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Cellular Localization of an HIV-1 Antigen in Subacute AIDS Encephalitis Using an Improved Double-labeling Immunohistochemical Method

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Among 102 brains obtained from patients with acquired immune deficiency syndrome (AIDS), 34 cases with subacute AIDS encephalitis were characterized by immunohistochemistry using an antibody that binds to a human immunodeficiency virus-1 (HIV-1) envelope glycoprotein, gp41. This glycoprotein was detected in mononucleated and/or multinucleated cells in 90% of adult and 50% of pediatric brains with subacute AIDS encephalitis. In addition, many gp41-positive cells with bipolar or multipolar processes were found in 10 cases, and these cells occurred most frequently in the basal ganglia and internal capsule. The phenotype of the gp41-positive cells was determined using an improved double-labeling immunohistochemical technique that employed beta-galactosidase and peroxidase conjugated reagents. Cell-type specific markers for double-labeling included: Ricinus communis ***agglutinin-1 (RCA-1) for macrophages and microglia;*** Ulex europaeus ***agglutinin-1 for endothelium; anti-glial fibrillary acidic protein (GFAP) for astrocytes; anti-amyloid precursor protein for neurons; and anti-leukocyte common antigen for leukocytes. Results of double-immunostaining revealed that gp41-positive cells of all morphologic types, including cells with bipolar or multipolar processes, were double-labeled with RCA-1, but not with markers for astrocytes, neurons, or endothelia. These findings support the contention that HIV-1 infection of the CNS is predominantly restricted to cells of the macrophage/microglia lineage. (Am J Pathol 1990, 136:1085–1092)***

Accumulating data indicate involvement of the central nervous system (CNS) as a characteristic of acquired immune deficiency syndrome (AIDS).[1] Subacute AIDS encephalitis, the constellation of neuropathologic features related to human immunodeficiency virus-1 (HIV-1), has been seen in 16 to 90% (30 to 40% in most large studies) of adults[2–6] and over 60% of children with AIDS.[7] Although it is still unclear how HIV-1 enters the CNS, viral epitopes have been demonstrated in the CNS by a variety of methods. The exact cellular localization of HIV-1, however, has been problematic because of lack of a satisfactory, precise double-labeling method. Several reports, which used *in situ* hybridization[7–15] or immunohistochemistry,[7,10,14–23] have suggested that HIV-1 is present not only in macrophages and multinucleated cells, but also in endothelia[10,12,16,18,21] and other glial cells.[9,10,14,18,21] Conversely, several convincing double-immunostaining studies[17,23] have demonstrated absence of HIV-1 epitopes in neuroglia. Previous double-labeling studies[10,11,17,21–23] have shown HIV-1 epitopes in mononucleated and multinucleated cells expressing markers for macrophages/microglia.

This report describes an improved double-labeling immunohistochemical method in paraffin sections and its use with a sensitive and specific anti–HIV-1 antibody and a battery of reliable cell markers for macrophage/microglia, astrocytes, neurons, endothelia, and leukocytes.

Materials

One hundred and two brains (81 adults and 21 children with AIDS) were obtained from the files of the Albert Einstein College of Medicine from August 1981 to May 1989. The brains had been fixed in 10% buffered formaldehyde for 2 weeks, embedded in paraffin, and examined histologically. Central nervous system tissue with subacute

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Table 1. *Immunohistochemical Reagents*

Viral and cell markers	Antibodies/lectins	Sources
Human immunodeficiency virus-1	Anti-gp41	Genetic Systems
Cytomegalovirus	Anti-cytomegalovirus	Dako
Microglia/macrophages	*Ricinus communis* agglutinin-1 (RCA-1)	Vector
	HAM-56	Enzo Biochem
	LN-1	ICN
	anti-HLA-DR	Becton Dickinson
Astrocytes	anti-GFAP	Dr. J. E. Goldman (Columbia University)
Endothelia	*Ulex europaeus* agglutinin-1	Vector
Leukocytes	Anti-leukocyte common antigen	Dako
Neurons	Anti-amyloid precursor protein	Drs. S. H. Yen and D. W. Dickson (Albert Einstein College of Medicine)

AIDS encephalitis (22 adult and 12 pediatric brains) was studied with immunohistochemistry.

Immunohistochemistry

Unstained 6-μ-thick paraffin sections from multiple cerebral regions were mounted on poly-L-lysine–coated glass slides for immunohistochemical studies. The sections were incubated with primary antibodies (Table 1) for 18 hours at 4°C. Detection of an HIV-1–associated antigen was determined using a mouse monoclonal anti-gp41 antibody (41.1; Genetic Systems, Seattle, WA). This antibody was raised against the HIV-1 envelope protein and gives reproducible and consistent binding to this HIV-1 antigen in routinely processed paraffin sections.[19,21-23] Other antibodies to HIV-1 (p17, p24; Du Pont, Wilmington, DE) also were tested, but did not work in paraffin sections. A monoclonal antibody to cytomegalovirus (CMV) (Dako, Santa Barbara, CA) was used to detect this virus in the cases with prominent microglial nodule formation (adult cases 4, 5, 7, 8, 11, 17, and 21; pediatric cases 4, 8, 11, and 12). A battery of reagents was used to define different cell types. The lectin *Ricinus communis* agglutinin-1 (RCA-1) (Vector, Burlingame, CA) is a reliable marker on paraffin sections for macrophages, microglia,[24] and multinucleated cells in AIDS.[20,23,25] *Ricinus communis* agglutin-1 was used at a 1:300 dilution in a specific buffer.[24] HAM-56 (Enzo Biochem, New York, NY), LN-1 (ICN ImmunoBiologicals, Lisle, IL),[26] and anti-HLA-DR (Becton Dickinson, Mountainview, CA),[27] other markers for macrophages/microglia, also were used. A rabbit polyclonal antibody to glial fibrillary acidic protein (anti-GFAP) (Dr. J. E. Goldman, Columbia University, NY) was used to identify astrocytes. *Ulex europaeus* agglutinin 1 (UEA-1) (Vector) was used to identify endothelia. Anti-leukocyte common antigen (anti-LCA) (Dako) was the marker for leukocytes. Although LCA is present on ramified microglia in vibratome sections,[28] in our hands it stains only mononuclear cells in paraffin sections. A rabbit polyclonal antibody to the beta-amyloid precursor protein[29] (obtained from Drs. S-H Yen/DW Dickson of our department) was used to identify neurons.

After incubation with primary antibodies, sections were processed using peroxidase-avidin-biotin-complex (ABC) (Vector), peroxidase-anti-peroxidase (PAP) (Sternberger-Meyer), or the streptavidin-beta-galactosidase (BRL, Gaithersburg, MD) detection systems. The chromogen used for peroxidase was diaminobenzidine (brown) or aminoethylcarbazole (red), and for beta-galactosidase, X-Gal (bright blue; BRL). For double-labeling, sections were first incubated with anti-gp41. Binding was usually detected by the beta-galactosidase-X-Gal system, which provides an insoluble blue reaction product at the site of antibody binding.[23,30] Subsequently, incubation with the second primary antibody or lectin (RCA-1, HAM-56, LN-1, anti-LCA, anti-GFAP, anti-amyloid precursor protein) was carried out and a brown color was developed with peroxidase-conjugated reagents and dimethylaminoazobenzene (DAB). For double-staining of anti-gp41 and UEA-1, the first label was detected by the peroxidase-ABC method with aminoethylcarbazole (red), and the chromogen for the second label was 4-chloro-1-naphthol (gray). If the ABC method was used in both first and second steps, the avidin-biotin complex from the first step was blocked by incubating sections with avidin and biotin solution (Avidin-Biotin blocking kit, Vector). For double-labeling of anti-gp41 and other mouse antibodies (HAM-56, LN-1, anti-LCA), anti-gp41 was removed by incubating the sections in 0.1 molar (M) glycine-HCl pH 2.2,[31] for 1 hour before adding the second mouse antibody.

Controls

HIV markers

Because anti-gp41 is IgG1, another mouse monoclonal antibody of the same subclass, but reactive with an irrelevant epitope in Alzheimer neurofibrillary tangles

(ALZ.NFT.64),[32] was chosen as a control. Anti-gp41 also was tested with HIV-1–infected (IIIb strain) and uninfected human T-lymphoblastic cell lines (H9), as well as with brain sections from an adult with neurosyphilis showing granulomas with multinucleated cells. Staining of macrophages/microglia was not detected by ALZ.NFT.64. Human immunodeficiency virus-1–uninfected H9 cells and sections from neurosyphilis were not stained by anti-gp41. Consistent positive staining was obtained with anti-gp41 in HIV-1–infected H9 cells.

Cell markers

Negative controls for other antibodies were unrelated antibodies of the same immunoglobulin subclasses. The control for RCA-1 and UEA-1 was staining in the absence of the lectin. No staining was seen in these controls.

Brain tissue

Control brains from three adult and two pediatric brains with AIDS, but without subacute AIDS encephalitis, were immunostained at the same time.

Results

Routine histologic examination of 102 brains showed subacute AIDS encephalitis, characterized by mononuclear cell infiltrates and microglial proliferation with or without multinucleated cells, in 22 adult (27%) and 12 pediatric brains (57%). Multinucleated cells were seen in 17 adults (21%) and nine children (43%). Immunohistochemistry with anti-gp41 antibody revealed positive mononucleated and/or multinucleated cells (Figure 1A) in 90% (20/22) of adult and 50% (6/12) of pediatric brains with subacute AIDS encephalitis (Table 2). The presence of subacute encephalitis and white matter changes (either diffuse or focal myelin pallor) appeared to correlate with presence of gp41 immunoreactivity in adult cases, but it was more difficult to demonstrate gp41-positive cells in pediatric cases. Interestingly, gp41-positivity seemed to be restricted to older children. No gp41 was detected in younger infants with subacute AIDS encephalitis.

Immunostaining of cases with prominent microglial nodules (cases with asterisk in Table 2) with a monoclonal antibody to CMV failed to reveal CMV antigen, except for one adult case (case 4), which had a positively stained Cowdry type A inclusion in the center of one of the microglial nodules. Even this lesion contained a few gp41-positive mononuclear cells. In other areas, microglial nodules and mononucleated and multinucleated cells were immunostained with anti-gp41 in the absence of CMV. Another adult case (case 8) showed only focal ependymal infection by CMV, but numerous gp41-positive cells in the parenchyma.

Among other possible associated findings, basal ganglia microinfarcts seemed to be common in adult cases with gp41-positive cells in the deep gray matter. Conversely, basal ganglia calcification, a characteristic neuropathologic feature of pediatric AIDS,[33] was present in the majority of pediatric brains irrespective of whether gp41 was detectable in the brain. Vascular proliferation[7] that resembles Leigh's syndrome was present in gray matter of two of the pediatric cases (cases 2 and 9). Neither case had coexistent gp41-positivity. One child (case 3) had multiple confluent aneurysms of the circle of Willis ("aneurysmal arteriopathy"). Not only cells in the brain, but also cells in the arterial wall were stained with anti-gp41.[23]

In general, gp41-positive cells were most frequently seen in the cerebral white matter and deep gray matter (basal ganglia and thalamus). Occasional gp41-positive cells with long processes were noted in these deep structures (Figure 1B). Although gp41-positive cells could be identified in and around blood vessels, perivascular localization was not a frequent feature. Perivascular cells were usually round or multinucleated and less often process-bearing. Gp41 was not detected in the leptomeninges, ependymal cells, or choroid plexus.

Single immunostaining with RCA-1 of adjacent sections (Figures 1C and D) revealed a staining pattern almost identical to that of anti-gp41, except that RCA-1 also stained blood vessels. Double-immunostaining with anti-gp41 and RCA-1 clearly demonstrated that gp41-positive cells were identical to cells stained with RCA-1 (Figures 1E and F). Gp41-positive cells of all morphologic types were double-labeled with RCA-1. This included round cells, process-bearing cells, and multinucleated cells. Dual-stained process-bearing cells sometimes resembled activated microglia with either long non-branched or short thickly branched processes. Cells resembling resting microglia, with delicate highly branched processes,[34] were only rarely gp41-positive. HAM-56 also double-stained macrophages and multinucleated cells, but was inferior to RCA-1 in staining of cells with processes (not shown). In our hands, LN-1 was not an effective marker for microglia in paraffin sections,[35] despite protease treatment, prolonged incubation times, and use of sensitive detection methods. The HLA-DR staining of paraffin sections sometimes showed cells consistent with microglia, but results were inconsistent. (In contrast, anti-HLA-DR gives consistent staining of microglia on vibratome sections).[35] Double-immunostaining of gp41 with anti-amyloid precursor protein (Figure 2A) and anti-GFAP (Figure 2B) revealed no evidence of gp41 in neurons or astrocytes, respectively. Anti-LCA stained only perivascular mononuclear cells

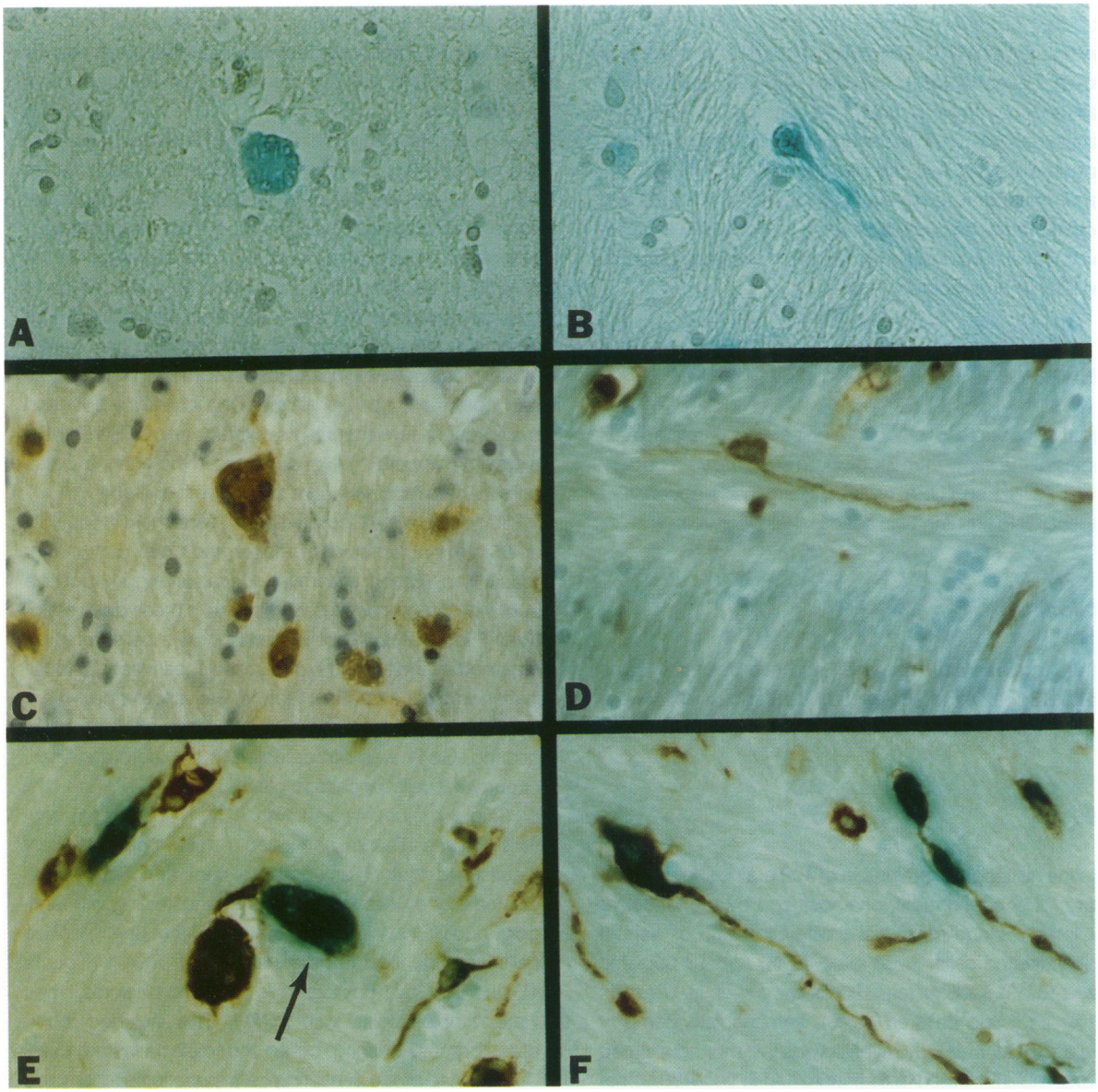

Figure 1. *Identification of HIV-1 gp41⁺ cells.* **A and B:** *Single immunostaining of the brain with subacute AIDS encephalitis with anti-HIV-1 gp41 antibody developed with beta-galactosidase method. A gp41-positive multinucleated cell* **(A)** *and a process-bearing cell* **(B)** *are stained bright blue.* **C and D:** *Single immunostaining of the adjacent section with microglia/macrophage marker RCA-1 developed with peroxidase-ABC method and DAB. Similar cells to those in A and B, as well as macrophages, are RCA-1–positive.* **(A–D** *counterstained with hematoxylin, ×450)* **E and F:** *Double immunostaining with anti-gp41 (blue) and RCA-1 (brown).* **E:** *Note a double-stained multinucleated cell (arrow) and a single-stained (RCA-1 only) similar cell, surrounded by single- or double-stained small process-bearing cells.* **F:** *Cells with long processes are also double stained with anti-gp41 and RCA-1.* **(E–F** *no counterstain, ×450).*

(Figure 2C), which were rarely double-labeled with anti-gp41. It was not difficult to find gp41-positive cells in and around vessels, but when UEA-1 was applied, these gp41-positive cells almost always appeared to be outside the vessel wall or within the lumen. Only rare equivocally double-stained endothelia were observed (Figure 2D).

Discussion

Our study demonstrates that cells expressing gp41 were exclusively co-labeled by RCA-1 and not by any of the other cell markers (glial, neuronal, endothelial). In addition, many of the cells had processes and features differing from resting microglia, but similar to activated microglia.[34] In the most severely affected cases, the major cell type containing the gp41 epitope was activated microglia in the basal ganglia. We do not know whether these cells are derived from resting microglia or blood-borne monocytes/macrophages, but it is noteworthy that such cells are common in subacute AIDS encephalitis and that they can be easily mistaken for astrocytes or neurons. *Ricinus communis* agglutinin-1 does not recognize astrocytes,

Table 2. *Summary of Histology and HIV-1 (gp41) Immunobistochemistry*

Age (yr)/Gender	Histology (MNC/MGC)	WM pallor	BG micro infarcts	gp41+ cells†	Other CNS pathology
Adult					
1. 37/M	+++/+++	++	+	+++	
2. 31/M	+++/+++	+++	+	+++	
3. 25/M	++/+++	+++	+	+++	
4. 27/M*	++/++	++	+	+++	CMV (focal)
5. 26/M*	+++/+	+	−	+++	
6. 35/M	++/+	++	−	+++	
7. 45/M*	++/+	+	+	+++	
8. 27/M*	++/−	+	+	+++	CMV (focal)
9. 26/F	++/−	++	+	+++	
10. 28/M	++/+	+	+	+++	Toxoplasmosis
11. 45/F*	++/++	++	+	++	
12. 33/M	++/++	++	−	++	
13. 39/M	++/+	+−	−	++	
14. 39/M	+++/+	+	−	++	PML
15. 29/M	++/+	+	−	++	1° lymphoma
16. 57/M	++/+	+	−	++	Cryptococcosis
17. 36/M*	++/−	+−	−	++	
18. 45/M	++/+	+	−	++	Toxoplasmosis
19. 31/M	+/−	+−	+	++	Toxoplasmosis
20. 24/M	++/+	+−	+	+	1° lymphoma
21. 28/M*	+/+	+	−	−	
22. 24/F	+/−	−	+	−	
23. 19/F	−/−	−	−	(+:a few in BG)	SDH
24. 39/F	−/−	−	−	(+:a few in PML)	PML
25. 25/M	−/−	−	−	−	1° lymphoma
Child (age in months)			BG calcification		
1. 132/M	++/++	+	+++	++	
2. 72/M	++/+++	++	+++	++	Vascular proliferation
3. 72/M	+/++	+	++	++	Aneurysms of the Circle of Willis
4. 35/M*	+++/+++	+++	++	++	
5. 36/F	++/+	++	+++	++	
6. 77/M	++/+	−	+++	+	
7. 23/F	++/+	+	+++	−	
8. 18/M*	++/++	+	++	−	
9. 36/F	+/−	++	++	−	Vascular proliferation
10. 6/M	+/−	−	++	−	
11. 6/F*	++/−	++	+++	−	
12. 4/M*	+/+	−	−	−	
13. 33/F	−/−	+−	++	−	
14. 14/M	−/−	+	++++	−	

* Cases with multiple microglial nodules; WM, white matter; BG, basal ganglia; CMV, cytomegalovirus; PML, progressive multifocal leukoencephalopathy; SDH, subdural hemorrhage.

† Intensity of gp41+ cells was graded in a 10× microscopic field as +++ (many), ++ (some), and + (a few). MNC, mononuclear cell infiltrate, MGC, multinucleated giant cell.

neurons, or oligodendroglia.[24,25] Furthermore, GFAP-positive astrocytes were never co-labeled with anti-gp41. Other workers using *in-situ* hybridization have suggested that HIV-1 genome is present in astrocytes,[9,14] oligodendroglia,[9,14] and neurons;[10] however, the exact cellular location of the grains indicating a positive result is often difficult to interpret in their published illustrations. Large inocula of HIV-1 have been shown to infect small numbers of glial cells in cell culture experiments,[35] but these results may not be relevant *in vivo*. In this connection, the most consistent result of a few double-labeling immunohistochemical studies including our results,[17,23] is that astrocytes are not infected by HIV-1. A newly developed marker for neurons, anti-amyloid precursor protein[28] also demonstrated absence of gp41 in neurons. However, latent low level HIV-1 infection of astrocytes and neurons may also be possible.

Ricinus communis agglutinin-1 does stain multinucleated cells[23,25] and process-bearing cells[20,23] in brains from AIDS patients. Single-immunolabeling studies on frozen sections with anti-HIV-1 antibodies showed staining of mononucleated and multinucleated cells,[16–24] as well as of process-bearing cells,[17,18,20,23] which were suggested to be macrophages/microglia, based on the similar appearance of cells on sections stained with HLA-DR[18] or RCA-1.[20] Double-labeling studies have demonstrated HIV-1 epitopes in macrophages (stained with Leu-M3,[17] CD4,[17] Leu-M5,[17] Mac-1[10]), multinucleated cells (stained

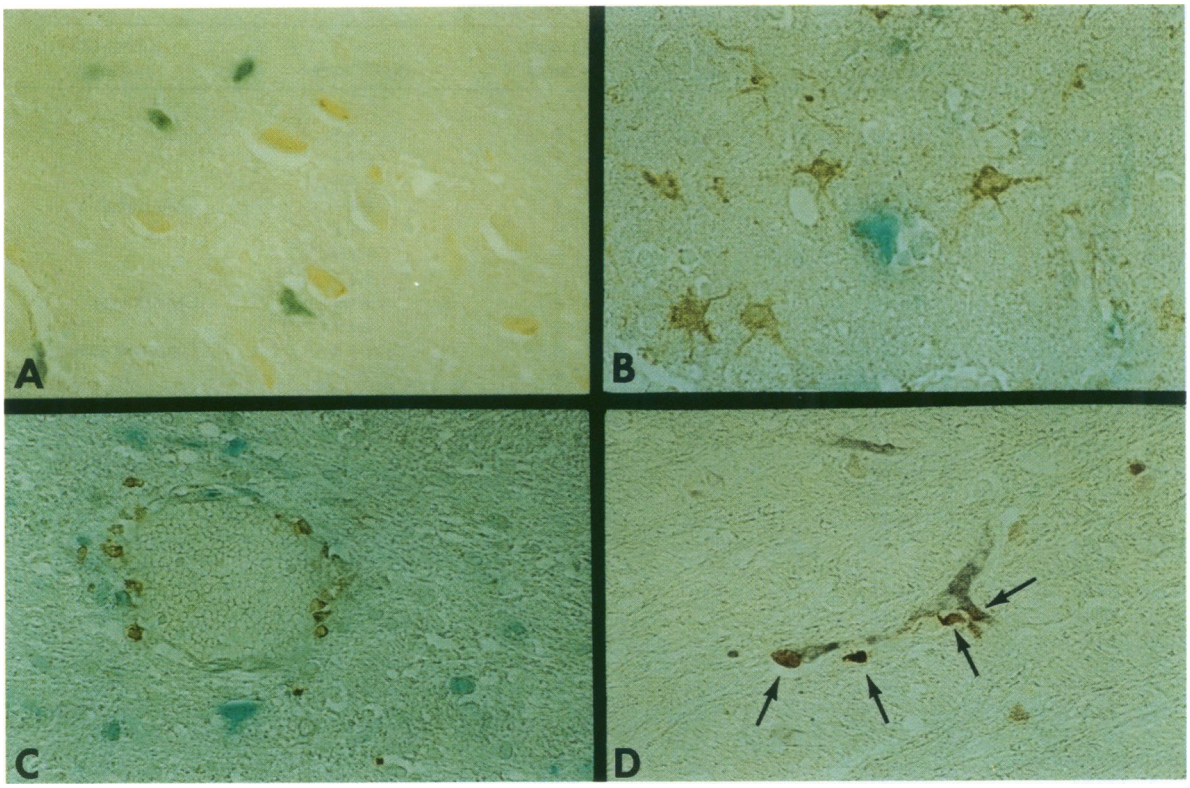

Figure 2. *Double immunostaining of gp41⁺ cells with various cell markers.* **A:** *A section from thalamus double immunostained with anti-gp41 (blue) and anti-amyloid precursor protein for neurons (brown). Note absence of double-labeled neurons.* **B:** *Double immunostaining using anti-gp41 (blue) and anti-GFAP (brown). The gp41⁺ cell in the center is not stained with anti-GFAP.* **C:** *Double immunostaining of perivascular cells with anti-gp41 (blue) and anti-leukocyte common antigen (brown). Note only single-stained leukocytes (brown) and scattered gp41⁺ cells (blue).* **D:** *Double immunostaining of perivascular gp41⁺ cells (red; arrow) and endothelia by UEA-1 (gray). Note the gp41⁺ cells adjacent to endothelia with, at most, equivocal double labeling.* (**A–E**, *no counterstain, ×250).*

with human macrophage marker,[21] negative with Leu-M3, CD4[17]), and process-bearing cells (stained with Leu-M5[17]); however, most of these studies used frozen sections, which gives less satisfactory morphologic detail than paraffin sections. The current study confirms and extends results using anti–HIV-1 antibody and RCA-1 double-immunostaining in paraffin sections, which demonstrated dual-positive multinucleated cells and process-bearing cells.[23]

The presence of HIV-1 epitopes in endothelia has only rarely been reported. Wiley and Nelson[21] found HIV-1 antigen in endothelia in over 20% of their cases. We were unable to obtain similar results using double-immunostaining with anti-gp41 and UEA-1. Equivocal endothelial staining by anti-gp41 was only rarely noted, and most often the positive cells were in the perivascular compartment. We cannot exclude other possibilities to account for this discrepancy, such as latent HIV-1 infection of endothelia below the limit of detection or transient infection of endothelia earlier in the course of disease. It should be noted, however, that we used the same anti-gp41 monoclonal antibody (41.1) in the present study as did Wiley et al.[21]

Cytomegalovirus infection of CNS has been postulated to be an important factor responsible for many cases of subacute ("microglial nodule encephalitis") AIDS encephalitis.[21] However, our results do not support this hypothesis. Although rare cases had microglial nodules that contained CMV antigen, these cases also had readily detectable nuclear and cytoplasmic inclusions. The majority of cases with microglial nodules had only HIV-1 antigen, and not CMV. Some of the differences between our results and previous studies may be related to sensitivity of the markers used. We used a mouse monoclonal antibody to CMV, whereas others[21,37] used a polyclonal goat antiserum. It is well known that polyclonal antibodies offer superior sensitivity compared with monoclonal antibodies in fixed tissue.

The frequent presence of gp41, often in microglia, and the paucity of endothelial involvement in brains with subacute AIDS encephalitis suggests a primary role for the HIV-1 infection of microglia and macrophages. Whether

intrinsic microglia are directly infected or whether infected monocytes/macrophages enter the brain ("Trojan horse" hypothesis[38]) cannot be currently resolved. These two possibilities are not mutually exclusive.

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