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Localization of HIV-1 Co-Receptors CCR5 and CXCR4 in the Brain of Children with AIDS

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The chemokine receptors CCR5 and CXCR4 are co-receptors together with CD4 for human immunodeficiency virus (HIV)-1 entry into target cells. Macrophage-tropic HIV-1 viruses use CCR5 as a co-receptor, whereas T-cell-line tropic viruses use CXCR4. HIV-1 infects the brain and causes a progressive encephalopathy in 20 to 30% of infected children and adults. Most of the HIV-1-infected cells in the brain are macrophages and microglia. We examined expression of CCR5 and CXCR4 in brain tissue from 20 pediatric acquired immune deficiency syndrome (AIDS) patients in relation to neuropathological consequences of HIV-1 infection. The overall frequency of CCR5-positive perivascular mononuclear cells and macrophages was increased in the brains of children with severe HIV-1 encephalitis (HIVE) compared with children with mild HIVE or non-AIDS controls, whereas the frequency of CXCR4-positive perivascular cells did not correlate with disease severity. CCR5- and CXCR4-positive macrophages and microglia were detected in inflammatory lesions in the brain of children with severe HIVE. In addition, CXCR4 was detected in a subpopulation of neurons in autopsy brain tissue and primary human brain cultures. Similar findings were demonstrated in the brain of adult AIDS patients and controls. These findings suggest that CCR5-positive mononuclear cells, macrophages, and microglia contribute to disease progression in the central nervous system of children and adults with AIDS by serving as targets for virus replication. *(Am J Pathol 1998, 152:167–178)*

Children with perinatal human immunodeficiency virus (HIV)-1 infection present with symptomatic disease at a median age of 8 months.[1] The most common initial manifestations are lymphoid interstitial pneumonia, encephalopathy, recurrent bacterial infection, and candida esophagitis.[1] Approximately 20 to 30% have rapid disease progression and develop severe immunodeficiency before 1 year of age.[2] The remaining 70 to 80% exhibit a slower course of disease, with a minority surviving into adolescence. Factors that are likely to determine disease progression include viral burden and phenotype as well as host factors, such as the immune response and genetic factors that affect virus replication.[2–4]

HIV-1 infects the central nervous system (CNS) and causes a progressive encephalopathy in 20 to 30% of infected children.[5–8] HIV-1 encephalopathy occurs almost exclusively in children with severe immunodeficiency and affects up to 80% of those with rapid disease progression. The most common clinical manifestations include developmental delays or loss of developmental milestones, cognitive impairment, motor deficits, and impaired brain growth.[5–8] The most common neuropathological abnormality is calcification or mineralization of deep cerebral blood vessels.[5,7,9–11] Children also develop the neuropathological changes that are characteristic of HIV-1 encephalitis (HIVE) in adults, including marked gliosis, diffuse microglial proliferation, focal microglial nodules, multinucleated giant cells, and focal demyelination.[5,7,9–11] The diffuse myelin pallor that is frequently observed may be a consequence of an abnormal blood-brain barrier.[12–14] The most commonly affected brain regions are the subcortical white matter and basal ganglia, followed by the cerebral cortex.[10,11,15,16] Increased neuronal loss has been demonstrated in the cerebral cortex of acquired immune deficiency syndrome (AIDS) patients but not in asymptomatic HIV-1-seropositive patients.[17–20] Apoptosis of neurons, astrocytes, and

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endothelial cells has been demonstrated in the brains of children[21] and adults[19,22–24] with AIDS, suggesting that apoptosis is likely to contribute to neuronal loss and possibly other neuropathological consequences of HIV-1 infection.

The mechanisms of CNS injury in HIV-1 infection are poorly understood. HIV-1 enters the brain through the passage of HIV-1-infected mononuclear cells into the perivascular space.[15,25,26] Most of the HIV-1-infected cells in the brain are macrophages and microglia.[11,15,25–29] Astrocytes are also infected but only at a very low level.[30–34] Astrocyte infection is more frequent in children than in adults.[30,31] Capillary endothelial cells in the brain may be infected at a very low level.[28,34,35] It has been proposed that HIV-1-infected macrophages and microglia secrete neurotoxic factors that lead to neuronal and glial cell death.[36] These factors may include the HIV-1 gp120 and Tat proteins, cytokines such as tumor necrosis factor-α, platelet-activating factor, nitric oxide, arachidonic acid metabolites, and excitatory amino acids. However, the *in vivo* role of these factors in contributing to CNS injury has not been established.

Recent studies have demonstrated that the chemokine receptors CCR5 and CXCR4 (also called FUSIN, HUMSTR, and LESTR) are co-receptors together with CD4 for HIV-1 entry.[37–42] Macrophage-tropic (M-tropic) HIV-1 viruses use CCR5 as a co-receptor, whereas T cell line-tropic (T-tropic) viruses use CXCR4.[37–42] Individuals with defective CCR5 alleles exhibit resistance to HIV-1 infection,[43–45] suggesting that CCR5 has an important role in HIV-1 replication *in vivo*. A subset of M-tropic viruses use CCR3 and CCR2b as co-receptors, in addition to CCR5.[39,42,46] CCR5 is expressed in activated/memory T cells, monocyte/macrophages, and granulocyte precursors.[38,40,47,48] CXCR4 is expressed in a broader range of tissues and cell types, including the brain, naive/memory T cells, and monocyte/macrophages.[37,47] HIV-1 viruses that infect the brain are M-tropic.[26,49,50] We previously demonstrated that CCR5 and CCR3 are both expressed in microglia and serve as co-receptors for HIV-1 infection of the CNS.[50] The expression of HIV-1 co-receptors in the brain of AIDS patients has not been examined.

In this study, we investigated the expression of CCR5 and CXCR4 in brain tissue from 20 pediatric AIDS patients in relation to neuropathological consequences of HIV-1 infection. The overall frequency of CCR5-positive perivascular mononuclear cells and macrophages was increased in the brain of children with severe HIVE compared with children with mild HIVE or non-AIDS controls, whereas the frequency of CXCR4-positive perivascular cells did not correlate with disease severity. CCR5- and CXCR4-positive macrophages and microglia were detected in inflammatory lesions in the white matter in children with severe HIVE. CXCR4 was also detected in a subpopulation of neurons. Similar findings were demonstrated in adult AIDS patients and controls. These findings together with the observation that HIV-1 viruses in the brain are M-tropic suggest a role for CCR5-positive mononuclear cells, macrophages, and microglia in contributing to disease progression in the CNS of children and adults with AIDS by serving as targets for virus replication.

Materials and Methods

Patients

Pediatric autopsy AIDS cases were obtained from Children's Hospital, Boston, MA; from University of Massachusetts Medical Center, Worcester, MA; from Dr. F. Gray's consultation file in Paris, France; and from Romania through Drs. J. Bell and C. Keohane. Autopsy tissues were fixed in 10% formalin and embedded in paraffin for routine histology. Pediatric non-AIDS control cases were obtained from the 1993 to 1996 autopsy files of Children's Hospital. Adult AIDS and non-AIDS control cases were obtained from Brigham and Women's Hospital, Boston, MA. Patients with hypoxic-ishemic encephalopathy were excluded.

Primary Brain Cultures

Primary human brain cultures were prepared from fetal abortuses at 13 to 18 weeks as described.[24] Tissue was procured using an approved protocol in compliance with institutional and federal regulations. Briefly, the tissues were minced and incubated briefly with 0.25% trypsin, dissociated by trituration, extensively washed, and cultured on polylysine-coated glass coverslips in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, penicillin, and streptomycin for 4 weeks before fixation. These cultures contain a mixture of astrocytes (70 to 90%), neurons (10 to 30%), microglia (1 to 5%), and fibroblasts (1 to 5%).[24]

TUNEL Histochemistry

Fragmented DNA was detected *in situ* by the terminal deoxynucleotidyl transferase-mediated duTP nick end labeling (TUNEL) method using a commercially available kit according to the manufacturer's protocol (Apoptag, Oncor, Gaithersburg, MD) followed by metal-enhanced diaminobenzidine color development (Pierce Chemical Co., Rockford, IL) as described.[24] Paraffin-embedded tissue sections were deparaffinized, rehydrated in graded ethanols, pretreated with 20 μg/ml proteinase K for 25 minutes at room temperature, and washed before TUNEL staining. A semiquantitative scale was used to estimate the frequency of TUNEL staining in tissue sections counterstained with methyl green to distinguish TUNEL-positive and -negative cells. The entire tissue section was reviewed by three of the authors (A.-V. Vallat, U. De Girolami, and D. Gabuzda), and the frequency of TUNEL staining was estimated and scored as follows: 0, negative; 1+, <10 positive cells; 2+, 10 to 20 positive cells; 3+, >20 positive cells.

Immunohistochemistry

Incubation with the primary antibody was performed for 1 hour at room temperature and detected using streptavidin alkaline-phosphatase and Fast Red color development according to the manufacturer's protocol (USA-Alk Phos detection system, Signet Laboratories, Dedham, MA) as described.[24] For staining with the rabbit anti-p24 antibody (1:800 dilution, donated to the National Institutes of Health AIDS Research and Reference Reagent Program by Dr. Julia Hurwitz), the slides were pretreated with proteinase K (20 μg/ml) for 25 minutes at room temperature followed by microwaving for 8 minutes in sodium citrate buffer, pH 6.0 (1.05 g of citric acid in 500 ml of dH_20). For staining with rabbit anti-CCR5 raised against an amino-terminal peptide (amino acids 1 to 36; 1:100 dilution) or rabbit anti-CXCR4 raised against an amino-terminal peptide (1:100 dilution; Biochain Institute, San Leandro, CA), slides were pretreated by microwaving for 8 minutes in citrate buffer and then incubated with 0.1% protease (Sigma type XXIV, Sigma Chemical Co., St. Louis, MO) for 5 minutes at 37°C. Immunostaining with anti-glial fibrillary acidic protein (anti-GFAP, prediluted; Signet Laboratories) or anti-CD68 (KP-1, 1:50 dilution; Dako, Carpinteria, CA) was performed without microwave pretreatment.

For immunohistochemistry combined with TUNEL staining, TUNEL staining was performed as described above. The sections were subsequently pretreated by microwaving for 8 minutes in citrate buffer. Incubation with rabbit anti-Nef antibody[24,51] (1:800 dilution) was then performed for 1 hour at room temperature and detected as described above. Immunohistochemistry combined with TUNEL staining followed by staining with the mouse monoclonal antibodies anti-CD68 (KP-1, 1:50 dilution; Dako), anti-CD31 (1:10 dilution; Dako), anti-GFAP (prediluted; Signet Laboratories), or anti-neurofilament (prediluted; Signet Laboratories) was performed without microwave pretreatment.

Immunofluorescence Staining

Double immunofluorescence staining of fixed primary brain cultures with the indicated primary antibodies followed by fluorescein-isothiocyanate- or rhodamine-conjugated antibodies (Sigma) was performed as described.[24,50] The dilutions for the primary antibodies were as follows: mouse anti-CD68 monoclonal (EBM 11; Dako), 1:10; mouse anti-GFAP monoclonal (Sigma), 1:50; mouse anti-microtubule-associated protein-2 (MAP-2) monoclonal (Sigma), 1:50; *Ricinis communis* agglutinin I (RCA-1) conjugated to rhodamine (Vector Laboratories, Burlingame, CA), 1:500; rabbit anti-Tau (Dako), 1:100; rabbit anti-CXCR4 (amino-terminal peptide; Biochain Institute), 1:20; rabbit anti-CCR5, 1:100; and mouse anti-CXCR4 monoclonal (125G5),[52] 1:20 (donated to the National Institutes of Health AIDS Research and Reference Reagent Program by Dr. James Hoxie).

Results

Twenty pediatric AIDS cases and eight non-AIDS controls were selected for this study. The clinical and neuropathological findings are summarized in Table 1. Systemic disease in the non-AIDS controls included three with neoplastic disease, three with neurological disorders, one with subglottic stenosis, and one with Marfan's syndrome; their mean age was 4.5 years (range, 2 to 14 years). The AIDS cases were classified into two broad categories. Group A consisted of 10 cases from the USA and France (cases 1 to 10). Group B consisted of 10 cases from Romania (cases 11 to 20). The mean age of group A was 2.75 years (range, 3 months to 7 years), and the mean age of group B was 5.5 years (range, 3 to 7 years). All patients in both groups had systemic manifestations of AIDS, including opportunistic infections and hematological disorders. The AIDS risk factors in group A were maternal intravenous drug use (n = 3), maternal blood transfusion (n = 1), maternal heterosexual contact with an infected spouse (n = 1), or unknown (n = 5). Patients in group B were infected through contaminated needles used in injectable therapies or transfusions. The neuropathology of the control and AIDS cases was reviewed. Neuropathological findings in control brains included Alzheimer type II astrocytosis secondary to metabolic disorder, CNS involvement by large cell lymphoma, infiltrating pontine glioma, mild cerebral edema, mild diffuse gliosis, and cerebellar vermis atrophy. The remaining two control brains were unremarkable. Of the 10 AIDS patients in group A, 4 had severe HIVE and 6 had mild or moderate HIVE. The patients with moderate or severe HIVE had clinical evidence of progressive HIV-1 encephalopathy (ie, microcephaly, developmental delays, loss of developmental milestones, and loss of motor function). Of the 10 AIDS patients in group B, none had HIVE. Mild lymphocytic meningitis was the only neuropathological abnormality observed in five cases and was associated with mild vasculitis (perivascular inflammation with transmural infiltration of inflammatory cells) in two cases (Figure 1). Four cases in group B were considered normal, and one case had evidence of postmortem autolysis.

To detect productive HIV-1 infection in the brain and further examine the neuropathological consequences of HIV-1 infection, HIV-1 Nef immunostaining was performed in combination with TUNEL staining to detect apoptotic cells *in situ*. Two or three different brain tissue sections from each case were examined. HIV-1 Nef is a marker for productive infection in macrophages and microglia as well as restricted infection in astrocytes.[30–32] Nef-positive cells were detected in 4 of 20 AIDS cases (Table 1). The majority of Nef-positive cells had morphological characteristics of macrophages, microglia, and multinucleated giant cells and were most often detected in subcortical white matter (Figure 2). A minor fraction of the Nef-positive cells (<5 or 10%) exhibited morphological characteristics of astrocytes. Immunostaining of serial sections with anti-Nef, the macrophage/microglial marker anti-CD68, or the astrocyte marker anti-GFAP demonstrated that the ma-

Table 1. Summary of Neuropathology and Detection of HIV-1 Nef Antigens in AIDS and Control Cases

Case	Age	PMI (hours)	Diagnosis	Neuropathology	Brain region*	Nef antigen†	Apoptosis‡ Neuron	Astrocyte	Endothelial
1	1.5 years	N/A	AIDS	HIV	BG (2+)	0	0	1+	1+
				Subcortical WM infarcts	CTX (2+)	0	1+	1+	0
					BG (2+)	0	1+	1+	1+
2	7 years	4	AIDS	HIVE	BG (2+)	0	0	1+	2+
				CMV encephalitis	CTX (2+)	0	0	1+	2+
3	8 months	20	AIDS	HIVE	CTX (2+)	0	1+	1+	2+
					BG (2+)	0	1+	1+	2+
					CTX (3+)	0	0	1+	1+
4	1 years	6	AIDS	Severe HIVE	CTX (3+)	1+	0	0	0
					CTX (3+)	2+	0	1+	1+
					CTX (3+)	2+	0	1+	0
5	2 years	N/A	AIDS	HIVE	CTX (3+)	2+	0	1+	2+
				CMV ventriculitis	BG (2+)	1+	0	2+	3+
6	2 years	N/A	AIDS	HIVE	BG (1+)	0	0	1+	1+
					CTX (1+)	0	0	0	0
7	11 months	14	AIDS	Severe HIVE	CTX (3+)	3+	1+	1+	1+
					CTX (3+)	2+	0	0	0
					BG (3+)	3+	0	0	1+
8	5 years	N/A	AIDS	Severe HIVE	CTX (3+)	2+	1+	2+	3+
					BG (3+)	2+	1+	3+	3+
9	11 months	30	AIDS	Severe HIVE	CTX (2+)	0	0	2+	2+
				CMV encephalitis	CTX (2+)	0	1+	1+	1+
10	3 months	16	AIDS	HIVE	CTX (2+)	0	0	0	0
					BG (2+)	0	0	0	0
11	5 years	N/A	AIDS	Meningitis	BG (1+)	0	0	0	0
				Mild vasculitis	CTX (1+)	0	0	0	0
12	6 years	N/A	AIDS	Meningitis	BG (2+)	0	0	1+	1+
				Mild vasculitis	CTX (2+)	0	0	0	1+
					CTX (2+)	0	0	0	3+
13	7 years	N/A	AIDS	Normal	BG (0)	0	0	0	1+
					CTX (0)	0	0	0	2+
14	6 years	N/A	AIDS	Meningitis	CTX (2+)	0	0	0	0
					BG (1+)	0	0	1+	1+
15	5 years	N/A	AIDS	Meningitis	CTX (1+)	0	0	0	0
					BG (0)	0	0	0	0
16	5 years	N/A	AIDS	Meningitis	BG (0)	0	0	0	0
					CTX (1+)	0	0	0	0
17	5 years	N/A	AIDS	Normal	BG (0)	0	0	0	0
18	5 years	N/A	AIDS	Normal	BG (0)	0	0	0	0
19	6 years	N/A	AIDS	Autolysis	CTX (2+)	0	0	0	0
					BG (1+)	0	0	0	0
20	6 years	N/A	AIDS	Normal	BG (0)	0	0	0	0
					CTX (0)	0	0	0	0
21	2.5 years	<24	Metabolic encephalopathy	Astrocytosis	BG (0)	0	0	0	0
					CTX (1+)	0	0	0	0
22	14 years	24	Lymphoma, intrathecal chemotherapy	CNS involvement by lymphoma	BG (0)	0	0	2+	1+
				Gliosis	CTX (0)	0	0	1+	0
23	3 years	<24	AML, total body irradiation	Normal	CTX (0)	0	0	1+	1+
					BG (0)	0	0	1+	2+
24	6 years	6	Pontine glioma, CNS irradiation	Pontine glioma	CTX (0)	0	0	0	0
					BG (0)	0	0	0	1+
25	1.5 years	<24	Subglottic stenosis	Mild cerebral edema	CTX (1+)	0	0	0	0
					BG (1+)	0	0	0	0
26	2 years	N/A	Cerebral palsy, seizures	Gliosis	CTX (1+)	0	0	0	0
					BG (1+)	0	0	0	1+
27	7 years	N/A	Marfan's syndrome	Normal	BG (0)	0	0	0	0
					CTX (0)	0	0	0	0
28	2 years	N/A	Developmental delays, seizures	Microcephaly	BG (0)	0	0	0	0
				Gliosis	CTX (0)	0	0	0	0

PMI, postmortem interval; N/A, not available; AML, acute myelogenous leukemia; WM, white matter; HIVE, HIV-1 encephalitis; BG, basal ganglia; CTX, cerebral cortex.

*Brain tissue sections were obtained at autopsy. Shown in parentheses is grading of HIVE (cases 1 to 10), lymphocytic meningitis and vasculitis (cases 11 to 20), or non-AIDS neuropathology (cases 21 to 28) as 0 (absent), 1+ (mild), 2+ (moderate), or 3+ (severe).

†HIV-1 Nef antigens were detected by immunostaining of brain tissue sections with rabbit anti-Nef and graded as 0 (absent), 1+ (rare foci of positive cells), 2+ (frequent foci of positive cells), and 3+ (abundant large foci of positive cells).

‡Apoptosis was detected by TUNEL staining of brain tissue sections. TUNEL staining of each cell type was graded as 0 (absent), 1+ (<10 cells per section), 2+ (10+ cells per section), and 3+ (>20 cells per section).

jority of Nef-positive cells showed similar localization and morphology as the CD68-positive cells. Only a minor fraction of Nef-positive cells (<5 or 10%) showed similar localization and morphology as the GFAP-positive cells. Nef-positive cells were detected in the brain only in cases from group A. All four cases in which Nef-positive cells were detected in brain tissue had severe HIVE on neuropathological examination (Table 1). Similar results were obtained using an HIV-1 p24 antibody, except that all of the p24-positive cells had

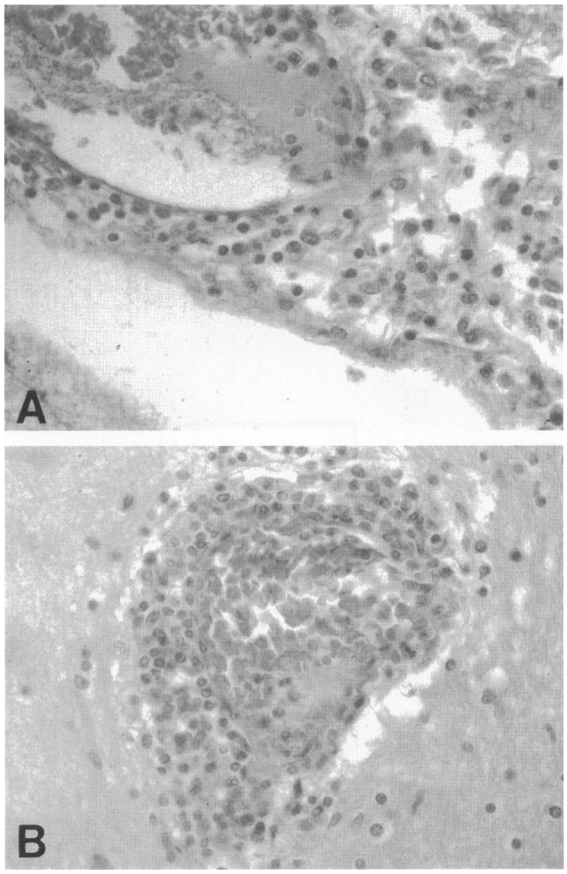

Figure 1. Lymphocytic meningitis (**A**) and perivascular inflammation (**B**) in cerebral cortex from a Romanian child with AIDS (case 12). H&E; original magnification, ×400.

morphological characteristics of macrophages, microglia, and multinucleated giant cells.

TUNEL staining was performed to detect apoptotic cells *in situ* and their relationship to HIVE and HIV-1-infected cells. We previously confirmed that detection of apoptotic cells by this method correlates with apoptotic nuclear morphology demonstrated by electron microscopy or staining with propidium iodide.[24] Apoptosis was detected by TUNEL staining in cerebral cortex and basal ganglia of 12 of 20 pediatric AIDS cases and 4 of 8 controls (Table 1). Neuronal apoptosis was surprisingly rare and was detected in only five AIDS cases. However, apoptotic cells of other types were present with variable frequency depending on the group of patients. In group A, there was an increased frequency of apoptotic cells with morphological characteristics of endothelial cells and astrocytes in six AIDS cases relative to non-AIDS controls. Less frequently, apoptotic perivascular cells with morphological characteristics of pericytes or macrophages were observed. Apoptosis of specific cell types was confirmed by TUNEL staining combined with immunostaining with the cell-specific markers anti-CD31, anti-GFAP, anti-CD68, or anti-neurofilament to detect endothelial cells, astrocytes, macrophages/microglia, and neurons, respectively. The most striking difference between AIDS patients in group A and controls was the increased frequency of apoptotic endothelial cells detected in the brain of AIDS patients (Figure 3). TUNEL-positive endothelial cells were detected in small and medium-sized blood vessels scattered throughout the gray and white matter. TUNEL-positive astrocytes were most frequently detected in the subcortical white matter and basal ganglia. The density of TUNEL-positive cells was nonuniform, and within a given patient there was variability in the number of apoptotic cells in different brain regions. There was no significant increase in the frequency of apoptotic cells in group B compared with

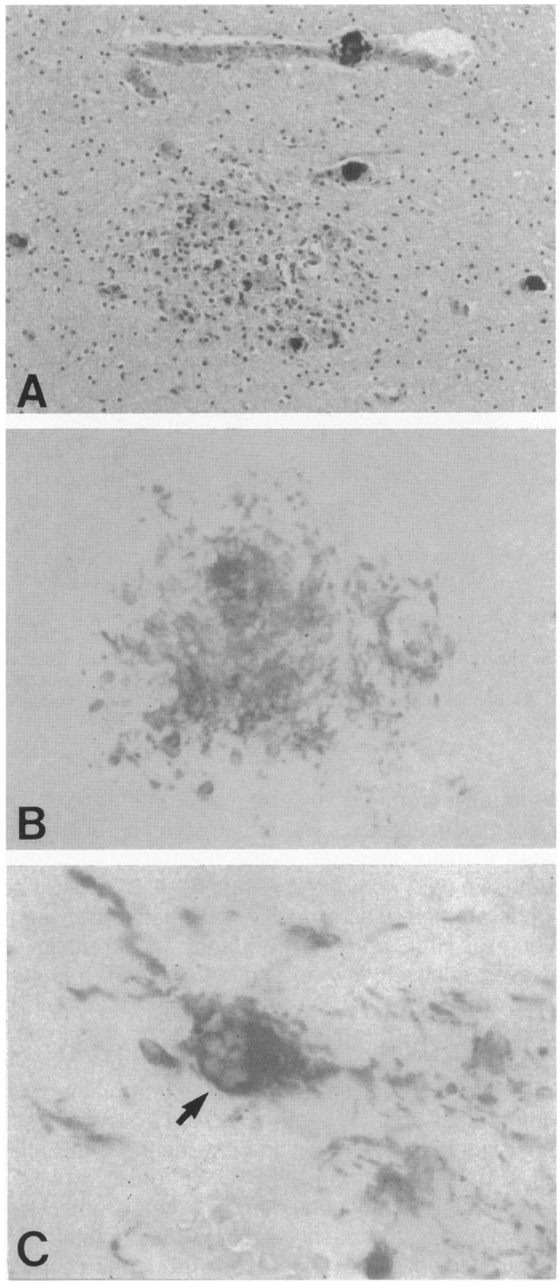

Figure 2. Detection of Nef antigens in brain tissue from a pediatric AIDS patient with severe HIV-1 encephalitis (case 7). **A:** Microglial nodule, inflammatory foci, and calcifications in cerebral cortex. H&E; original magnification, ×100. **B** and **C:** Immunostaining with anti-Nef. **B:** Nef staining in a microglial nodule. Original magnification, ×200. **C:** Nef staining in a multinucleated giant cell (**arrow**). Original magnification, ×400.

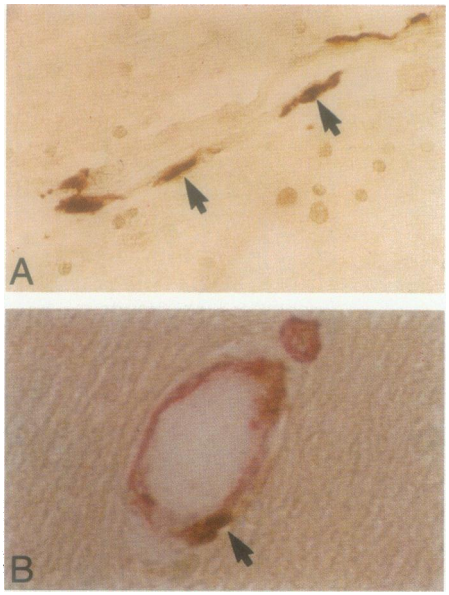

Figure 3. Endothelial cell apoptosis in cerebral cortex from a pediatric AIDS patient. TUNEL staining was performed followed by light methyl green counterstain (**A**) or immunostaining with anti-CD31 (**B**). **A:** TUNEL-positive endothelial cells. Original magnification, ×400. **B:** TUNEL-positive endothelial cells double stained with anti-CD31 (**arrows**). Original magnification, ×600.

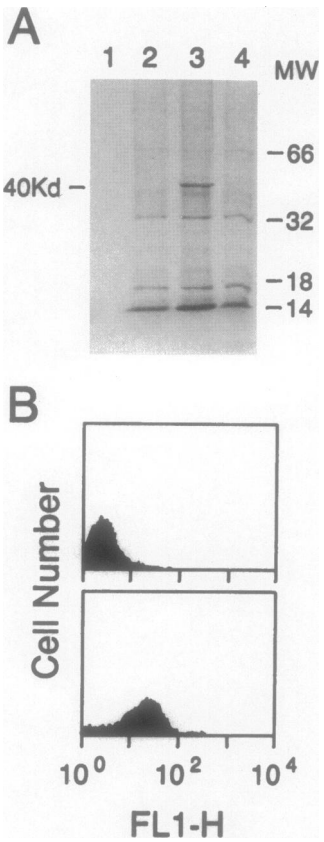

Figure 4. Characterization of anti-CCR5 antibody. **A:** Immunoprecipitation of lysate from Cf2.Th canine thymocyte cells transfected with no DNA (**lane 1**) or expression plasmids for CXCR4 (**lane 2**), CCR5 (**lane 3**), or CCR3 (**lane 4**) using DEAE-dextran as described.[39] Cells were metabolically labeled with [^{35}S]cysteine at 48 hours after transfection, lysed, and immunoprecipitated with rabbit anti-CCR5 (1:500 dilution). The 40-kd marker on the left indicates the position of the CCR5 protein shown in **lane 3**. Molecular weight markers are indicated on the right. **B:** Flow cytometry analysis of PM1 cells.[48] Cells were stained with no primary antibody (**top**) or with rabbit anti-CCR5 (**bottom**), followed by fluorescent secondary antibody. The fluorescence intensity (FL1-H) is shown on the *x* axis.

HIV-negative controls, except in case 12 (Table 1). This patient had mild vasculitis and meningitis and demonstrated a minor increase in the frequency of apoptotic endothelial cells compared with non-AIDS control cases. In general, there was a positive correlation between the severity of HIVE and the frequency of apoptotic cells. However, relatively few apoptotic cells were detected in cases 4 and 7, although HIVE was very severe. There was an increased frequency of apoptotic cells in all four patients with Nef-positive cells in the brain relative to control patients that had not received cranial irradiation or intrathecal chemotherapy (Table 1). However, there was no significant association between the localization of the Nef-positive cells and the TUNEL-positive cells. Apoptotic nuclei were not detected or were extremely rare in cerebral cortex and basal ganglia of six pediatric control patients. However, an increased frequency of TUNEL-positive astrocytes and endothelial cells was detected in two pediatric control patients that had received cranial irradiation or intrathecal chemotherapy (cases 22 and 23).

Based on the preceding studies, nine pediatric AIDS patients with mild or severe HIVE and eight non-AIDS controls were selected for immunohistochemical studies to detect CCR5 and CXCR4 expression in the brain. A rabbit anti-CCR5 serum was produced by immunization with a CCR5 amino-terminal peptide (amino acids 1 to 36). The rabbit anti-CCR5 serum specifically recognized CCR5 but not CCR3 or CXCR4 as determined by immunoprecipitation and flow cytometry analysis (Figure 4). Immunohistochemical staining of brain tissue from pediatric AIDS and non-AIDS controls was performed with anti-CCR5 or anti-CXCR4. CCR5- and CXCR4-positive cells were detected in all cases examined (Figures 5 and 6). CCR5-positive perivascular mononuclear cells were detected in brain tissue from all AIDS and non-AIDS cases examined and were most frequently detected in the white matter (Figure 5A). The overall frequency of CCR5-positive perivascular mononuclear cells and macrophages was increased in the brains of AIDS patients with severe HIVE compared with AIDS patients with mild HIVE or HIV-1-negative controls (Figure 7). However, the frequency of CCR5-positive perivascular cells was variable among individuals within each group and there was considerable overlap between the three groups. CCR5-positive macrophages and microglia were detected in microglial nodules (Figure 5B) and in scattered foci in the white matter of AIDS patients with severe HIVE but not in patients with mild HIVE or HIV-1-negative controls. In general, the CCR5-positive cells were more intensely stained and more frequent in AIDS patients with detectable Nef antigens in the brain compared with AIDS patients with absent Nef staining. However, most of the CCR5-positive cells did not appear to be infected as determined by immunostaining of serial sections with anti-CCR5 and anti-Nef. We performed a similar analysis of CXCR4 expression. CXCR4-positive perivascular

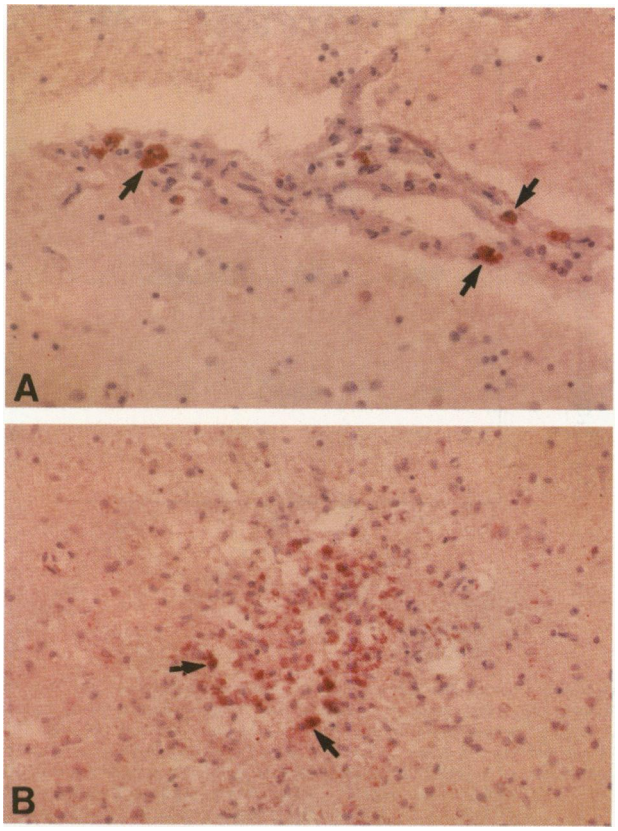

Figure 5. Immunostaining of CCR5 in brain tissue from AIDS patients. **A:** CCR5-positive perivascular mononuclear cells and macrophages (**arrows**) in white matter. **B:** CCR5-positive cells (**arrows**) in a microglial nodule. Original magnification, ×200.

mononuclear cells were detected in brain tissue from all cases examined and were most frequently detected in the white matter (Figure 6A). The frequency of CXCR4-positive perivascular mononuclear cells was variable among individuals within each group of patients and did not correlate with the severity of HIVE (Figure 7). CXCR4-positive macrophages and microglia were occasionally detected in microglial nodules and scattered foci of inflammatory cells in the white matter in AIDS patients with severe HIVE (Figure 6B). Additionally, CXCR4-positive neuronal subpopulations were detected in regions of cerebral cortex and basal ganglia in all cases examined (Figure 6C). Similar analysis of brain tissue sections from adult AIDS patients (n = 5) and non-AIDS controls with normal brain (n = 4) demonstrated that the pattern of CCR5 and CXCR4 expression was similar to that observed in the corresponding groups of pediatric patients (Figure 7 and not shown). However, CCR5- and CXCR4-positive perivascular mononuclear cells, macrophages, and microglia were generally more frequent in brain tissue from adult AIDS patients compared with pediatric AIDS patients.

We previously demonstrated that CCR5 and CCR3 are both expressed in microglia in primary human fetal brain cultures as determined by reverse transcriptase polymerase chain reaction (RT-PCR) analysis and double immunofluorescence staining.[50] Although CXCR4 mRNA was detected in primary human brain cultures by RT-PCR, the

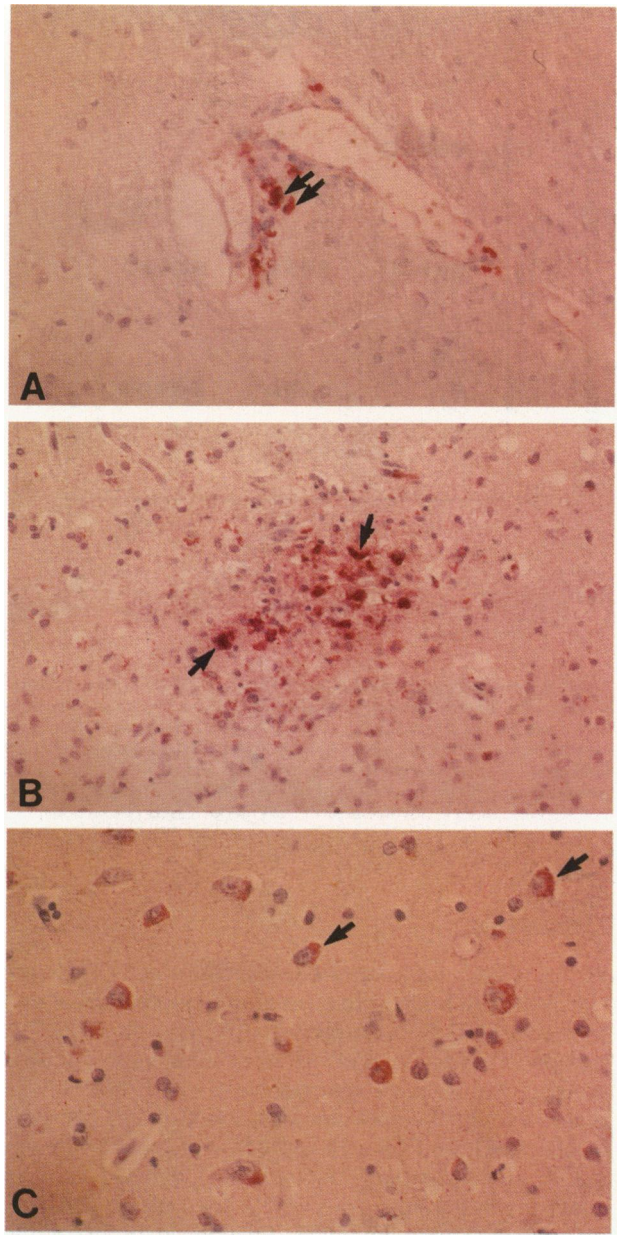

Figure 6. Immunostaining of CXCR4 in brain tissue from AIDS patients. **A:** CXCR4-positive perivascular mononuclear cells (**arrows**) in white matter. **B:** CXCR4-positive cells (**arrows**) in a microglial nodule. **C:** CXCR4-positive neurons (**arrows**) in cerebral cortex. Original magnification, ×200 (**A** and **B**) and ×400 (**C**).

localization of CXCR4 expression in different cell types was not determined. To determine which cell types express CXCR4 in primary human brain cultures, double immunofluorescence staining of fixed cultures was performed with anti-CXCR4 and cell-specific markers for neurons, astrocytes, and microglia. CXCR4 was detected in microglia (Figure 8, A and B). In addition, CXCR4 was detected in a subset of primary human neurons (approximately 5 to 10%; Figure 8, C and D). These results provide additional evidence that CXCR4 is expressed in microglia and in a subpopulation of human neurons, consistent with the preceding *in vivo* studies of autopsy brain tissue.

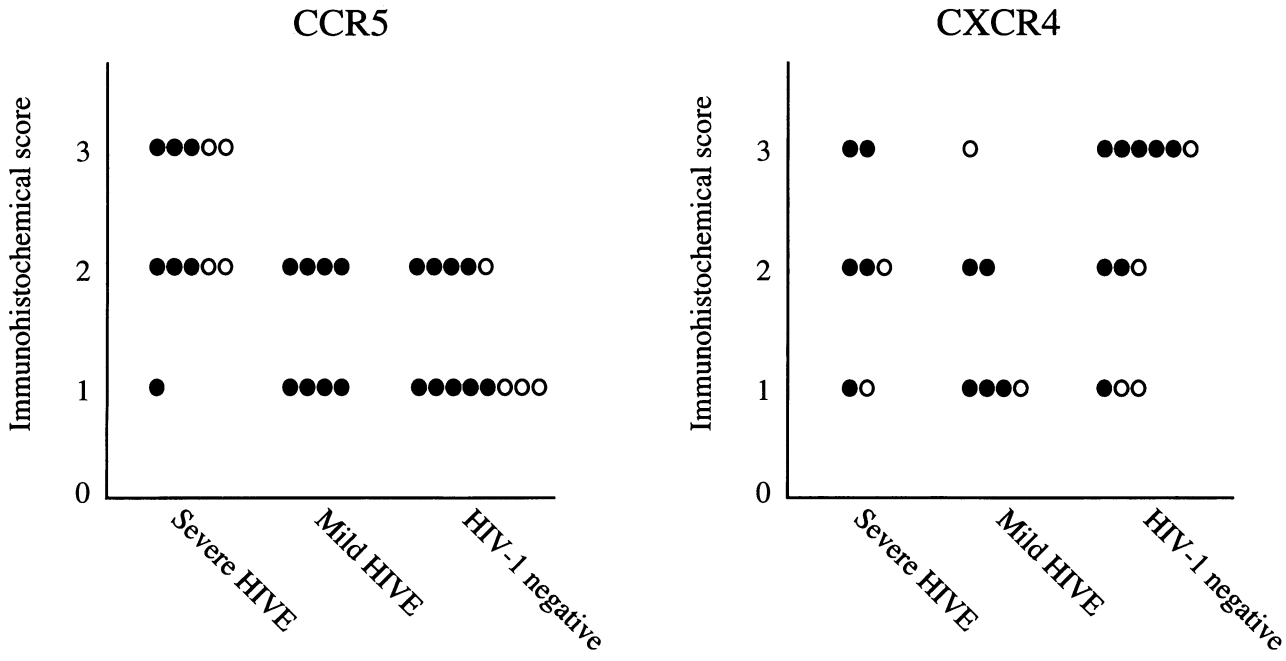

Figure 7. Immunohistochemical score of CCR5- or CXCR4-positive perivascular mononuclear cells and macrophages in brain tissue from AIDS patients. Symbols represent the immunohistochemical score of CCR5- or CXCR4-positive perivascular mononuclear cells and macrophages in brain tissue sections from each group of patients. The group of patients with mild HIVE included two Romanian children with mild meningitis and vasculitis. ●, brain tissue sections from pediatric AIDS or HIV-1-negative control cases; ○ brain tissue sections from adult AIDS or HIV-1-negative control cases. CCR5- or CXCR4-positive cells were detected by immunostaining as described in the Materials and Methods. The immunohistochemical score represents the frequency of positively stained perivascular mononuclear cells and macrophages and was graded as 0 (absent), 1+ (involvement of <10 blood vessels per section), 2+ (involvement of 10 to 20 blood vessels per section), and 3+ (involvement of >20 blood vessels per section).

Discussion

In this study, we examined expression of CCR5 and CXCR4 in the brain in relation to neuropathological consequences of HIV-1 infection in children with AIDS. Two groups of pediatric patients were examined. Group A consisted of patients with perinatal HIV-1 infection acquired through maternal transmission. Group B consisted of children from Romania with HIV-1 infection acquired during infancy through contaminated needles used for injections and transfusions.[53,54] A third group consisted of adult patients with AIDS. Patients in group A had moderate or severe HIVE and were younger at the time of death than patients in group B (2.75 *versus* 5.5 years). Neuropathological abnormalities in group B were absent or very mild, consisting of lymphocytic meningitis and vasculitis.[53,55] Similar findings have been described in early HIV-1 infection in adults[20,56] but to our knowledge have not been previously described in HIV-1-infected children. The absence of HIVE in Romanian children may also reflect death at an earlier stage of disease, as these children are frequently malnourished, have a high incidence of infections (mostly pneumonia and gastroenteritis), and rarely receive antiretroviral therapy.[53–55] It is also possible that patients in group B may be less susceptible to HIVE than those in group A as a consequence of acquiring HIV-1 infection at a later age, when the immune and central nervous systems are more mature.

Our studies demonstrate that CCR5 and CXCR4 are expressed in the brain of pediatric AIDS patients in groups A and B as well as in HIV-1-negative controls. The overall frequency of CCR5-positive perivascular mononuclear cells and macrophages was increased in the brain of children with severe HIVE compared with children with mild HIVE or HIV-1-negative controls, whereas the frequency of CXCR4-positive perivascular cells did not correlate with disease severity. However, the frequency of CCR5-positive perivascular cells was variable among individuals within each group of patients and there was considerable overlap between the three groups. CCR5- and CXCR4-positive macrophages and microglia were detected in microglial nodules and scattered foci in the white matter in children with severe HIVE but not in children with mild HIVE or HIV-negative controls. In addition, CXCR4 was detected in a subpopulation of neurons. CCR5-positive cells were more frequent and more intensely stained in AIDS patients with detectable Nef antigens in the brain. However, most of the CCR5-positive cells were not infected, as determined by immunostaining of serial brain tissue sections with anti-CCR5 and anti-Nef. The expression pattern of CCR5 and CXCR4 in the brain of adult AIDS patients and controls was similar to that observed in pediatric patients. The frequency of CCR5- and CXCR4-positive mononuclear cells, macrophages, and microglia was generally higher in the brains of adult AIDS patients than in pediatric AIDS patients. Whether this finding may help to explain why productive HIV-1 infection is more frequently detected in the brains of adults com-

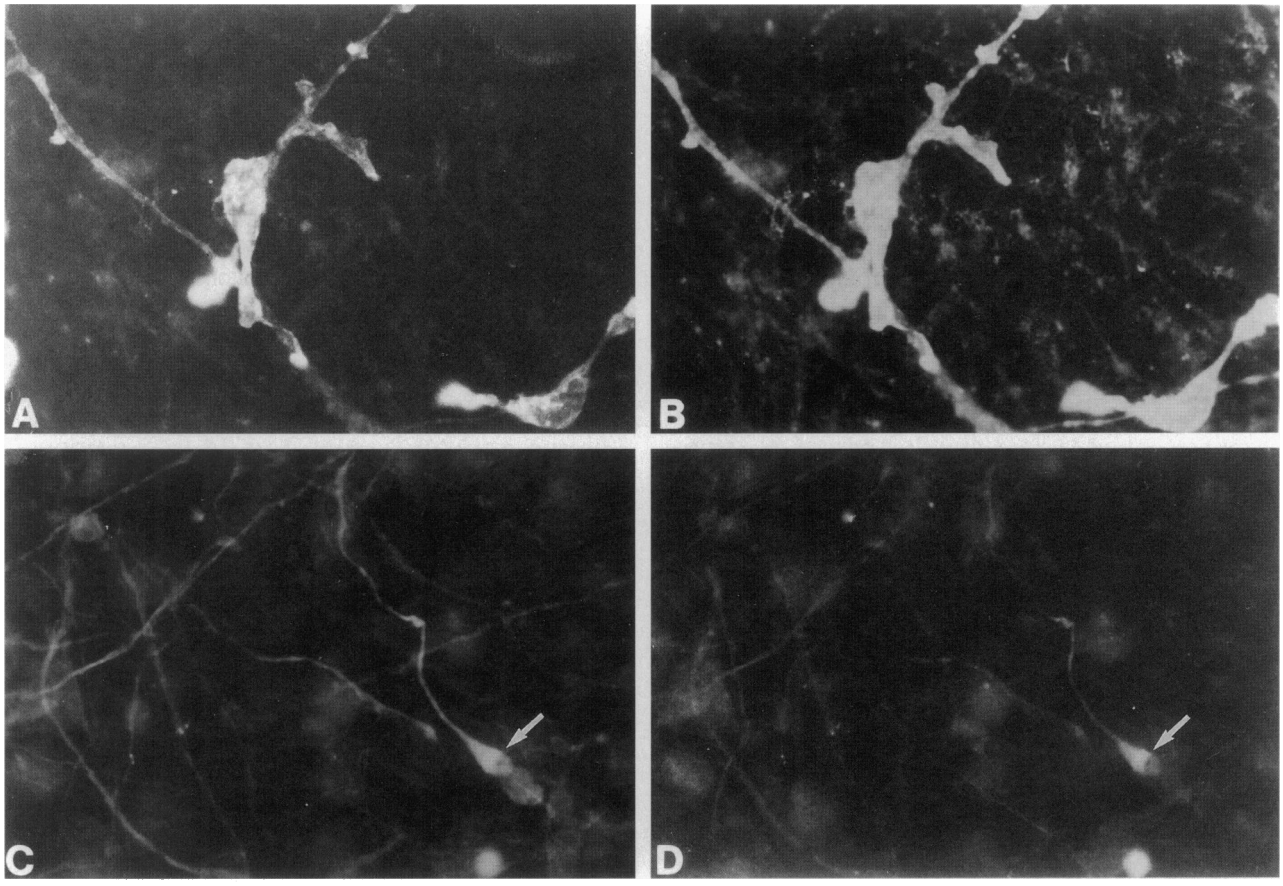

Figure 8. Double immunofluorescence staining of CXCR4 in microglia (**A** and **B**) and neurons (**C** and **D**) in primary human fetal brain cultures. **A** and **B**: Co-localization of mouse anti-CXCR4 (**A**) and RCA-1 (**B**) staining in microglia demonstrated by fluorescein-specific (**A**) or rhodamine-specific (**B**) fluorescence. **C** and **D**: Co-localization of rabbit anti-CXCR4 (**C**) and mouse anti-MAP-2 (**D**) in neurons (**arrow**) demonstrated by rhodamine-specific (**C**) or fluorescein-specific (**D**) fluorescence.

pared with children with AIDS[10,11,57] remains to be determined.

HIV-1 viruses isolated from the brain are M-tropic[24,49] and use CCR5 and CCR3 as co-receptors.[50] CCR5 and CCR3 are both expressed on microglia.[50] The present study demonstrates that CXCR4 is also expressed on microglia *in vitro* and *in vivo*, consistent with recent work by Lavi et al.[58] However, HIV-1 viruses that use only CXCR4 as a co-receptor do not enter microglia efficiently.[50] The CCR3 co-receptor was not examined in the present study due to the lack of availability of an antibody suitable for use on formalin-fixed autopsy tissue. These findings suggest that increased entry of CCR5-positive mononuclear cells into the brain and an increased frequency of CCR5-positive macrophages and microglia within the brain parenchyma together may contribute to disease progression in the CNS of children and adults with AIDS by serving as targets for infection. Consistent with this possibility, the increased frequency of CCR5-positive perivascular mononuclear cells and macrophages in the brain of children with severe HIVE correlated with the clinical course, as all of these patients had clinical evidence of progressive HIV-1 encephalopathy. The level of CCR5 expression on peripheral blood mononuclear cells is variable among normal individuals[48] and is up-regulated by interleukin-2.[47,48] It will be of interest to determine whether individuals that express higher levels of CCR5 on cells of the immune or central nervous systems are at increased risk for developing HIVE after HIV-1 infection. In this regard, the role of host factors and cytokines in regulating the level of CCR5 expression in the brain of AIDS patients is an important area for future investigation.

Our studies show that CXCR4 is expressed in subpopulations of neurons in some regions of the cerebral cortex and basal ganglia in all pediatric and adult cases examined. Consistent with this observation, we also detected CXCR4 expression in a subpopulation of human neurons in primary human brain cultures. These findings are consistent with previous studies that demonstrated CXCR4 expression in brain and brain-derived cell lines.[37,59] A recent study examined the anatomical localization of CXCR4-positive neurons in adult autopsy brain tissue and found that CXCR4-positive subpopulations of neurons were predominantly localized in the hippocampus, regions of cerebral cortex, amygdala, thalamus, basal ganglia, and brainstem nuclei.[58] SDF-1, the CXCR4 ligand, has been shown to induce signaling in primary human neurons *in vitro*.[59] This effect was inhibited by soluble HIV-1 gp120, raising the possibility that gp120 neurotoxicity[36] may involve binding of gp120 to CXCR4. The chemokine receptors CXCR2 (a receptor for interleu-

kin-8) and the Duffy antigen (which binds many different chemokines) are also expressed in subpopulations of human neurons *in vivo*.[59–61] The biological roles of chemokine receptors in neuronal development, brain function, and CNS disease remain to be determined.

Previous studies have shown that the detection of HIV-1 by immunohistochemistry or *in situ* hybridization in the brain of children with AIDS is highly variable. The brains of children with a rapidly progressive course of neurological disease frequently show detectable HIV-1 antigens, whereas those of children with a plateau course show little or no detectable HIV-1 antigens.[10] However, severe HIVE can also exist in children without evidence of active HIV-1 replication in brain.[10,11,57] These observations are consistent with our finding that Nef antigens were detected in the brains of only 4 of 20 pediatric AIDS patients and were detected only in patients with severe HIVE. We found that the vast majority of Nef-positive cells in brain were macrophages and microglia, consistent with previous studies.[10,11,57] However, a minor fraction of the Nef-positive cells demonstrated morphological characteristics of astrocytes as judged by GFAP immunostaining of serial sections. Consistent with this observation, previous studies have suggested that astrocytes are more susceptible to HIV-1 infection in children than in adults.[30,31] Thus, astrocytes may be a reservoir for HIV-1 in the developing nervous system. The receptors that mediate CD4-independent HIV-1 entry into astrocytes have not been identified. We did not detect significant levels of CCR5 or CXCR4 expression in astrocytes. However, we cannot exclude the possibility that our methods were not sufficiently sensitive to detect low levels of expression.

Our studies demonstrate an increased frequency of apoptotic neurons, astrocytes, and endothelial cells in children with severe HIVE. We found that apoptosis of neurons as well as other cell types was relatively infrequent in pediatric compared with adult AIDS patients.[19,22–24] Neuronal apoptosis was detected in the brains of 5 of 20 pediatric AIDS patients. All five patients were in group A, consistent with the finding that neuronal loss usually occurs in the later stages of disease.[17,19,20] The detection of apoptotic neurons in the brains of children with AIDS is consistent with a previous study.[21] However, we did not detect apoptotic neurons as frequently as Gelbard et al,[21] which may reflect differences in the patient populations examined or the TUNEL staining method used. Rather, we found that the majority of apoptotic cells detected in the brains of pediatric AIDS patients were endothelial cells and astrocytes. Apoptotic perivascular cells with morphological characteristics of pericytes and macrophages were rarely detected in some patients, as previously described.[21] In general, the frequency of apoptotic neurons, astrocytes, and endothelial cells correlated with the severity of HIVE. There was no significant difference in the frequency of apoptotic cells in group B AIDS patients compared with non-AIDS controls, except for one patient in group B with lymphocytic meningitis and vasculitis (case 12). The two control patients that had an increased frequency of apoptotic endothelial cells in the brain relative to the other controls had received cranial irradiation or intrathecal chemotherapy. The demonstration that exposure to ionizing radiation and chemotherapeutic agents can induce apoptosis of endothelial cells and other cell types[62,63] provides a possible explanation for this finding. Apoptosis of neurons, astrocytes, and endothelial cells has been demonstrated in adults with AIDS,[19,22–24] and in macaques infected with SIV.[64] As in adults with AIDS,[19,24] we found that there was no significant association between the localization of the apoptotic cells and HIV-1 Nef-positive cells. Furthermore, cells undergoing apoptosis were generally not CCR5 or CXCR4 positive. These findings suggest that the apoptotic stimuli are likely to be soluble factors acting at a distance rather than direct viral infection. It will be important to identify the apoptotic stimuli that induce apoptosis in the brains of AIDS patients and to determine whether CCR5-positive cells elaborate pro-apoptotic soluble factors.

Our studies suggest that increased trafficking of CCR5-positive mononuclear cells across the blood-brain barrier may contribute to HIV-1 replication and disease progression in the CNS in children and adults with AIDS. Several factors may contribute to the increased entry of CCR5-positive mononuclear cells into the brains of AIDS patients. These include abnormal blood-brain barrier permeability,[12–14] increased expression of chemokines such as MIP-1α and MIP-1β,[65] which attract CCR5-positive mononuclear cells to sites of inflammation, and increased expression of adhesion molecules involved in monocyte trafficking through the blood-brain barrier.[66] Furthermore, *in vitro* studies suggest that activated or HIV-1-infected monocytes are more likely to migrate through the blood-brain barrier.[66,67] Our studies raise the additional possibility that endothelial cell apoptosis may also contribute to increased entry of mononuclear cells into the CNS by disrupting the integrity of the blood-brain barrier. Chemokine ligands for CCR5 inhibit HIV-1 infection of microglia in primary brain cultures.[50] These findings together with the results of the present study suggest that inhibition of CCR5 and the entry of CCR5-positive mononuclear cells into the CNS are potential therapeutic strategies for neurological disease in children and adults with HIV-1 infection.

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