tissue from age-matched patients without neurologic disease served as negative controls. Tissue sections formerly diagnosed as being JCV-positive by ISH and H&E staining (von Einsiedel *et al*, 1993) were included as positive controls. A triplicate of samples was included in each run, and every run was repeated once. We tried to rule out cross-contamination and false-positive results due to diffusion artifacts, and added template DNA (plasmid containing the complete genome of JCV strain GS cloned over its unique Eco RI site, obtained from K. Dörries, Würzburg, Germany) to the master mix. Solution phase PCR using the same primers as for *in situ* PCR was performed on extracted DNA from parallel section. Furthermore, we omitted the enzyme-labeled antibody inimmunohistochemical staining, and solution phase PCR with plasmid JCV DNA was performed on blank slides. Omission of DNA polymerase was used as an amplification control.

Immunohistochemistry for CD68 and GFAP

Immunohistochemistry for the detection of GFAP (Eng, 1981), indicating astrocytic phenotype, and CD68 for detection of microglial macrophages (Micklem *et al*, 1989; Fiala *et al*, 2002) were performed on PML cases using selected tissue blocks from the cerebrum, cerebellum, and spinal cord. For this purpose, primary parallel sections were investigated by *in situ* PCR and ISH, respectively, either followed by immunochemistry for CD68 or GFAP.

Immunohistochemistry for CD68 for monoclonal antibodies to macrophages

After *in situ* PCR and ISH for the detection of JCV DNA has been performed, selected brain tissue sections with no, mild, or severe involvement of PML were double stained with CD68 antibodies, clone KP1, Dako Cytomation (DAKO Corporation,

Carpinteria, USA). Slides were incubated in 3% H_2O_2 in phosphate-buffered saline (PBS) for 20 min, and rinsed in water. The slides were exposed to 0.1 M sodium citrate buffer (pH 6.0) for 45 min at 120°C, and rinsed three times with PBS, thereafter. Five percent normal horse serum (NHS) and 5% bovine serum albumin (BSA) in PBS were applied for 30 min. The slides were blotted off, but not rinsed or allowed to dry out. Primary antibodies made in "blocking" solution were applied at a dilution of 1:75 and incubated at 4°C overnight. The slides were rinsed three times in PBS. The secondary antibody "biotinylated HxM" was applied at a dilution of 1:200, incubated for 30 min at reverse transcription (RT), and rinsed in PBS for three times. The vector alkaline phosphatase–avidin biotin complex was applied for 30 min, followed water rinse in PBS. NBT/BCIP stock solution, a chromogenic substrate for alkaline phosphatase staining tissue blue (Roche Diagnostic Corporation, Indianapolis, USA), was added onto the brain tissue sections for 5 min, and rinsed in running tap water for 10 min. Finally the brain tissue sections were coverslipped, using aqueous mounting medium.

Immunohistochemistry for GFAP

Again, after *in situ* PCR and ISH has been carried out, some brain tissue sections with different involvement of PML were investigated with additive immunohistochemistry. For the detection of gliosis, polyclonal rabbit anti-cow GFAP was utilized introducing the commercial kit DAKO Z033 (DAKO Corporation). The procedure for immunohistochemistry was the same as described for CD68. Blocking solution consist of 5% normal goat serum and 5% BSA in PBS, GFAP primary antibody was diluted 1:500 in "blocking" solution. The secondary antibody "biotinylated G × R #1000" was diluted 1:200. Reagents A, B, and C were diluted 1:100, alkaline phosphatase–avidin biotin complex was added, and NBT/BCIP solution (Roche Diagnostic Corporation) applied.

To visualize double-staining results, JCV DNA was marked by DAB, revealing a brownstaining product, and CD68-positive cells or GFAP-positive cells, correspondingly, revealing a blue staining product using NBT/BCIP.

Evaluation of slides were obtained by light microscopy (Nikon Optiphot-2, Nikon GmbH, Düsseldorf, Germany).

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