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Human immunodeficiency virus type 1 in spinal cords of acquired immunodeficiency syndrome patients with myelopathy: Expression and replication in macrophages

(*in situ* hybridization/immunohistochemistry/vacuolar myelopathy)

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ABSTRACT **Spinal cord disease is common in patients infected with human immunodeficiency virus type 1 (HIV-1), and a characteristic vacuolar myelopathy is present at autopsy in approximately one-fourth of acquired immunodeficiency syndrome patients. Pathologic examination of the spinal cord shows vacuolation of white matter and infiltration by macrophages, a process distinct from HIV-1 encephalopathy. To determine the presence and localization of HIV-1 RNA in the spinal cords of acquired immunodeficiency syndrome patients with vacuolar myelopathy, we used the technique of combined *in situ* hybridization and immunohistochemical staining on the same slide. Spinal cord tissue sections were stained with markers for macrophages, endothelial cells, oligodendroglia, astrocytes, and myelin and then hybridized *in situ* with HIV-1-specific RNA probes. Combined *in situ* hybridization and immunohistochemical staining on three spinal cords showed HIV-1 expression in mononuclear and multinucleated macrophages localized mainly to areas of myelopathy in spinal cord white matter. Immunohistochemical staining and electron microscopy showed myelin within macrophages and electron microscopy revealed HIV-1 budding from macrophages. These data suggest a role for HIV-1-infected macrophages locally in the pathogenesis of vacuolar myelopathy and add to the body of evidence that these cells play a role systemically in the development of HIV-1-related disease.**

Neurologic disease often occurs as a result of infection with human immunodeficiency virus type 1 (HIV-1) (1, 2), a lentivirus that is the etiologic agent of the acquired immunodeficiency syndrome (AIDS) (3). Other lentiviruses, including visna (3) and simian immunodeficiency virus (4), are associated with chronic, persistent infection of the central nervous system. HIV-1 has been identified in the brains of AIDS patients with encephalopathy by using Southern blot analysis (5), *in situ* hybridization (5–9), immunohistochemistry (8, 10), and electron microscopy (EM) (11). Spinal cord disease is also common in HIV-1-infected patients, and vacuolar myelopathy is present at autopsy in approximately one-fourth of patients with AIDS (12). The myelopathy syndrome, a clinical consequence of spinal cord pathology, is associated with spastic paraparesis, ataxia, and incontinence (12). Postmortem examination shows vacuolation of the spinal cord white matter and infiltration by macrophages, a process that is pathologically distinct from HIV-1 encephalopathy (12). Although AIDS encephalopathy has been etiologically linked to direct infection with HIV-1, the etiology of vacuolar myelopathy has not been previously elucidated. Isolation of HIV-1 from the lumbar spinal cord of an AIDS patient with myelopathy (2) and immunohistochemical demonstration of HIV-1 antigen in three spinal cords (8, 10) suggest that HIV-1 is directly involved.

The predominant cell types in the brain that are infected by HIV-1 are monocyte/macrophage derived, as demonstrated by immunohistochemistry (7, 8) and combined *in situ* hybridization/immunohistochemistry (6). In addition, based upon morphology, it has been suggested that astrocytes, oligodendroglia, endothelial cells, and neurons may be infected (7, 9). To investigate whether HIV-1 is expressed in the spinal cords of AIDS patients with myelopathy and to identify precisely the types of cells infected, we used the combined technique of *in situ* hybridization for HIV-1 RNA and immunohistochemical staining for cell type on the same slide; we also used EM. By using these methods, the results show that HIV-1 is present in spinal cords with myelopathy, localized mainly to regions of vacuolar myelopathy, and expressed in cells of monocyte/macrophage lineage.

MATERIALS AND METHODS

Tissue and Cell Specimens. Spinal cords from three AIDS patients with clinical myelopathy were obtained at autopsy and fixed in 10% phosphate-buffered formalin. Cross sections from representative levels were embedded in paraffin and sections were cut, attached to silanated slides (13), and processed for immunohistochemistry and *in situ* hybridization. Sections of brain obtained from the three patients at autopsy were processed for routine neuropathologic evaluation. Cell blocks were made from HIV-1-infected and uninfected peripheral blood mononuclear cells. Normal donor cells were infected with prototype HIV-1 (14), harvested 4 days later, and fixed in 10% neutral buffered formalin. A cell pellet was embedded into a cell block and processed as described above.

Immunohistochemistry. Immunohistochemical staining was performed using the horseradish peroxidase-labeled avidin-biotin technique (15), with diaminobenzidine employed as the chromogen. Seven primary reagents were used: (*i*) *Ricinus communis* agglutinin 1 (RCA-1) (Vector Laboratories), a biotinylated lectin that reacts with monocyte-derived cells, microglia, and endothelial cells (16); (*ii*) Leu-M1 (Becton Dickinson), a mouse monoclonal antibody recognizing a population of circulating monocyte/macrophages

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Abbreviations: HIV-1, human immunodeficiency virus type 1; AIDS, acquired immunodeficiency syndrome; RCA-1, *Ricinus communis* agglutinin 1; EM, electron microscopy.

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and granulocytes (17); (*iii*) MAC-387 (Dako, Santa Barbara, CA), a mouse monoclonal antibody directed against cells of the monocyte/macrophage series and peripheral granulocytes (18); rabbit polyclonal antisera (Dako) to: (*iv*) lysozyme, which identifies a variety of cells including monocytes and tissue histiocytes (19); (*v*) glial fibrillary acidic protein, directed against astrocytes (20); (*vi*) myelin basic protein, which demonstrates myelin and developing oligodendroglia (21); and (*vii*) factor VIII-related antigen, which is present in endothelial cells (22).

Preparation of RNA Probes. Three fragments of HIV-1 DNA representing portions of the *gag, pol, env*, and long terminal repeat regions (23) were subcloned into SP6/T7 bidirectional transcription vectors (Promega Biotec). SP6 or T7 RNA polymerase (New England Biolabs) was used based upon whether the antisense or sense RNA transcript was generated. The three individual antisense transcripts were combined to make a pooled ^{35}S-labeled RNA antisense probe; the same procedure was followed for the three sense transcripts, which were used as a control probe. Specific activity of antisense probes and sense control probes was 2–3 $\times 10^8$ cpm/μg.

***In Situ* Hybridization.** Procedures were primarily those outlined by Gendelman and coworkers (23) with some minor modifications. After rendering the sections permeable using hydrochloric acid followed by proteinase K, the slides were acetylated to reduce nonspecific background binding, dehydrated in graded ethanols, and prehybridized overnight. The slides were then hybridized in the following mixture: 80×10^6 cpm of ^{35}S-labeled RNA probe per ml/10% dextran sulfate (Sigma)/50% deionized formamide/300 mM sodium chloride/10 mM Tris·HCl, pH 8.0/0.5 mM EDTA/100 mM dithiothreitol/0.1% Triton X-100/500 μg of tRNA per ml/0.02% polyvinylpyrrolidone/0.02% Ficoll/1 mg of bovine serum albumin per ml. Hybridization mixture was applied to slides, siliconized coverslips were mounted, and hybridization took place at 50°C overnight. The slides were then washed extensively and treated with RNase A and RNase T1 as described (24) and dehydrated in a graded ethanol series containing 0.3 M ammonium acetate. Finally, the slides were dipped in NTB-2 emulsion (Kodak) diluted 1:1 with 0.6 M ammonium acetate and exposed for 10 days at 4°C for autoradiography. After development with D-19 (Kodak) diluted 1:1 with water, the slides were fixed in 30% thiosulfate and counterstained with Mayer's hematoxylin for histologic exam.

Specificity of the *in situ* hybridization procedure was demonstrated by hybridizing both the HIV-1-specific antisense probe and control sense probe to tissue sections derived from HIV-1-infected and uninfected cell blocks. When slides derived from infected cells were hybridized to the pooled HIV-1 antisense probe, ≈20% of the cells exhibited abundant silver grains distributed in a characteristic stellate pattern. By contrast, sections from uninfected cells hybridized to the same probe exhibited less than one grain per cell, as did HIV-1-infected cells hybridized to the control (sense) probe. Thus, the observation of silver grains overlying cells after hybridization with an HIV-1-specific antisense probe correlated with the presence of HIV-1.

Combined Immunohistochemistry and *in Situ* Hybridization. Specimens were first processed for immunohistochemistry and then for *in situ* hybridization by using the procedures described above. Since the *in situ* hybridization signal is reduced in specimens that have been first processed for immunohistochemistry, parallel sections were processed for *in situ* hybridization alone to quantitate more accurately the degree of HIV-1 RNA expression (23, 24).

Transmission EM. Portions of spinal cord from patients 1 and 2, originally fixed in 10% phosphate-buffered formalin, were cut into small pieces and transferred for further fixation to 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). After a minimum of 24 hr in glutaraldehyde, the tissue was placed in 1% osmium tetroxide, block-stained for 1 hr in 50% ethanol saturated with uranyl acetate, dehydrated in a graded ethanol series and propylene oxide, and embedded in Spurr's plastic. Thin sections were stained with uranyl acetate and lead citrate and examined on a Zeiss model 10A electron microscope (11).

RESULTS

Clinical and Neuropathologic Findings in Three AIDS Patients. All three AIDS patients, referred to as patients 1–3, showed clinical and pathologic findings of HIV-1 myelopathy and encephalopathy. They each had dementia and spastic paraparesis; one of the patients, patient 1, also had incontinence. Histologic examination of the spinal cords from these patients showed vacuolar myelopathy, which was characterized by large vacuoles and mononuclear cell infiltration of the white matter (Fig. 1*A*). The mononuclear cells appeared morphologically to be macrophages and were primarily located either in a perivascular distribution or adjacent to or within vacuoles (Fig. 1*A*). The spinal cord of patient 1 exhibited the most severe myelopathy and was notable for extensive infiltration of the white matter with multinucleated giant cells. The brains of these patients demonstrated microglial nodules, multinucleated giant cells, white matter pallor, neuronal loss, and gliosis, findings typical of HIV-1 encephalopathy.

***In Situ* Hybridization.** HIV-1 RNA, indicated by silver grains overlying cells, was demonstrated in the spinal cords of all three patients by *in situ* hybridization. Localization of HIV-1 RNA expression was primarily confined to regions of active myelopathy and detected in mononucleated inflammatory cells and multinucleated cells that appeared on histology to be macrophages (Fig. 1 *B* and *C*). The spinal cord of patient 1 was notable in that virtually all of the mononucleated and multinucleated cells extensively infiltrating the white matter expressed HIV-1 RNA (Fig. 1*C*). In the other two patients, by contrast, there were some macrophages located in areas of myelopathy that did not exhibit silver grains. HIV-1 RNA was detected rarely in mononuclear cells in the gray matter of all three patients as well as in the subarachnoid space and spinal nerve root entry and exit zones in patient 1. Neuronal cell bodies did not express detectable HIV-1 RNA. Hybridization of spinal cord sections using control RNA probes of the sense polarity showed less than one silver grain per cell (Fig. 1*D*), no more than that seen when sections from uninfected control cells were hybridized.

Immunohistochemistry. Immunohistochemical analyses of spinal cord mononucleated inflammatory cells and multinucleated giant cells using the macrophage markers RCA-1, Leu-M1, MAC-387, and lysozyme demonstrated that these cells were macrophages (Table 1; Fig. 1 *E* and *F*). The factor VIII-related antigen reagent clearly identified endothelial cells and blood vessels (Table 1) and emphasized the perivascular distribution of the macrophage infiltrate. Though intact axons were seen, staining with antiserum to myelin basic protein showed numerous regions of white matter demyelination, primarily in areas containing vacuoles and macrophages. The cytoplasm of many macrophages stained positively for myelin basic protein (Table 1; Fig. 1*G*), indicating phagocytosis of myelin by these cells. Antiserum to glial fibrillary acidic protein demonstrated minimal reactive astrocytosis; none of the mononucleated or multinucleated cells stained with this antibody (Table 1).

Combined Immunohistochemistry and *in Situ* Hybridization. Double labeling with immunohistochemical staining followed by *in situ* hybridization provided precise definition of cells expressing HIV-1 RNA. In all three patients, the cells

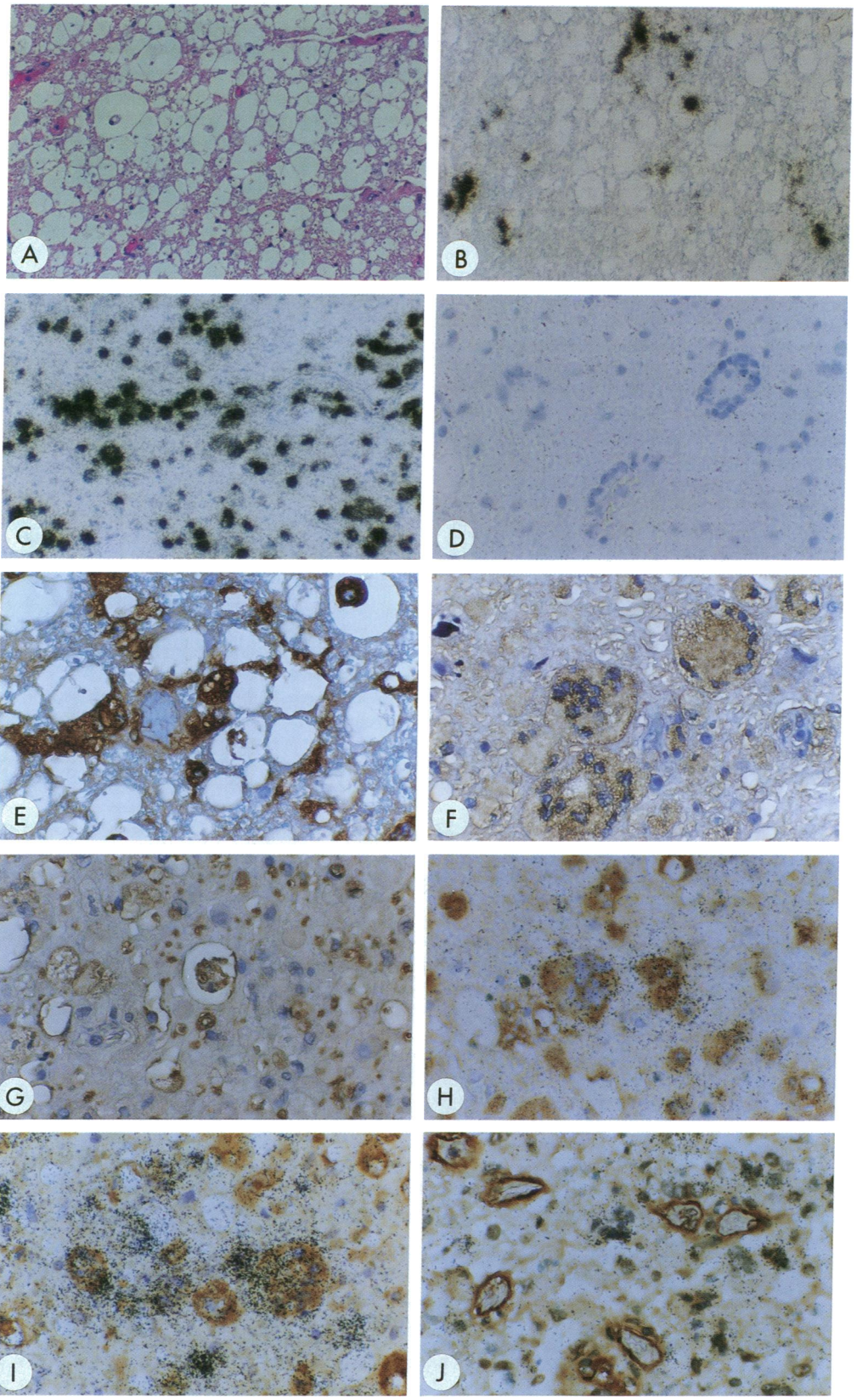

FIG. 1. (*A*) Histopathology of vacuolar myelopathy. [Patient (Pt) 2; hematoxylin/eosin, ×100.] (*B*) *In situ* hybridization detected HIV-1 RNA in mononuclear cells in a perivacuolar distribution. (Pt 2; ×100.) (*C*) *In situ* hybridization detected HIV-1 RNA in mononuclear cells and multinucleated giant cells in a perivascular distribution. (Pt 1; ×100.) (*D*) Background hybridization signal using control sense probe. (Pt 1; ×400.) (*E*) Mononuclear and multinucleated cells stained positively with the lectin RCA-1. (Pt 2; ×400.) (*F*) Mononuclear cells and multinucleated giant cells stained positively with Leu-M1 antibody. (Pt 1; ×400.) (*G*) Macrophages stained positively for myelin basic protein. (Pt 1; ×400.) (*H*) *In situ* hybridization detected HIV-1 RNA in mononuclear macrophages and multinucleated giant cells first stained for lysozyme. (Pt 1; ×400.) (*I*) *In situ* hybridization detected HIV-1 RNA in mononuclear macrophages and multinucleated giant cells first stained with RCA-1. (Pt 1; ×400.) (*J*) *In situ* hybridization detected HIV RNA in mononuclear cells in a perivascular distribution. No HIV-1 RNA was detected in endothelial cells first stained with antibody to factor VIII-related antigen. (Pt 1; ×400.)

Table 1. Immunohistochemical analyses of mononucleated and multinucleated giant cells

Marker	Patient 1		Patient 2	Patient 3
	MC	MNGC	MC	MC
Macrophage				
RCA-1	+	+	+	+
MAC-387	+	+	+	+
Leu-M1	+	+	−	ND
Lysozyme	+	+	+	+
Neuronal and endothelial cell				
Glial fibrillary				
Acidic protein	−	−	−	−
Myelin basic protein	+	+	+	+
Factor VIII related				
Antigen	−	−	−	−

MC, mononucleated cells; MNGC, multinucleated giant cells; ND, not done.

that clearly expressed HIV-1 RNA as detected by *in situ* hybridization and immunohistochemistry were mononucleated or multinucleated cells staining with the macrophage marker RCA-1, MAC-387, or lysozyme (Fig. 1 *H* and *I*). Many mononucleated inflammatory cells and multinucleated cells that stained with antiserum to myelin basic protein showed HIV-1 expression by *in situ* hybridization as well. Mononucleated and multinucleated macrophages that exhibited HIV-1 RNA were frequently present immediately adjacent to, as well as occasionally within, the walls of blood vessels, but HIV-1 RNA expression was not detected in the factor VIII-related antigen-labeled endothelial cells of these vessels (Fig. 1*J*). HIV-1 RNA expression was not detected in any cell labeled with antiserum to glial fibrillary acidic protein or factor VIII-related antigen. The double-label procedure occasionally showed cells that expressed HIV-1 RNA but were not identified either by immunohistochemistry or by morphology (Fig. 1 *H* and *I*).

Transmission EM. Examination of the spinal cords of patients 1 and 2 by EM showed that the number of macrophages and viral particles in each cord paralleled the degree of tissue damage. Small clusters of mature virions associated with rare macrophages were seen in the spinal cord of patient 2, which showed moderate damage. The spinal cord of patient 1, by contrast, showed extensive necrosis and was characterized by abundant viral particles associated with mononucleated and multinucleated giant cells, the predominant type of intact cell observed in the tissue (Fig. 2 *A* and *B*). Macrophages were identified by several criteria: a prominent perinuclear Golgi zone, short profiles of rough endoplasmic reticulum, and, where the plasma membrane remained intact, subplasmalemmal linear densities (Fig. 2 *A* and *B*). The macrophages had from one to several irregular, often eccentric nuclei, had an irregular surface, and contained abundant lipid vacuoles as well as phagocytized myelin and cellular debris.

Typical mature virions containing cone-shaped nucleoids were observed scattered within the debris and clustered along the plasma membrane of macrophages (Fig. 2 *B–E*). Particles were seen in varying stages of budding from the plasma membrane. Mature particles were seen in apparent coated pits and partially coated cytoplasmic vacuoles near the cell surface. Although free mature particles were seen within the thickened basement membrane of many vessels, they were not observed either budding from or intimately associated with the surface of endothelial cells or free in vessel lumina.

DISCUSSION

We used *in situ* hybridization to demonstrate the presence of HIV-1 RNA in the spinal cords of three AIDS patients with

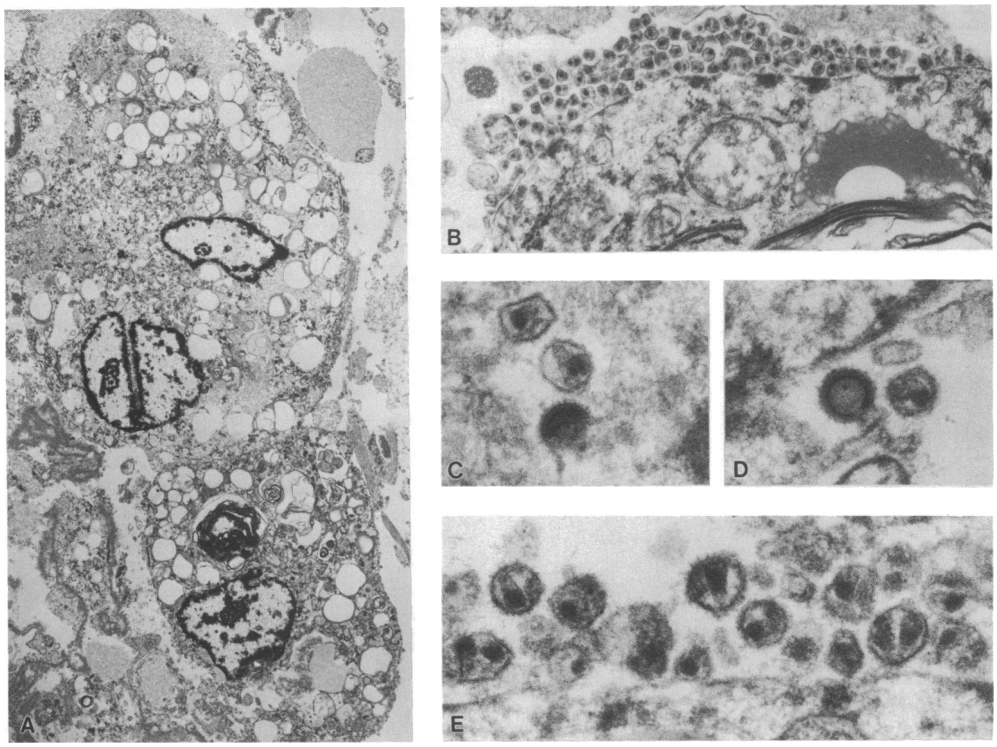

FIG. 2. Transmission electron micrographs of macrophages producing HIV-1 in the spinal cord of patient 1. (*A*) Typical mononuclear and binucleated macrophages are present. Both cells are rich with lipid vacuoles; the mononuclear cell also contains phagocytized myelin. (×2500.) (*B*) Aggregate of mature virions on the surface of a macrophage that contains internalized myelin and demonstrates subplasmalemmal linear densities. (×17,400.) (*A–C*) Virions in varying stages of maturation from mid-budding (*C*; ×61,600) to late budding (*D*; ×61,600) to mature (*E*; ×61,600). Spikes are discernible on budding virus and some of the mature particles.

vacuolar myelopathy. Combined *in situ* hybridization for HIV-1 RNA and immunohistochemical staining to identify cell type showed that HIV-1 was expressed in cells of monocyte/macrophage lineage. These cells were found primarily in areas of myelopathy in spinal cord white matter. EM supported the *in situ* hybridization and immunohistochemical staining results by showing retroviral particles associated with and budding from macrophages. Immunohistochemistry and EM both showed myelin within macrophages as well. This work extends the studies showing HIV-1 infection (5–11) and phagocytized myelin (6, 11) within macrophages in the brains of patients with HIV-1 encephalopathy. These data strongly suggest a role for HIV-1-infected macrophages locally in the pathogenesis of vacuolar myelopathy and systemically in the development of disease in HIV-1-infected patients.

The spinal cord of one patient described here had abundant HIV-1-infected multinucleated giant cells. This finding supports the demonstration of such cells in the brains of AIDS patients and the proposal that these cells may be markers of HIV-1 infection in tissue (25). Previous studies using morphology and immunohistochemistry to identify cell type suggested that these cells were macrophage derived (6, 25). This study showed that the multinucleated cells stained positively with four macrophage markers, thereby confirming their derivation from macrophages.

Combined *in situ* hybridization for HIV-1 RNA and immunohistochemistry using markers for a variety of cell types showed that HIV-1 expression in spinal cord was localized predominantly to cells of monocyte/macrophage lineage, as it is in the brain (6–11). This double-label procedure, however, occasionally showed spinal cord cells that were clearly expressing HIV-1 but were not identified by immunohistochemical staining or morphology. It is therefore possible that primary neural or glial cells of the spinal cord may express HIV-1 but were not labeled by the cell markers we employed. It is also possible that low levels of viral replication may occur in primary neural or glial cells, result in pathologic changes, but escape detection by the *in situ* hybridization technique.

The frequent demonstration of HIV-1-infected, myelin-containing macrophages within or adjacent to vacuoles suggests that active ingestion of myelin by macrophages contributes directly to the formation of the vacuoles, as either a part of a primary process or a secondary response to tissue damage. In two spinal cords examined by EM the number of macrophages and viral particles paralleled the degree of pathologic change. There are several possible mechanisms for tissue destruction by macrophages. Infection by HIV-1 may stimulate macrophages to phagocytize myelin. HIV-1-infected macrophages may secrete factors that directly damage neural tissue. Finally, macrophages are likely to ingest myelin that has been damaged by any mechanism.

The demonstration of infected macrophages in spinal cord supports the "Trojan horse" theory of HIV-1 dissemination via circulating monocytes. This theory is based on data from sheep infected with visna virus. Visna-infected monocytes show restricted viral expression and evade host immune surveillance in the periphery (26), but when these monocytes enter target tissues, they mature into macrophages which support enhanced viral replication (27). HIV-1-infected monocyte/macrophages have been described in peripheral blood (28), lymph nodes (29), lung (30), and skin (31) as well as brain and spinal cord. Therefore a monocyte may become infected with HIV-1, circulate to a distant organ, and then mature into a macrophage that serves as a reservoir of HIV-1 and mediator of tissue damage. The frequent perivascular distribution of HIV-1-infected macrophages seen in the present study and reported by others (6, 8, 10) supports the concept of egress of HIV-1-infected circulating monocytes from tissue blood vessels into parenchyma. Additional studies are necessary to elucidate the traffic of infected macrophages into the central nervous system.

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