Progressive Multifocal Leukoencephalopathy and Apoptosis of Infected Oligodendrocytes in the Central Nervous System of Patients With and Without AIDS

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Context: Progressive multifocal leukoencephalopathy (PML) is a demyelinating disease of the central nervous system (CNS) caused by JC virus (JCV) that occurs in immunocompromised patients. Demyelination of the CNS is a consequence of virus-induced killing of oligodendrocytes, although the exact mechanism of cell death is unknown.

Objective: To examine archival autopsy and surgical pathologic specimens from 8 patients with PML, including 6 patients with human immunodeficiency virus (HIV)-associated PML and 2 patients with non-HIV–associated PML, for evidence of apoptosis.

Design: Apoptotic cells were identified by TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end in situ labeling) or immunohistochemical detection of activated caspase 3. The JCV-infected cells were identified by in situ hybridization for viral transcripts or immunohistochemical analysis for JCV T antigen.

Results: Apoptosis of JCV-infected oligodendrocyte apoptosis was a prominent feature in all cases of both HIV- and non–HIV-associated PML. There were no differences between number or distribution of apoptotic cells identified by TUNEL or immunohistochemical analysis for activated caspase 3. Bizarre astrocytes were occasionally positive for JCV but were not apoptotic. Neurons, astrocytes, macrophages, and oligodendrocytes remote from lesions were neither apoptotic nor JCV infected.

Conclusions: Our study demonstrates that apoptosis occurs in oligodendrocytes associated with demyelinated lesions of patients with both HIV-associated and non–HIV-associated PML. There were no differences in degree, location, or type of infected or apoptotic cells between patients with HIV-associated and non–HIV-associated PML. The extent of apoptosis did not correlate with the presence or intensity of host inflammatory response. Accumulation of viral particles in nuclei of infected cells made it difficult to identify morphologic changes in the nucleus typically associated with apoptosis.

Arch Neurol. 2002;59:1930-1936

Progressive multifocal leukoencephalopathy (PML) is a fatal, demyelinating central nervous system (CNS) disease caused by JC virus (JCV), a human polyoma virus. Serological studies indicate that approximately 80% of the US population has developed antibodies to JCV by adulthood. Initial infection with JCV is usually asymptomatic and is followed by viral latency. Clinically, symptomatic infection that results in PML is thought to be caused by immunosuppression–associated reactivation of latent virus either within the CNS or at extraneural sites with subsequent spread to the CNS.

Progressive multifocal leukoencephalopathy is characterized by multiple foci of demyelination, located predominantly in the white matter near the gray-white matter junction. The cardinal pathologic features of PML include enlarged hyperchromatic nuclei of oligodendroglia with viral inclusions, atypical enlarged bizarre astrocytes, and a variable degree of lymphocytic inflammatory response. Clinically, PML often presents with motor weakness, speech disturbances, visual impairment, and cognitive abnormalities.

In recent years, HIV infection has become the dominant underlying disease associated with PML, which develops in approxi-
mately 3% of patients with acquired immunodeficiency syndrome (AIDS).2,8-10

The demyelination associated with PML is secondary to death of JCV-infected oligodendroglia. The mechanism of JCV-induced oligodendrocyte cell death is unknown. It is also unclear whether only infected oligodendrocytes die or whether virus can also induce death in neighboring uninfected cells (the bystander effect).

Apoptosis is a distinct form of cell death in which affected cells undergo characteristic morphologic and biochemical changes, including cytoplasmic shrinkage, condensation and fragmentation of nuclear chromatin, membrane alterations, and changes in gene and protein expression.11,12 Almost all forms of apoptosis are associated with sequential activation of cysteine-aspartyl proteases (caspases) by extracellular and/or intracellular stimuli. These proteases cleave numerous cellular substrates, leading to oligonucleosomal DNA fragmentation and the other morphologic hallmarks of apoptosis.13,14

Apoptosis has been implicated in glial loss in demyelinating diseases, including multiple sclerosis15-18 and experimental allergic encephalomyelitis.19 Apoptosis also occurs in virus-induced demyelinating diseases as exemplified by Theiler virus infection20,21 and measles virus–associated subacute sclerosing panencephalitis.22 Apoptosis of glia and neurons also occurs following infection with a variety of other neurotropic viruses.23-30 Given the propensity for several neurotropic viruses to cause apoptosis, we attempted to determine whether PML was also associated with apoptosis. Knowing the pivotal role of oligodendrocyte damage in demyelinating disorders, we hypothesized that apoptosis might be the mechanism of oligodendrocyte death in PML.

**METHODS**

The autopsy, surgical pathologic specimens, and consultation files in the pathology department of the University of Colorado Health Sciences Center, Denver, were searched for autopsy and surgical pathologic cases of PML diagnosed during the past 20 years (Table 1). Cases were excluded based on (1) insufficient volume of tissue available for analysis (as was the case for many stereotactic biopsy specimens) or (2) the lack of availability of paraffin tissue blocks for preparation of freshly cut sections. Eight cases were identified that met clinical and pathologic criteria for PML, including typical clinical and neuroimaging findings and the presence of demyelination, inclusion-bearing oligodendrocytes, and bizarre astrocytes. In all cases, JCV transcript was detected in brain tissue by in situ hybridization (ISH). One of the patients (case 1) had both biopsy and autopsy tissue available for study. Cases included 2 patients with non–HIV-associated PML (cases 1-2) and 6 patients (cases 3-8) with HIV-associated PML. Only one of the AIDS patients (case 4) was receiving highly active antiretroviral therapy (HAART) at the time of diagnosis of PML. Two patients with HIV-associated PML (cases 3 and 5) had pathologic evidence of inflammatory PML characterized by the presence of significant perivascular lymphocytic infiltration.2

**JCV IN SITU HYBRIDIZATION**

Sections were deparaffinized as described for immunohistochemical analysis. Tissue was digested with the enzyme Pronase I (Ventana Medical Systems Inc, Tucson, Ariz) (20 minutes at 37°C), washed in diethylpyrocarbonate-treated water, and then incubated with 3% water (4 minutes at 37°C) prepared according to the manufacturer’s instructions. Slides were then washed in Tris-buffered saline (TBS), immersed in prewarmed stringency wash (Dako Corporation, Carpinteria, Calif) (20 minutes at 37°C), washed in deionized water and counterstained with nuclear fast blue (Dako), dehydrated in graded ethanol followed by xylene, and then permanently mounted.

**IMMUNOHISTOCHEMICAL ANALYSIS FOR ACTIVATED CASPASE 3 AND CELL-TYPE SPECIFIC MARKERS**

Brain tissue sections were deparaffinized by baking for 5 minutes at 57°C, immersion in mixed xylenes, then rehydrated in graded alcohols. Tissue sections then underwent antigen retrieval (10 mM citrate buffer, 10 minutes at 90°C) followed by incubation with 3% water (4 minutes at 37°C) and blocking with 5% normal goat serum in phosphate-buffered saline (PBS). Immunocytochemical analysis was performed using a pri-

| Table 1. Summary of Patient Information* |
|-------------------|-------------------|-------------------|-------------------|
| Case No./Sex/Age, y | Predisposing Factor | Autopsy (A) or Biopsy (B) | Duration† | Comments |
| 1B/M/39 | Chronic myelogenous leukemia, immunosuppressant drugs | B | 1 mo | Autopsy was 7 mo after biopsy |
| 1A | | A | 7 mo | Cord blood transplantation in February 1999; GVHD‡; biopsy for PML in July 2000 |
| 2/M/65 | Large cell undifferentiated lung carcinoma | A | 10 d | |
| 3/M/52 | HIV positive | B | ND | Inflammatory PML |
| 4/M/40 | HIV positive for 13 y | A | 3 mo | Undergoing HAART for 4 mo |
| 5/M/35 | HIV positive | A | ND | Inflammatory PML |
| 6/F/23 | HIV positive for 4 y | A | 2 mo | Died of bronchopneumonia |
| 7/M/27 | HIV positive | A | 5 mo | |
| 8/A/42 | HIV positive | A | 8 mo | |

*HIV indicates human immunodeficiency virus; ND, no data available; GVHD, graft-vs-host disease; PML, progressive multifocal leukoencephalopathy; and HAART, highly active antiretroviral therapy.
†Duration of illness after first clinical presentation with PML symptoms before biopsy or autopsy.
‡Patient developed GVHD while taking immunosuppressant medication.

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ary antibody specific for the activated form of caspase 3 (Cell Signaling Technology Inc, Beverly, Mass) diluted 1:25 in PBS containing 3% bovine serum albumin (BSA; Sigma-Aldrich Corporation, St Louis, Mo) for 30 minutes at 37°C. Binding of primary antibody was detected using biotinylated secondary antibody followed by avidin-HRPO (Ventana Medical Systems Inc), using diaminobenzidine (DAB; Ventana Medical Systems Inc) as substrate. Immediately following the DAB reaction, sections were incubated with the secondary primary antibody against CD3 or glial fibrillary acid protein (GFAP) (Dako), both at a 1:100 dilution. Binding of second primary antibodies was detected using biotin-avidin–alkaline phosphatase secondary antibody and labeling substrate. Sections were counterstained with Gills 2 (1:9 dilution, Ventana Medical Systems Inc). All reactions were performed using the automated staining system (Ventana Medical Systems Inc).

TUNEL
A biotin-streptavidin–based TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end in situ labeling) kit optimized for neuronal tissues was used (NeuroTACS II; Trevigen Inc, Gaithersburg, Md). Deparaffinized brain tissue sections were permeabilized with Neuropore (Trevigen Inc) (30 minutes at room temperature), then samples were incubated at 37°C with a mixture of terminal deoxynucleotidyl transferase (TdT), deoxyuridinetriphosphates conjugated to biotin, Mn++, and TdT reaction buffer for 1 hour. After stopping the TdT reaction and washing, the tissues were incubated 30 minutes at room temperature with streptavidin–conjugated horseradish peroxidase, washed, immersed in DAB diluted in PBS solution for 5 minutes at room temperature, then counterstained with blue counterstain (Trevigen Inc). The DAB–stained samples were dehydrated in a series of ascending ethanol concentrations followed by mixed xylenes, air-dried, permanently mounted, and stored at room temperature until imaging.

DUAL-LABEL FLUORESCENCE WITH TUNEL AND A SINGLE ANTIBODY
Tissue sections were permeabilized with Neuropore, a non-proteolytic permeabilization and blocking reagent (Trevigen Inc) for 30 minutes at room temperature then washed in TBS (140 mM sodium chloride, 20 mM Tris, pH 7.6), and nonspecific binding was blocked with 2% BSA (Sigma-Aldrich Corporation) diluted in Neuropore for 30 minutes at room temperature, then incubated (overnight at 4°C) with antibody against SV40 large T antigen (Oncogene Research Products, San Diego, Calif) diluted in 1% BSA containing Neuropore. The next day, TUNEL reactions were performed as described herein, except that after stopping the TdT reaction and washing, the samples were incubated with a mixture of streptavidin–Cy3 (Jackson Immunoresearch Laboratories Inc, West Grove, Pa) to detect TUNEL and secondary antibody (1:100) conjugated to fluorescein isothiocyanate (Jackson Immunoresearch) to detect the SV40 antibody. Finally, samples were incubated with 100 ng/mL of Hoechst 33342 (Molecular Probes Inc, Eugene, Ore) for 10 minutes at room temperature in the dark as counterstain, washed in PBS, mounted with anti-fade media, and stored in the dark at −20°C until imaging.

RESULTS
In each patient, we assessed the presence or absence of apoptosis in cells from brain regions affected by PML as identified by histologic criteria and from brain regions that were remote from demyelinating lesions and appeared histologically normal. The CNS cells infected with JCV were easily identified by ISH using a JCV riboprobe in all 8 patients (Figure 1A and B). Apoptotic cells were detected in areas of PML lesions in all 8 patients but not in brain regions remote from these lesions (data not shown). There were no differences between the number and the distribution of apoptotic cells identified by TUNEL or the presence of activated caspase 3. Accumulation of viral particles in the nuclei of infected cells made it impossible to identify morphologic changes in the nucleus typically associated with apoptosis, such as nuclear pyknosis or karyorrhexis. Staining results from all patients studied are summarized in Table 2.

In all 8 cases, most apoptotic cells were identified as oligodendrocytes by their morphologic structure, location, and absence of GFAP immunohistochemical staining. These cells were most abundant at the periphery, rather than in the center, of demyelinated lesions (Figure 1C and D). The number of JCV-infected oligodendrocytes corresponded to the number of apoptotic cells, suggesting that nearly all apoptotic cells were JCV infected and that infection with JCV induced apoptosis. Both HIV-associated and non–HIV-associated cases were otherwise similar in the distribution and cell-type specificity of infection and apoptosis.

In case 1, from which both biopsy and autopsy tissue was available, the biopsy tissue showed many oligodendrocytes containing inclusions on hematoxylin-eosin–stained sections, which were positive for JCV by ISH and often apoptotic (Figure 2A and B). In contrast, in sections prepared from tissues obtained at autopsy 7 months later, significantly fewer infected oligodendrocytes were present, and apoptotic cells were more rare. In the autopsy samples, numerous bizarre astrocytes were identified in the lesions and about half of these were positive for JCV by ISH but were not apoptotic (Figure 2C and D). Neurons, normal astrocytes, and macrophages were not found to be JCV infected by ISH or apoptotic in any of the 8 cases examined.

In an effort to better define the nature of the apoptotic cells in PML lesions, we performed dual-label immunohistochemical analysis with caspase 3 and GFAP. Bizarre atypical astrocytes, reactive astrocytes, and normal–appearing astrocytes were all GFAP positive, yet these cells were not apoptotic. Atypical bizarre astrocytes but not normal–appearing or reactive astrocytes were frequently found to be positive for JCV infection by ISH (Figure 2D). We also performed co-labeling for apoptosis (caspase 3 or TUNEL) and JCV infection. Serial tissue sections (4 mm apart) were examined for JCV-infected cells using antibody to SV40 large T-antigen and apoptotic cells (TUNEL). Unfortunately, because of technical incompatibility between the required protocols, it was not possible to perform TUNEL or caspase 3 immunocytochemical analysis on sections in which JCV infection was identified by ISH. Using combined SV40 T-antigen and apoptosis staining, we found several examples of dual-positive (antigen and TUNEL) cells (Figure 3). In all cases in which we performed dual staining, most cells positive for JCV T antigen were also TUNEL positive, sug-
suggesting that most productively infected cells were undergoing apoptosis. The rare examples of cells positive for T antigen and negative for TUNEL may represent a population of infected cells not yet undergoing apoptosis. The relative insensitivity of SV40 T-antigen staining compared with ISH (data not shown) for identifying JCV-

Figure 1. JC virus (JCV)-infected and apoptotic cells in progressive multifocal leukoencephalopathy (PML). Brain tissue sections from 2 patients with PML demonstrating numerous JCV-infected cells by in situ hybridization for JCV. Brain tissue cells that were JCV positive (dark blue) from case 5 (A) (original magnification ×100) and case 6 (B) (original magnification ×400). Tissue sections from 1 patient (D) (case 5) (original magnification ×400), demonstrating apoptotic oligodendrocytes (brown; arrows) detected by TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end in situ labeling), which are predominantly found at the periphery of lesions rather than in the demyelinated center where oligodendrocytes are absent (C) (original magnification ×100).

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<th>Case No.</th>
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*JCV indicates JC virus; ISH, in situ hybridization; IHC, immunohistochemical analysis; plus sign, positive; plus and minus sign, rare positive; minus sign, negative; and ND, not done.
†All cases showed apoptotic cells as identified by both the presence of the active fragment of caspase 3 and TUNEL (see Figure 1 footnote for expansion).
infected cells prevented accurate quantification of the percentage of infected cells that were also apoptotic and vice versa.

We next attempted to determine whether inflammatory cells were also undergoing apoptosis. We performed dual-label immunohistochemical analysis to detect CD3 and active caspase 3. The extent of inflammation was variable in AIDS and non-AIDS patients, although one AIDS patient (case 5) had a significant perivascular lymphocytic response consistent with the diagnosis of inflammatory PML. Neither CD3 cells nor infiltrating macrophages were found to be JCV infected by ISH in any patient. Rare CD3+ cells and CD3− lymphocyte-appearing cells (presumably B lymphocytes) were positive for activated caspase 3, indicating that apoptosis of these cells was occurring (data not shown). Apoptotic macrophages were not seen. There was no increase in the number of apoptotic cells in areas adjacent to lymphocytic collections, suggesting that the host immune response, regardless of its intensity, did not contribute significantly to apoptosis in other cells.

Our study demonstrates that apoptosis occurs in brain tissue of patients with both HIV-associated and non-HIV-associated PML. Apoptosis was limited almost exclusively to oligodendrocytes associated with demyelinated lesions. The number and distribution of apoptotic oligodendrocytes closely paralleled the number and distribution of infected oligodendrocytes as determined by JCV ISH, suggesting that apoptosis was the major mechanism responsible for cell death. Apoptosis as determined by TUNEL and immunohistochemical analysis for activated caspase 3 was rarely seen in the bizarre astrocytes present in PML lesions, even though these cells were JCV infected. Because only oligodendrocytes support productive JCV infection, this suggests that productive infection may be required for JCV-induced apoptosis.

Figure 2. Oligodendrocytes containing viral inclusions were positive for JC virus (JCV) and apoptosis; in contrast, bizarre atypical astrocytes were occasionally JCV infected but were not apoptotic (original magnification ×400). Biopsy tissue sections from case 1, demonstrating oligodendrocytes positive for JCV transcripts (dark purple) (A) and apoptotic oligodendrocytes positive for activated caspase 3 (brown) (B). Autopsy tissue sections from case 1, demonstrating bizarre atypical astrocytes that were JCV infected (dark purple) (C). These bizarre atypical astrocytes were not apoptotic as demonstrated by dual-label immunohistochemical analysis showing no colocalization between glial fibrillary acid protein–positive (red; blue arrows) bizarre astrocytes and cells positive for activated caspase 3 (brown; black arrows) (D).
from demyelinative lesions. This indicates that apoptosis occurs as a direct consequence of viral infection and is not an indirect consequence of the severity of patient illness or other associated factors, such as hypoxia, hypotension, or ischemia. This is further supported by a previous study that indicated that JCV-infected oligodendrocytes overexpress p53 and Bax (a proapoptotic protein) and do not express Bcl-2 (an antiapoptotic protein).\textsuperscript{32} Apoptosis also did not correlate with the degree of the host inflammatory response, although occasional infiltrating lymphocytes were found to be apoptotic.

Although our study clearly indicates that apoptosis is a major mechanism of oligodendrocyte death in PML, there are some potential limitations in our study. For example, we used biochemical rather than morphologic markers to identify apoptotic cells, because it was difficult to accurately evaluate nuclear pyknosis and karyorrhexis in JCV-infected cells due to the concomitant presence of potentially confounding virus-induced changes in the nucleus. In addition, the absence of specific markers for necrotic cell death comparable to those used to detect apoptosis meant that it was not possible to accurately quantify the relative contribution of apoptosis and necrosis to oligodendrocyte death. However, our finding that most cells positive for JCV T antigen are also undergoing apoptosis strongly suggests that it is apoptosis rather than necrosis that is the primary mechanism of oligodendrocyte cell death in JCV-infected cells in PML lesions.

We did not find significant differences in apoptosis between AIDS and non-AIDS patients or among patients with variable PML-associated inflammatory responses. Two patients with prolonged survival, including an AIDS patient who received HAART (case 4) and a non-AIDS patient (case 1), had increased numbers of infected atypical bizarre astrocytes and a reduced number of infected oligodendrocytes as determined by JCV ISH in autopsy tissue. The reduction in infected oligodendrocytes may be a consequence of HAART and/or prolonged survival with the disease.\textsuperscript{33-35}

Oligodendrocyte death is the key initiating event responsible for the demyelination that is the cardinal pathologic feature of PML. We now show that JCV-infected oligodendrocytes die as a result of apoptosis. Elucidating the specific cellular pathways associated with JCV-induced oligodendrocyte apoptosis may reveal novel targets for therapy of this devastating disease. Most apoptotic signaling pathways involve activation of caspase cascades that originate with pathway-specific initiator caspases (eg, caspase 8 and 9), which converge on common downstream effector caspases (eg, caspase 3).\textsuperscript{11,13,14,36} Effector caspases in turn act on cellular substrates to induce the morphologic changes in cellular membranes, cytoskeletal proteins, and chromatin that are the hallmarks of irreversible commitment to cell death. It is conceivable that if caspase activation could be inhibited at an early stage then some JCV-infected oligodendrocytes may be able to recover and survive. Support for the feasibility of this strategy comes from studies in which caspase inhibitors have been used successfully to protect neurons from apoptosis triggered by a variety of inciting stimuli, both in vivo and in vitro.\textsuperscript{37-42}
Accepted for publication August 1, 2002.

Author contributions: Study concept and design (Ms Richardson-Burns and Drs Kleinschmidt-DeMasters, DeBiasi, and Tyler); acquisition of data (Ms Richardson-Burns); analysis and interpretation of data (Ms Richardson-Burns and Drs Kleinschmidt-DeMasters, DeBiasi, and Tyler); drafting of the manuscript (Ms Richardson-Burns and Drs DeBiasi and Tyler); critical revision of the manuscript for important intellectual content (Ms Richardson-Burns and Drs Kleinschmidt-DeMasters, DeBiasi, and Tyler); statistical expertise (Ms Richardson-Burns and Drs Kleinschmidt-DeMasters, DeBiasi, and Tyler); obtained funding (Dr Tyler); administrative, technical, and material support (Ms Richardson-Burns and Drs Kleinschmidt-DeMasters, DeBiasi, and Tyler); study supervision (Drs Kleinschmidt-DeMasters, DeBiasi, and Tyler).

This study was supported by grant DAMD 17-98-1-8614 from the US Army Medical Research and Material Command (Fort Detrick, MD), MERIT and REAP Awards from the Department of Veterans Affairs (Washington, DC), grant 1RO1AG14071 from the National Institutes of Health (Bethesda, MD), and the Reuler-Lewin Family Professorship of Neurology (Denver, Colo).

We thank Traci D. Sachs, BA, for expert histologic assistance and Ron Bouchard, BA, for microscopy and digital imaging assistance.

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