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ORIGINAL ARTICLE

Impaired Astrocytes and Diffuse Activation of Microglia in the Cerebral Cortex in Simian Immunodeficiency Virus-Infected Macaques Without Simian Immunodeficiency Virus Encephalitis

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Abstract

Various types of neuronal damage have been reported in acquired immunodeficiency syndrome (AIDS) dementia. We previously demonstrated that inflammation and cortical damage occur independently according to viral tropism in a simian immunodeficiency virus (SIV)-infected macaque model of AIDS dementia. To elucidate the pathogenesis of cortical degeneration, we examined the frontal cortex of SIV-infected macaques and found apoptosis and decreased expression of the excitatory amino acid transporter 2 in astrocytes and diffuse activation of microglia in association with limited neuronal damage. Some activated microglia also expressed excitatory amino acid transporter 2 but not proinflammatory cytokines. No inflammatory changes were seen in the cortex or the white matter, and SIV-infected cells were not detected in or around cortical lesions either by immunohistochemistry or by the polymerase chain reaction detection of SIV genomes of extracted DNA from microdissected tissue samples. These results indicate that an astrocytic abnormality and a compensatory activation of microglia might provide a protective effect against neuronal degeneration in the frontal cortex of SIV-infected macaques without SIV encephalitis.

Key Words: AIDS encephalopathy, Animal model, Cerebral cortex, Immunohistochemistry, Injury of astrocytes, Neuroprotection by microglia

INTRODUCTION

Human immunodeficiency virus 1 (HIV-1) can induce acquired immunodeficiency syndrome dementia complex (ADC), a clinical triad of progressive cognitive decline, motor dysfunction, and behavioral abnormalities, which eventually affects 15% to 20% of AIDS patients (1, 2). Although the introduction of highly active anti-retroviral therapy has reduced progression of AIDS, inconsistent results have been reported regarding the effects of highly active anti-retroviral therapy on central nervous system (CNS) involvement (3–8), thus suggesting that the prevalence of dementia may eventually increase corresponding to longer life spans of people with HIV-1 infection.

One of the histopathologic correlates of ADC is diffuse and nodular inflammatory infiltrates with formation of multinucleated giant cells (MNGCs) in the brain white matter (9, 10). Myelin pallor (11) and axonal damage (12–14) with abundant HIV-1-infected macrophages and microglia have been mainly demonstrated in the white matter (15, 16), but poor correlations between these findings and the clinical manifestations of ADC have been repeatedly reported (17, 18). On the other hand, Budka et al (10, 11) described astrocytic gliosis, a reduction of neurons and proliferation of rod cells in the cerebral cortex of many cases with HIV-1 infection and have identified this diffuse poliodystrophy (DPD) as an additional histopathologic feature of ADC. Furthermore, a variety of pathologic findings, including neuronal loss (19–21), apoptosis (22), and synaptic and dendritic simplification (23–25), have been reported in the cortex in HIV-AIDS. Because of the complexity of the histopathologic findings in human autopsy brains, however, a precise relationship between these histopathologic changes, namely, the inflammatory process in the white matter and degenerative process in the cortex, has not been elucidated.

Simian immunodeficiency virus (SIV) infection in rhesus macaques is considered to be a suitable animal model of human HIV-1 infection and has been used in various studies as a model for AIDS encephalopathy. Desrosiers et al (26) reported that macrophage-tropic variants of SIV were associated with the appearance of encephalitis. Previously, we inoculated macaques with 3 SIV strains and investigated the relationship between the lymph node and brain pathology. The animals infected with macrophage virus tropic-SIV239env/MERT chymeric virus, did not develop AIDS 3 to 4 years after infection, but microglial nodules with MNGCs were demonstrated in the white matter, and no pathologic changes were noticed in the cerebral cortex. The other animals infected with T-cell-tropic viruses SIVmac239 and SIV/HIV-1-(SHIV)-RT

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developed typical simian AIDS pathology in the lymph nodes within 3 years after infection; the cerebral cortex of these animals showed astrocytic gliosis and electron microscopic abnormalities without evidence of microglial nodules or MNGCs in the white matter. From these observations, we hypothesized that there are 2 independent pathogenetic processes in simian AIDS encephalopathy, that is, immune response against virus-infected macrophage/microglial cells in the white matter without immunodeficiency and cortical degeneration caused in the late stage of AIDS (27).

The roles of macrophage infiltration and microglial activation in the pathogenesis of HIV encephalitis have been extensively studied. With respect to the cortical pathology, the expression of viral neurotoxins or neurotoxic cytokines from microglia and/or astrocytes has been reported to induce neuronal dysfunction and death (28–31). On the other hand, recent reports suggest that activated microglia express excitatory amino acid transporters (EAATs) and glutamine synthetase, and may be neuroprotective in the early stages of the disease (32, 33). In our SIV model, degenerative changes were observed in the cerebral cortex of macaques infected with T-cell-tropic viruses, and microglial nodules with MNGCs were absent (27); therefore, we further examined the frontal cortex of rhesus macaques infected with T-lymphocyte-tropic SIV and focused on microglial activation, apoptosis, and EAAT-2 expression, as well as localization of virus-infected cells.

MATERIALS AND METHODS

Virus

Molecularly cloned SIVmac239 is a T-lymphocyte-tropic virus, the pathogenic properties of which have been previously described. This virus causes immunosuppression and eventually leads to the development of AIDS in macaques. A chimeric virus, SHIV-RT, consists of a SIVmac239 virus backbone in which the *SIV RT* gene was replaced by the HIV-1 *HxB2 RT* gene, as previously described (27, 34). In experimentally infected rhesus monkeys, SHIV-RT has been shown to induce AIDS (34, 35).

Animals

Eleven rhesus macaques were screened and found to be seronegative for SIV, simian T-lymphotropic virus, B virus, and Type D retroviruses. Four macaques (532, 627, 682, and 730) were inoculated intravenously with SIVmac239 and killed 133, 46, 115, and 463 weeks after inoculation, respectively. The other 3 (631, 677, and 700) were inoculated with SHIV-RT and killed 108, 156, and 263 weeks after inoculation, respectively. Four uninfected macaques (671, 630, 778, and 780) were used as controls (Table 1). The animals were housed in individual cages and maintained according to the rules and guidelines of the National Institute for Infectious Diseases for experimental animal welfare. The animals were killed at various times after infection when they became moribund.

$CD4^+$ Cell Counts and Viral RNA Loads

$CD4^+$ cell counts were performed on peripheral blood samples at the time of autopsy. To measure the level of virus replication in the periphery, viral RNA was quantified in plasma at autopsy. Viral RNA in the plasma of inoculated macaques was measured by real-time reverse transcriptase-polymerase chain reaction (PCR).

Histopathology and Immunohistochemistry

The routine histopathologic methods used in this study have been described elsewhere (27). Brain tissue specimens were embedded in paraffin, sectioned, and mounted on glass slides. The EnVision system (DAKO, Carpinteria, CA) was used for immunohistochemistry except for a guinea pig anti-glial glutamate transporter 1, EAAT-2 antibody with which the avidin-biotin-peroxidase complex method (Vector, Burlingame, CA) was applied. Immunoreactivity was visualized using either diaminobenzidine/peroxidase (brown) or the 3-amino-9-ethylcarbazole substrate-chromogen system (DAKO; red). Light counterstaining was done with hematoxylin.

TABLE 1. Clinical Data

Animal No.	Sex	Age at Virus Inoculation, weeks	Age at Death, weeks	Duration of Infection, weeks	Viral Inocula	Viral RNA Load in Plasma at Autopsy, copies/ml	$CD4^+$ Cell Count in PBMCs at Autopsy, per μl	Clinical Information
532	M	260	393	133	SIVmac239	214,300	380	Weight loss and moribund
627	M	312	358	46	SIVmac239	25,000	90	Weight loss and moribund
682	M	100	215	115	SIVmac239	480,000	140	Weight loss and self-biting
730	M	156	619	463	SIVmac239	67,000	275	Weight loss and moribund
631	F	156	264	108	SHIV-RT	2,500,000	200	Weight loss and inactivity, inguinal B-cell lymphoma
677	M	104	260	156	SHIV-RT	6,900	100	Weight loss and moribund
700	M	104	367	263	SHIV-RT	530,000	192	Weight loss and moribund
630					Control			
671					Control			
778					Control			
780					Control			

PBMC, peripheral blood mononuclear cell; SHIV, simian immunodeficiency virus/human immunodeficiency virus-1.

Antibodies

To identify activated microglia, we used a mouse monoclonal antibody to human macrophage CD68 (KP1, 1:50; DAKO) and a rabbit anti-ionized calcium-binding adaptor molecule 1 antibody (Iba1; 1:500; Wako Chemicals, Osaka, Japan) (36). To characterize astrocyte abnormalities, we used a guinea pig anti-glial glutamate transporter 1, EAAT-2 antibody (1:6000; Chemicon, Temecula, CA). For SIV-infected cells, we used an anti-SIV envelope gp160/gp32 antibody (KK41; 1:50; Dr K. Kent and the National Institute for Biological Standards and Control), which has been previously described (27). Lymph nodes of SIV-infected and uninfected animals were used as positive and negative controls, respectively.

A mouse anti-human Ki-67 antibody (1:300; DAKO) that can detect cells in all active phases (G1, S, G2, and M) of the cell cycle was used to detect dividing cells. Sections of lymph nodes and small intestines were used as positive controls for proliferating cells. A mouse anti-human tumor necrosis factor antibody (TNF-α; 1:400; Abcam, Cambridge, MA) and a rabbit polyclonal antibody against interleukin 1β (IL-1β; 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) were used to detect the respective cytokines. A tonsil with chronic inflammation was used as a positive control. We also performed glial fibrillary acidic protein (GFAP), CD3, and CD20 immunohistochemical staining for routine cell characterization.

Double Label Immunohistochemistry

Double label immunohistochemistry was performed for GFAP or Iba1 and Ki-67 to determine the phenotype of the proliferating cells by first performing immunohistochemistry for Iba1 or GFAP using the EnVision system (DAKO) and then for Ki-67 using avidin-biotin-peroxidase complex (Vector). Double labeling was performed using diaminobenzidine/peroxidase, followed by Vector blue/alkaline phosphatase. We also performed fluorescence microscopy for double label staining of EAAT-2 and Iba1 using fluorescein isothiocyanate and rhodamine-based detection methods.

Apoptosis

In situ terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling of fragmented DNA (TUNEL) was done using the ApopTag in situ apoptosis detection kit (Chemicon). We also performed immunohistochemistry using an affinity-purified polyclonal rabbit immunoglobulin G directed specifically against the active form of caspase 3 (1:1000; R and D Systems, Minneapolis, MN) and anti-single-stranded DNA antibody (ssDNA; 1:250; DakoCytomation, Kyoto, Japan) for the identification of apoptotic cells. Lymph nodes and small intestines were used as positive controls. To examine the phenotype of apoptotic cells, we performed double label immunohistochemistry for GFAP and activated caspase 3 or ssDNA using the same method.

Electron Microscopy

Pieces of the frontal cortex from animals 531, 627, 682, and 630 were postfixed in 1% osmium tetroxide and embedded in epoxy resin. One-micrometer semithin sections of Epon-embedded samples were stained with toluidine blue and safranine. For electron microscopy, sections were stained with uranium acetate and lead citrate and examined using a Hitachi H-7000 electron microscope.

Quantitative and Semiquantitative Analysis

Ionized calcium-binding adaptor molecule 1 antibody-positive cells were counted in 10 200×-magnified light microscopic fields of cortical layers 2 to 5 of the middle frontal gyrus. These findings were considered to indicate an increase in the activated microglia when more than 700 Iba1-positive cells were counted in these 10 fields. We also performed semiquantitative assessments for the following immunohistochemical findings: astrocytic gliosis, EAAT-2 expression, Ki-67 or CD68-positive cells, TNF-α, and IL-1β expression.

Laser Microdissection and PCR-Based Detection of *SIV gag* and *SIV env* Genes

Polymerase chain reaction-based molecular detection of SIV genomes was performed to detect SIV-infected cells in the brain lesions of SIV-infected animal 532, which showed representative clinical and pathologic features. Paraffin-embedded sections of the frontal lobe were dehydrated, stained with hematoxylin, and air-dried. The parenchyma of the frontal cortex, perivascular areas of the frontal cortex, and white matter were identified based on cellular staining patterns and separately dissected using a laser microdissection system (AS LMD; Leica, Wetzlar, Germany). From each dissected sample, genomic DNA was extracted by a DNeasy tissue handbook kit (Qiagen, Tokyo, Japan). We used 2 sets of nested oligonucleotide primer pairs for the PCR detection of SIV provirus (37). The sequences of the primer

TABLE 2. Oligonucleotides Used as Primers in Nested PCR to Detect SIV gag and SIV env Proteins

Region of Amplification	Sequence of Outer Primers	Sequence of Inner Primers
SIV gag	5′-ACTGTCTGCGTCATCTGGTGC	5′-CACGCAGAAAAAGTGAAAC
	5′-GTCCCAATCTGCAGCCTCCTC	5′-CTCTGATAATCTGCATAGCCGC
SIV env	5′-TTATGGTGTACCAGCTTGGAGGAATGC	5′-CGATACTTGGGGAACAACTCAGTGCCTAC
	5′-CCAAACCAAGTAGAAGTCTGTGTCTCCATC	5′-GAGACCACCACCTTAGAACATTTAGGC
β-Globin	3′-TCCCAGTTTCTCCAGTTTCC	
	3′-AGACCATCCTGGCTAACACG	

PCR, polymerase chain reaction; SIV, simian immunodeficiency virus.

pairs used to detect SIV gag, SIV env, and β-globin as genomic control are listed in Table 2. The relative detection efficiency of all the primer sets was determined in a series of preliminary experiments. The DNA from SIV plasmid and paraffin sections of lymph node from SIVmac239-infected macaque 682 with abundant SIV-Env-positive cells was amplified as positive controls, and DNA from a lymph node of uninfected macaque 671 was amplified as a negative control in all assays to monitor potential PCR contamination. Each cycle consisted of 1 minute denaturation at 94°C, 1 minute primer annealing at 55°C, and 1 minute extension at 72°C. After 30 cycles, 4 ml of the amplified DNA was taken, and 30 additional cycles of amplification were carried out using the nested primers. The same primers for β-globin were used in a second PCR. After the second round of amplification, a 13-μl aliquot of the reaction products was applied for 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

RESULTS

Clinical Manifestations

Table 1 summarizes the clinical data, including the viral RNA loads and $CD4^+$ cell counts in the peripheral blood, at the time of autopsy from the 7 SIV-infected rhesus macaques. Among the 4 macaques infected with SIVmac239, macaque 627 showed the most rapid decrease in the $CD4^+$ cell counts and became moribund within 46 weeks after infection. Macaque 532 had a prolonged clinical course and showed very high viral loads and decreased $CD4^+$ cell counts at 133 weeks after infection; thereafter, it became moribund and was diagnosed to have AIDS because of very low $CD4^+$ CD29 high T cells (less than 1% of peripheral blood mononuclear cells). Macaque 682 also had a prolonged clinical course and showed very high viral loads and decreased $CD4^+$ cell counts. This animal was killed for autopsy 115 weeks after infection because of self-biting

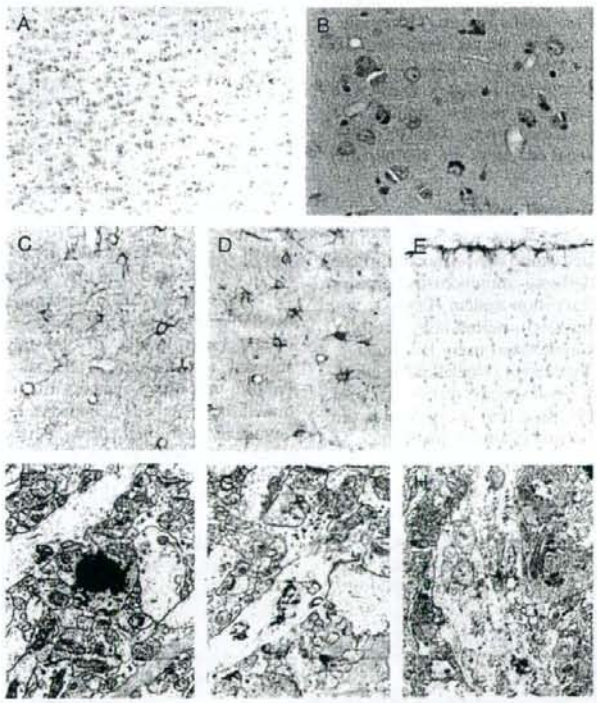

FIGURE 1. Representative findings in the frontal cortex of simian immunodeficiency virus (SIV)-infected macaques. Loss of neurons is not apparent **(A)**, but there is an increase in small glial cells and satellitosis around neurons **(B)** in a SIVmac239-infected macaque. Diffuse gliosis in SIVmac239-infected macaque **(C)** and in a SIV/human immunodeficiency virus-1-RT-infected macaque **(D)** contrasts with the uninfected control **(E)**. By electron microscopy, in the SIVmac239-infected macaque, there is deposition of glycogen-like granules **(F)** and an increase in lamellar bodies in the dendrites **(G)**. Foamy changes are detected in the cytoplasm of dendritic trunks **(H)**. Some astrocyte processes contain glial fibrils **(F, G)**. **(A)** Klüver-Barrera; **(B)** hematoxylin and eosin; **(C–E)**: anti-glial fibrillary acidic protein immunohistochemistry; **(F–H)** Electron microscopy. Original magnifications: **(A, E)** 100×; **(B)** 200×; **(C, D)** 400×; **(F)** 9,000×; **(G)** 15,000×; **(H)** 9,000×. **(A, B, F, G)** from macaque 627; **(C)** from macaque 532; **(D)** from macaque 631; **(E)** from macaque 630.

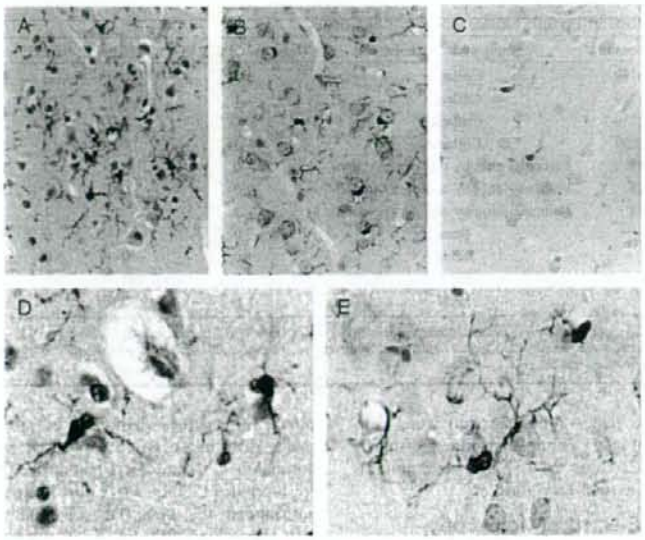

FIGURE 2. Ionized calcium-binding adaptor molecule 1 antibody (Iba1) immunohistochemical staining of activated microglia in the cerebral cortex. Increased Iba1-positive activated microglia are evident in simian immunodeficiency virus (SIV)-infected macaques **(A, B)** compared with the control **(C)**. Some Iba1-positive cells are close to neurons and surround neuronal cell bodies with their extended processes **(D, E)**. **(A, D)** SIVmac239-infected macaque 532; **(B, E)** SIV/human immunodeficiency virus-1-RT-infected macaque 631; **(C)** uninfected control (630). Original magnification: **(A, B)** 400×; **(C)** 100×; **(D, E)** 800×.

behavior. Macaque 730 had the longest clinical course and showed decreased $CD4^+$ cell counts; it was diagnosed as having AIDS at autopsy at 463 weeks. The 3 macaques infected with SHIV-RT also showed decreased $CD4^+$ cell counts and were diagnosed as having AIDS at the time of autopsy. Macaque 631 developed a B-cell lymphoma. All

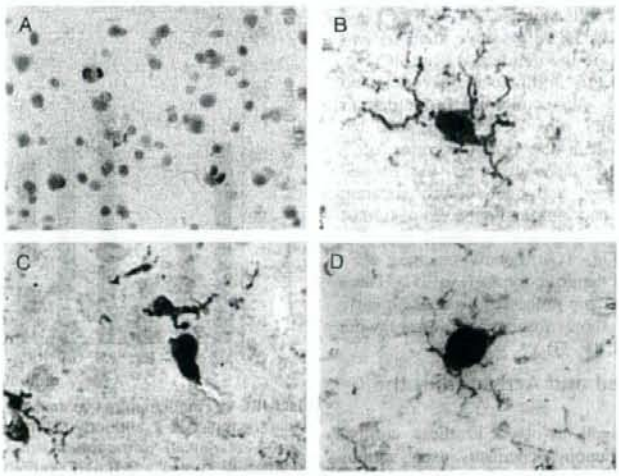

FIGURE 3. Proliferation and activation of microglia in the frontal cortex of animal 631 infected with simian immunodeficiency virus/human immunodeficiency virus-1-RT. Ki-67-positive cells are scattered in the cortical parenchyma **(A)**. Ionized calcium-binding adaptor molecule 1 antibody (binding adaptor molecule 1 antibody (Iba1)-positive microglia with branches **(B)**, amoeboid shape **(C)**, and a large nucleus **(D)** have Ki-67-positive nuclei. **(A)** Anti-Ki-67; **(B–D)** double label of Ki-67 (dark blue) and Iba1 (brown). Original magnification: **(A)** 400×; **(B–D)** 800×.

TABLE 3. Pathologic Findings in Lymph Nodes and Frontal Cortex*

Animal No.	Sex	Viral Inocula	Lymph node Pathology	Decrease of EAAT-2	CD68	GFAP	Ki-67	IL-1β	TNF-α
532	M	SIVmac239	Collapse	1	0–1	2	0–1	0	0
627	M	SIVmac239	Collapse	1	1	1	2	0	1
682	M	SIVmac239	Collapse	0	0–1	1	1	0	0
730	M	SIVmac239	Collapse	3	0–1	3	2	0	0
631	F	SHIV-RT	Follicular atrophy and degeneration	0	0–1	2	2	0	0
677	M	SHIV-RT	Follicular atrophy and degeneration	0	0–1	3	2	0	0
700	M	SHIV-RT	Follicular atrophy and degeneration	2	1	1	3	0	1
630		Control	None	0	1	0	1	0	0
671		Control	None	0	0–1	0	0	0	0
778		Control	None	0	0–1	0	0–1	0	0
780		Control	None	0	0–1	0	0–1	0	0

*, Immunohistochemical staining for each antigen was assessed semiquantitatively by scoring from 0 to 3 in each animal.
EAAT, excitatory amino acid transporter; F, female; GFAP, glial fibrillary acidic protein; IL, interleukin; M, male; SIV, simian immunodeficiency virus; SHIV-RT, SIV/human immunodeficiency virus-1; TNF-α, tumor necrosis factor-α.

SHIV-RT-infected animals had weight loss and were moribund at the time of autopsy. Other than the self-biting behavior in macaque 682, none of the infected animals showed apparent neurologic manifestations.

Histopathology and Electron Microscopy

The animals infected with SIVmac239 and SHIV-RT showed neuropathologic findings as described previously in the cerebral cortex by routine histopathologic examination (27). Briefly, no apparent loss of neurons was detected in the cerebral cortex of any macaques (Fig. 1A). The density of small glial cells seemed to be increased, and they formed apparent perineuronal satellitosis. There were no obvious abnormalities such as pyknosis or chromatolysis evident in the neurons (Fig. 1B). Patchy or diffuse astrocytic gliosis was noted in macaques infected with SIVmac239 (Fig. 1C) and SHIV-RT (Fig. 1D), whereas GFAP-positive staining was limited to the subpial region in the uninfected controls (Fig. 1E). There were no inflammatory infiltrates in the cortex, and there was no evidence of either microglial nodules or MNGCs in the white matter of infected macaques. No abnormalities were observed in the cerebral cortex of 3 uninfected controls. By electron microscopy, the frontal cortex of macaques 532, 627, and 682 showed apparent degenerative changes in the neuropil, including deposition of glycogen-like granules (Fig. 1F), and increased lamellar bodies in the dendrites (Fig. 1G). Foamy changes were detected in the cytoplasm of dendritic trunks (Fig. 1H). There were scattered swollen astrocytic processes because an early reaction of astrocytes and some astrocytic processes were filled with glial fibrils (Figs. 1F, G).

Microglia Are Increased and Activated in the Cerebral Cortex

To characterize the cell involved in these cortical changes, we performed immunohistochemistry using various antibodies, including Iba1, which is restricted to macrophages/microglia (36), and Ki-67, a specific marker of cell proliferation. The cells that increased in number in the cortex were negative for CD3, CD20, or CD68 (data not shown). In contrast, Iba1 immunostaining of SIVmac239- and SHIV-RT-infected macaques demonstrated increased numbers and a wider distribution of Iba1-positive microglia (Figs. 2A, B) compared with uninfected controls (Fig. 2C). Some of the Iba1-positive cells were located close to neurons and surrounded the neuronal cell bodies with their extended processes (Figs. 2D, E). Ki-67-positive cells were increased in the cerebral cortex of SIVmac239 (627 and 730) and all SHIV-RT-infected macaques (Fig. 3A; Table 3). Most of the Ki-67-positive cells were located in the parenchyma, and some positive cells were also closely attached to neuronal cell bodies. Some of the Iba1-positive microglia with ramified processes (Fig. 3B), amoeboid shapes (Fig. 3C), or a large nucleus (Fig. 3D) were also Ki-67 positive by double label immunohistochemistry. Ki-67–positive cells were not

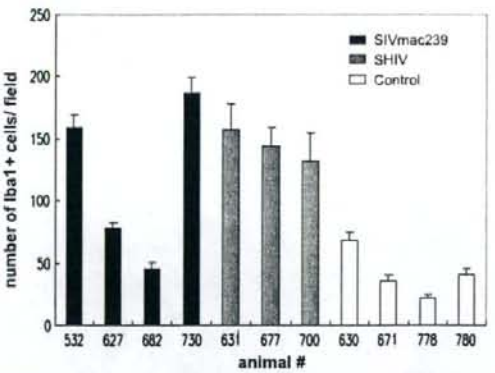

FIGURE 4. Semiquantitative analysis of ionized calcium-binding adaptor molecule 1 antibody (Iba1)-positive cells in the cerebral cortex of simian immunodeficiency virus (SIV)-infected and uninfected control macaques. Ionized calcium-binding adaptor molecule 1 antibody-positive cells were counted in ten 200× magnified light microscopic fields. There were more than twice as many Iba1-positive microglia in the SIV/human immunodeficiency virus-1-RT-infected animals and in 3 of 4 SIVmac239-infected animals as in the controls.

observed among GFAP-positive astrocytes by double label immunohistochemistry with anti-GFAP and anti-Ki-67. Only a few Ki-67-positive cells were found in the cerebral cortex of uninfected controls, and most of them were located near vessels.

In counts of stained cells in 10 microscopic fields at 200× magnification (Fig. 4), the numbers of Iba1-positive cells in all 4 control animals tended to be less than 70 per field. We therefore considered 70 or more cells per field to indicate more Iba1-positive cells. Among the SIVmac239 and SHIV-infected animals, 6 of 7 showed increased Iba1-positive cells. Five showed more than 130 Iba1-positive cells per field, and 1 animal infected with SIVmac239 (627) had 78.2 Iba1-positive cells per field. Another animal infected with SIVmac239 (682) showed no increase in the number of Iba1-positive cells (45.2 per field).

Apoptosis of Astrocytes in SIVmac239- and SHIV-RT-Infected Animals

Our previous study demonstrated astrocytic gliosis, increase in lamellar bodies in the dendrites, and swelling of astrocytic processes in the frontal cortex of macaques infected with SIVmac239 and SHIV-RT (27). To analyze which cell types were predominantly affected, we performed the in situ TUNEL method (Figs. 5A, C, E) and immunohistochemical staining for activated caspase 3 (Figs. 5B, D, F) and ssDNA (Figs. 6E, G) to detect apoptosis. Although the staining results tended to vary in each animal sample, consistent results such as comparable staining patterns in all 3 methods in duplicate can be obtained in samples from SIVmac239-infected (532, 682, 627, and 730), SHIV-RT-infected (700 and 631), and uninfected control (671 and 778) animals.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling of fragmented DNA-positive cells were mainly demonstrated in the second layer of the cortex and mostly in glial cells (Figs. 5A, C, E). Some of the positive cells were located close to neurons in SIVmac239-infected (Fig. 5C) and in SHIV-RT-infected animals (Fig. 5E). In activated caspase 3 staining, positive cells showed intracytoplasmic and nuclear labeling (Figs. 6A, C). The numbers of positive cells tended to be high, but most of

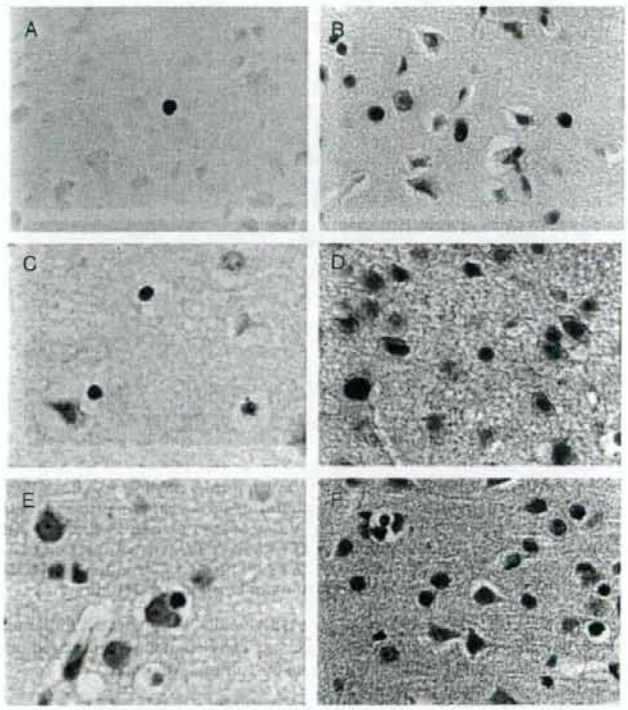

FIGURE 5. ApopTag in situ and anti-activated caspase 3 immunohistochemical staining in the cerebral cortex. Stained glial cells are seen in the simian immunodeficiency virus (SIV)mac239-infected **(A–D)** and SIV/human immunodeficiency virus-1 (SHIV)-RT-infected **(E, F)** macaques. **(A, C, E)**: ApopTag in situ; **(B, D, F)** anti-activated caspase 3. **(A, B)** SIVmac239-infected macaque 532, **(C, D)** SIVmac239-infected macaque 730; **(E, F)** SHIV-RT-infected macaque 700. Original magnification: **(A–F)** 400×.

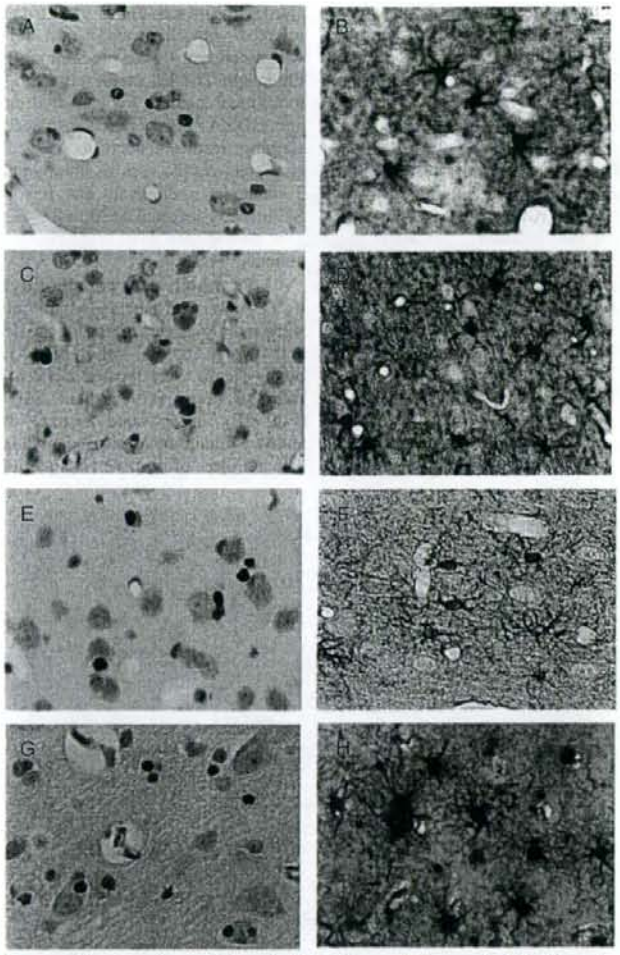

FIGURE 6. Double label immunohistochemistry with anti-glial fibrillary acidic protein (GFAP) and anti-activated caspase 3 or single-stranded DNA antibody (ssDNA) in the cerebral cortex of the simian immunodeficiency virus (SIV)mac239- and SIV/human immunodeficiency virus-1 (SHIV)-RT-infected animals. Most of the doubly positive cells are astrocytes with small round nuclei. **(A, C)** Anti-activated caspase 3; **(E, G)** anti-ssDNA; **(B)** double label anti-activated caspase 3 (brown) and GFAP (dark blue); **(D)** double label anti-activated caspase 3 (red) and GFAP (dark blue); **(F)** double label ssDNA (red) and GFAP (brown); **(H)** double label ssDNA (dark blue) and GFAP (brown); **(A, B, E, F)** from SIVmac239-infected macaque 682; **(C, D)** from SHIV-RT-infected macaque 631; **(G, H)**from SHIV-RT-infected macaque 700. Original magnification: **(A–H)** 400×.

the positive cells seemed to be glia (Figs. 5B, D, F). The ssDNA-positive cells showed nuclear labeling and also seemed to be glia (Figs. 6E, G). Based on double label immunohistochemistry (Figs. 6B, D, F, H), more than half of the activated caspase 3 and ssDNA-positive cells were also positive for GFAP. Some of the activated caspase 3 and ssDNA-positive cells seemed to be microglia according to the shape of their nuclei. No apparent neuronal staining with these markers of apoptosis was observed. Only very few positive cells were detected in the uninfected controls by the in situ TUNEL method and based on activated caspase 3 and ssDNA immunostaining.

EAAT-2 Expression

The expression of Na^+-dependent glutamate transporters (EAAT-1 and EAAT-2) primarily on astrocytes is thought to keep the extracellular glutamate concentration low in the brain and prevent excitotoxicity to neurons. In all

animals studied, EAAT-2 expression was predominantly in the neuropil of the cerebral cortex. A diffuse decrease in EAAT-2 expression and scattered astrocyte staining in the neuropil were observed in SIVmac239-infected animal 730 (Fig. 7A) and SHIV-RT-infected animal 700 in contrast to the diffuse staining in the control animals (Fig. 7B). The 2 infected animals had very long durations of SIV infection: 463 and 263 weeks, respectively. A patchy decrease in EAAT-2 expression was observed in SIVmac239-infected animals 627 (Fig. 7C) and 532. In addition, we observed a strong expression of EAAT-2 by microglial cells, some of which came in close contact with neurons and blood vessels in SIV-infected animals (Fig. 7D), as demonstrated by double label immunofluorescence with anti-Iba1 and anti-EAAT-2 (Figs. 7E–G). The decrease in EAAT-2 in the neuropil seemed to be mild when the activated microglia expressed EAAT-2 (Fig. 7D).

Diffusely Activated Microglia Do Not Express TNF or IL-1β

To clarify the role of microglial activation in the frontal cortex, we examined expression of the potentially harmful proinflammatory cytokines. Cells positive for IL-1β and TNF were detected in the marginal zone of follicles of the positive control tonsil, but IL-1β and TNF were not detected in the cortex where the microglia were diffusely activated. However, TNF was detected in a few perivascular cells of SIVmac239-infected (627) and SHIV-RT-infected (700) macaques (Table 3).

SIV Infection Is Undetectable in the Frontal Cortex of SIV-Infected Animals

We performed immunohistochemistry for SIVenvgp160/gp32 to detect virus-infected cells in the frontal cortex in which astrocytic gliosis and microglial activation were observed. No SIVenvgp160/gp32-positive cells could be detected in the cerebral cortex of any SIV-infected animals. Only a few mononuclear cells were positive in the meninges of the macaque 682 infected with SIVmac239. To confirm the absence of SIV-infected cells in these cortical lesions, nested PCR was carried out on genomic DNA extracted from 3 different parts of paraffin-embedded frontal lobe sections, that is, the frontal cortex parenchyma, perivascular areas of the frontal cortex, and the white matter, using AS LMD. Our PCR system detected a single copy of *SIV gag*/*SIV env* genes in 100 cells by a sensitivity assay using SIV plasmid DNA diluted with DNA from paraffin-embedded lymph node sections of an uninfected macaque. Although strong bands can be easily detected by PCR of SIV DNA in infected lymph nodes, no positive bands were obtained from the

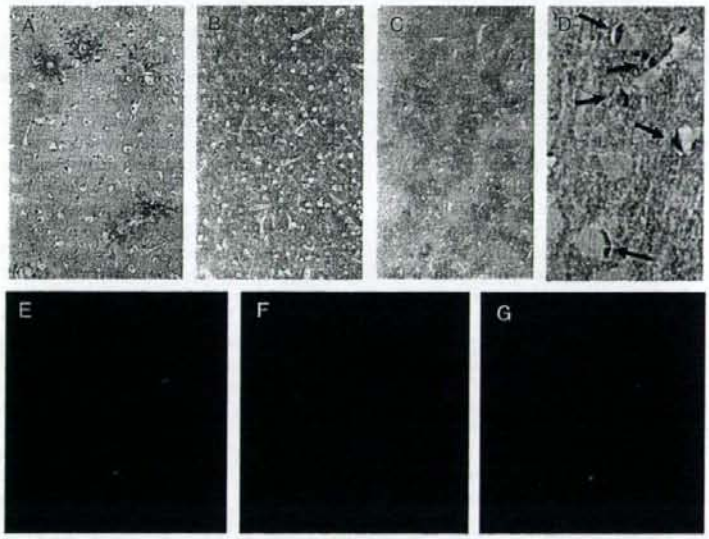

FIGURE 7. Decreased excitatory amino acid transporter (EAAT) 2 expression and EAAT-2 expression by microglia in the cortex of simian immunodeficiency virus (SIV)mac239-infected animals. A diffuse decrease in EAAT-2 (**A**; 730) and patchy decrease in EAAT-2 (**C**; 627) contrast with diffuse staining in the uninfected control animal (**B**; 671). In animal 627, strong EAAT-2 expression was noted on perineuronal and perivascular cells (arrows), and the decrease in the expression of EAAT-2 in the neuropil seemed mild (**D**; 627). Activated microglia expression of EAAT-2 is demonstrated by double label immunofluorescence with anti-ionized calcium-binding adaptor molecule 1 antibody (Iba1) and anti-EAAT-2 (**E–G**; 627). **(A–E)** Excitatory amino acid transporter 2; **(F)** Iba1; **(G)** merged. Original magnification: **(A–C)** 100×; **(D–G)** 400×.

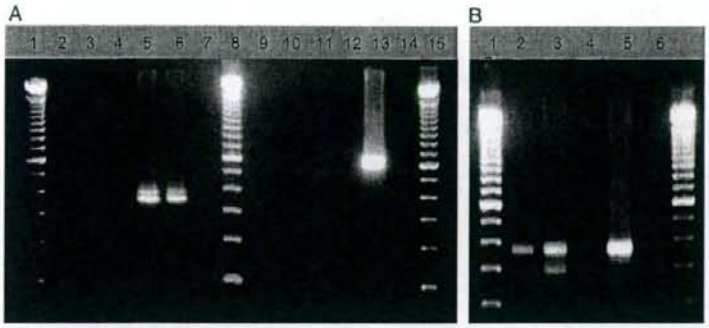

FIGURE 8. Detection of simian immunodeficiency virus (SIV) gag and env DNA in SIVmac239-infected macaque 532 in LCM microdissected tissues by PCR. The blots illustrate SIV gag (Lane A-2 to A-7) and env (Lanes A-9 to A-14), and β-globin (Lanes B-2 to B-6) amplified DNA from parenchymal (Lanes A-2 and A-9; B-2) and perivascular areas (Lanes A-4 and A-11; B-4) of the frontal cortex, and white matter (Lanes A-3 and A-10; B-3) of macaque 532. Lanes A-5, A-6, A-12, and A-13 are positive controls with 1 copy of plasmid DNA (Lanes A-5 and A-12) and 4 copies of plasmid DNA (Lanes A-6 and A-13). Lane B-5 is a positive DNA control from lymph node sections of 682. Lanes A-7, A-14, and B-6 indicate distilled water used as a polymerase chain reaction control without template DNA. Lanes A-1, A-8, A-15, B-1, and B-7 are molecular weight standards.

DNA samples of any of the 3 regions of the frontal lobe of the SIVmac239-infected animal (Fig. 8).

DISCUSSION

In this study, we examined the cortical pathology seen in animals infected with T-cell-tropic SIV, especially focusing on the change in the astrocytes and microglia. We observed abnormalities of astrocytes, including apoptosis and a decreased expression of EAAT-2 in the neuropil. We used 3 different methods, TUNEL and immunohistochemistry for activated caspase 3 and ssDNA to analyze apoptosis, and found that most positive cells were astrocytes by all 3 methods. Although the numbers of caspase 3- and ssDNA-positive cells seemed higher than expected, the concordance of the results of all 3 methods and double immunohistochemistry suggested a predominant involvement of astrocytes in the frontal cortex of SIV-infected animals. The apoptosis of astrocytes might be observed under physiologic conditions whereby the brain removes any excessive astrocytes that have proliferated after certain types of brain injury (38). Proliferation of astrocytes was not a plausible explanation in this study because Ki-67-positive astrocytes could not be detected by double label immunohistochemistry (data not shown). Another astrocytic change observed was a remarkable decrease in the expression of EAAT-2 in the neuropil in animals with a prolonged duration of SIV infection because a major cellular component of the brain astrocyte have important effects on neuronal biology by buffering the extracellular milieu, providing cytoskeletal support, and protecting neurons during CNS injury. The neuroprotective role of astrocytes has been described in connection with the expression of glutamate transporters (EAAT-1 and EAAT-2). The astrocytes maintain a low extracellular glutamate concentration in the brain. Glutamate, the major neurotransmitter in the CNS, induces excitotoxic neuronal cell death when its extracellular concentration increases, and it is also believed to be an important factor in the pathogenesis of many CNS disorders, including amyotrophic lateral sclerosis, Huntington disease, Alzheimer disease, and multiple sclerosis (39–43). The present findings suggest that astrocytes in the cerebral cortex are also primarily involved in the pathogenesis of AIDS encephalopathy.

We found diffuse activation of microglia in the cortex of infected animals and some of the activated microglia expressed EAAT-2; expression of TNF and IL-1β was not detected by immunohistochemistry. In general, microglia are distributed ubiquitously throughout the CNS and become activated in response to harmful stimuli (44). Activated microglia release proinflammatory cytokines such as IL-1β and TNF and thus mediate a neurotoxic function (29, 30). On the other hand, activated microglia may also secrete neurotrophic factors and provide neuroprotective functions (45). The expression of EAAT-2 by microglia has been reported in both AIDS brains (33, 34) and in SIVmac251-infected macaques (46). We confirmed the EAAT-2 expression by activated microglia in our model. The decrease in the expression of EAAT-2 in the neuropil seemed to be mild where activated microglia expressed EAAT-2. These data suggest that microglia might, like astrocytes, clear extracellular glutamate, thereby playing a neuroprotective role in the cortical degeneration seen in AIDS brains.

The involvement of the cerebral cortex in ADC is one of the major pathologic changes, and this phenomenon is called DPD (11). Neuron loss and apoptosis are believed to be the primary lesion in DPD. In our model, however, we observed only mild neuronal damage, that is, ultrastructural changes of dendrites. Because, with only 1 exception, our animals did not show any neurologic signs, we suspect that neuroprotection by activated microglia was efficient, and that this may also explain the absence of neuronal loss. Another possible explanation might be the difference in the stage of DPD. In human ADC, an autopsy is usually performed at the advanced stages such as in patients demonstrating a

consciousness disturbance or who are in a vegetative state. If our animals were in a subclinical stage and the findings observed in this model were the early changes of DPD, abnormalities of astrocytes and microglial activation might precede neuronal damage, indicating that astrocytes are primarily involved in DPD.

In human AIDS encephalopathy, HIV-infected macrophages and microglia produce viral neurotoxins or neurotoxic cytokines that lead to neuronal dysfunction and death (30, 31). The presence of activated microglia has been discussed regarding their roles as effectors of neuronal degeneration. In the present study, SIV encephalitis was not observed in any of the T-cell-tropic SIV-infected animals, and we did not detect any SIV-infected cells in or around cortical lesions. Moreover, expression of IL-1β and TNF were not detected in activated microglia in the frontal cortex of SIV-infected animals. These findings suggested that reduced expression of EAAT-2 and activation of microglia in the cerebral cortex occur independently from SIV encephalitis. Similarly, Gray et al (47) reported an interesting human case of ADC with prominent cortical atrophy and severe neuronal loss with minimal changes in the white matter and basal ganglia.

With respect to the question as to how the decrease in EAAT-2 may occur, Wang et al (48) demonstrated that HIV-1 and gp120 induce transcriptional downmodulation of the EAAT-2 transporter gene in human astrocytes and attenuate glutamate transport by the cells in vitro. Although we could not detect any SIV-infected cells in and around the cortical lesions analyzed, our animals did develop systemic AIDS and showed high viral loads in their plasma. It might therefore be possible that an increase in the soluble form of virus antigens such as gp120 and Tat in the blood and cerebrospinal fluid (as may occur in the late stages of AIDS) might induce an astrocytic abnormality, thus inducing subsequent neuronal damage.

In conclusion, we demonstrate the presence of abnormalities in astrocytes, increase in activated microglia, and a compensatory expression of EAAT-2 by microglia in the frontal cortex of SIV-infected animals without evidence of SIV encephalitis. These findings suggest that a degeneration of the cerebral cortex might occur in human ADC independently from HIV-1 encephalitis.

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Original Article

Expression of proinflammatory cytokines and its relationship with virus infection in the brain of macaques inoculated with macrophage-tropic simian immunodeficiency virus

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The pathogenesis of acquired immunodeficiency syndrome dementia complex (ADC) is still poorly understood. Many studies suggest that proinflammatory cytokines such as IL-1β and TNF-α released by microglia/macrophages or astrocytes play a role in CNS injury. A microscopic finding of a microglial nodule with multinucleated giant cells (MNGCs) is a histopathologic hallmark of ADC and named HIV encephalitis. However, in vivo expression of these cytokines in this microenvironment of HIV encephalitis is not yet clarified. One of the main reasons is complexities of brain pathology in patients who have died from terminal AIDS. In this study, we infected two macaques with macrophage-tropic Simian immunodeficiency virus SIV239env/MERT and examined expression of TNF-α and IL-1β in inflammatory lesions with MNGCs and its relation to virus-infected cells using immunohistochemistry. One macaque showed typical inflammatory lesions with MNGCs in the frontal white matter. Small microglial nodules were also detected in the basal ganglia and the spinal cord. SIVenv positive cells were detected mainly in inflammatory lesions, and seemed to be microglia/macrophages and MNGCs based on their morphology. Expression of IL-1β and TNF-α were detected in the inflammatory lesions with MNGCs, and these positive cells were found to be negative for SIVenv by double-labeling immunohistochemistry or immunohistochemistry of serial sections. There were a few TNF-α positive cells and almost no IL-1β positive cells in the area other than inflammatory lesions. Another macaque showed scattered CD3+ cells and CD68+ cells in the perivascular regions of the white matter. SIVenv and TNF-α was demonstrated in a few perivascular macrophages. These findings indicate that virus-infected microglia/macrophages do not always express IL-1β and TNF-α, which suggests an indirect role of HIV-1-infected cells in cytokine-mediated pathogenesis of ADC. Our macaque model for human ADC may be useful for better understanding of its pathogenesis.

Key words: cytokines, HIV encephalitis, macaque model, macrophage-tropic, simian immunodeficiency virus.

INTRODUCTION

Human immunodeficiency virus-1 (HIV-1) can induce a clinical triad of progressive cognitive decline, motor dysfunction, and behavioral abnormalities named acquired immunodeficiency syndrome dementia complex (ADC). Although the introduction of highly active anti-retroviral therapy (HAART) has been successful to reduce progression of acquired immunodeficiency syndrome (AIDS), controversial results have been reported that the prevalence of dementia may eventually increase, corresponding to a longer life span of people with HIV-1 infection.[1-3]

Acquired immunodeficiency syndrome dementia complex is histopathologically identified by diffuse and

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nodular microgliosis with formation of multinucleated giant cells (MNGCs) in the white mater of the brain and is termed HIV encephalitis.[4,5] Myelin pallor[6] and axonal damage[7-9] with abundant HIV-infected macrophages and microglial cells have been demonstrated in the white mater.[5,10] Many studies suggest proinflammatory cytokines such as IL-1β and TNF-α released by microglia and macrophages which play a role in CNS injury.[11,12] On the other hand, infection of astrocytes may also occur with limited virus replication,[13] and astrocytes could cause CNS injury by secreting cytokines.[14-17] These observations suggest a role of proinflammatory cytokines in ADC. However, in vivo expression of these cytokines in the microenvironment of HIV encephalitis, microglial nodules with MNGCs, the histopathologic hallmark of ADC, is not yet clarified. One of the main reasons is presumed to be the complexities of brain pathology in patients who have died from terminal AIDS.

Simian immunodeficiency virus (SIV) infection of macaques has been shown to recapitulate key features of HIV infection of the human CNS, including the development of encephalitis with characteristic histopathological changes and psychomotor impairment.[18,19] Our previous study demonstrated that a macrophage-tropic SIV, SIV239env/MERT, caused typical microglial nodules with MNGCs without development of AIDS. Using this animal model of HIV-1 encephalitis, we explored which cell types expressed TNF-α and IL-1β and whether it is related to viral infection in the microenvironment of SIV encephalitis.

MATERIALS AND METHODS

Virus and animal

SIV239env/MERT is a macrophage-tropic virus the pathogenic properties of which have been previously described. This virus comprises four amino acid substitutions in the env of SIVmac239 backbone, and replicates as efficiently as the highly macrophage-tropic virus SIVmac316 in the alveolar macrophages.[20]

The rhesus macaques were screened and found to be seronegative for SIV, STLV, B virus, and type D retroviruses. Two macaques, #531 and #626, were infected intravenously with 100 TCID50 of SIV239env/MERT. Three uninfected macaques were used as controls. The animals were housed in individual cages and maintained according to the rules and guidelines of the National Institute for Infectious Diseases (NIID) for experimental animal welfare.

CD4+ cell count and viral RNA load

CD4+ cell counts were performed in peripheral blood specimens at the time of autopsy. To measure the level of virus replication in the periphery, viral RNA was quantified in plasma at autopsy. Viral RNA in the plasma of inoculated macaques was measured by real-time RT_PCR.

Histopathological examination

Routine histopathological methods applied are described elsewhere.[21] In brief, coronal sections of the brain and spinal cord were embedded in paraffin. For routine light microscopy, the paraffin sections were stained with HE and KB.

We used EnVision system (Dako, Carpinteria, CA, US) for immunohistochemistry. The following antibodies were used as the first antibodies: a mouse monoclonal antibody (mAb) anti-human TNF-α (1:400, abcam K. K., Tokyo, Japan), a rabbit polyclonal antibody IL-1β (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, US), a mAb anti-GFAP (1:100; Chemicon International, Temecula, CA, US), a rabbit anti-human CD3 (1:50; Dako, Glostrup, Denmark), a mAb anti-human macrophage CD68 (KP1; 1:50; Dako), a rabbit anti-Iba1 antibody (1:500, Wako Pure Chemical Industries, Osaka, Japan), and a mAb anti-SIV envelope gp160/gp32, KK41 (1:50; National Institute for Biological Standards and Control, Herts, UK). Immunoreactivity was visualized using either diaminobenzidine/peroxidase or 3-amino-9-ethylcarbazole (Dako, Carpinteria, CA, US) substrate–chromogen system (Dako). Light counterstaining was done with hematoxylin. For the SIV envelope gp160/gp32 immunostaning, a lymph node section from an SIV-infected rhesus macaque was used as a positive control. In the same way, for the TNF-α and IL-1β immunostaining, a tonsil section was used as a positive control.

Double-label immunohistochemistry

We performed double-label immunohistochemistry for IL-1β and SIVKK41 using the same section to examine the expressions of IL-1β correlating with the SIVenvgp160/gp32- positive cells. This entailed performing immunohistochemistry for IL-1β, followed by immunohistochemistry for SIVKK41. Double labeling was performed using AEC/peroxidase followed by Vector blue/alkaline phosphatase.

RESULTS

Clinical manifestation

Macaque #531 showed very slow progression of clinical course. A CD4+ cell count remained moderately decreased, 270/μL, even long after infection, and was sacrificed for autopsy 154 weeks after infection. Plasma viral load was relatively high, 277 800 copies/mL, at autopsy. Macaque #626 also showed also progression of clinical

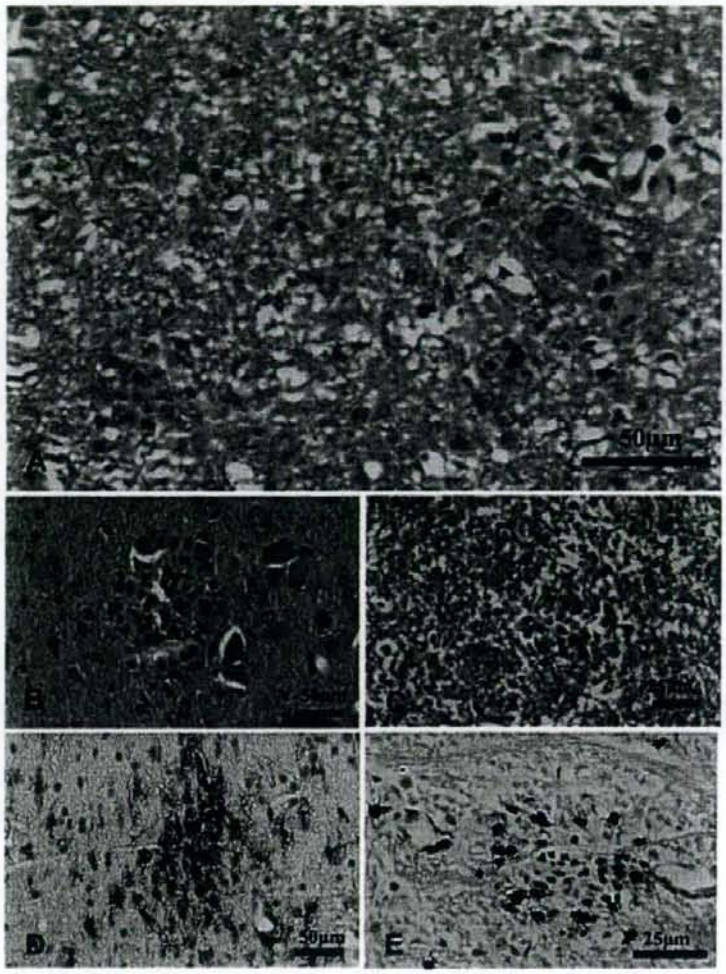

Fig. 1 Histopathological findings of the brain in macaque #531. A typical inflammatory lesion with microglial nodule with multinucleated giant cells (MNGCs) in the frontal white matter (A). Small microglial nodules seen in the basal ganglia (B), and the spinal cord (C). Microglial nodules were mainly composed of microglia/macrophages (D), and CD3+ T-cells (E). A, B, and C: HE; D: CD68; E: CD3. A: frontal white matter, B and D: basal ganglia, C and E: spinal cord.

course. CD4+ cell count remained moderately decreased, 220/μL, even long after infection. It was sacrificed for autopsy 218 weeks after infection. Plasma viral load remained low, 1000 copies/mL, at autopsy. These two macaques did not show obvious neurological symptoms or behavior abnormality.

Histopathological findings of the brain and lymph nodes

Macaque #531 showed a pathological hallmark of AIDS encephalopathy such as typical inflammatory lesions with MNGCs in the frontal white matter, (Fig. 1A). To a lesser extent, microglial nodules were also detected in the basal ganglia and spinal cord (Fig. 1B–C). Microglial nodules were mainly composed of microglia/macrophages (Fig. 1D), and CD3+ T-cells were scattered in the surrounding areas (Fig. 1E). Astrocytic gliosis was not accentuated in the areas of microglia nodules. On the other hand, no abnormality was observed in the cerebral cortex and cerebellum. Another macaque, #626, showed scattered CD3+ cells and CD68+ cells in the perivascular regions of the white matter and the meninges. However, microglial nodules with MNGCs could not be found. No other pathologic abnormality was found in the brain.

Virus-infected cells were detected by the immunostaining of the SIVenvgp160/gp32. In macaque #531, positive cells were detected mainly in inflammatory lesions

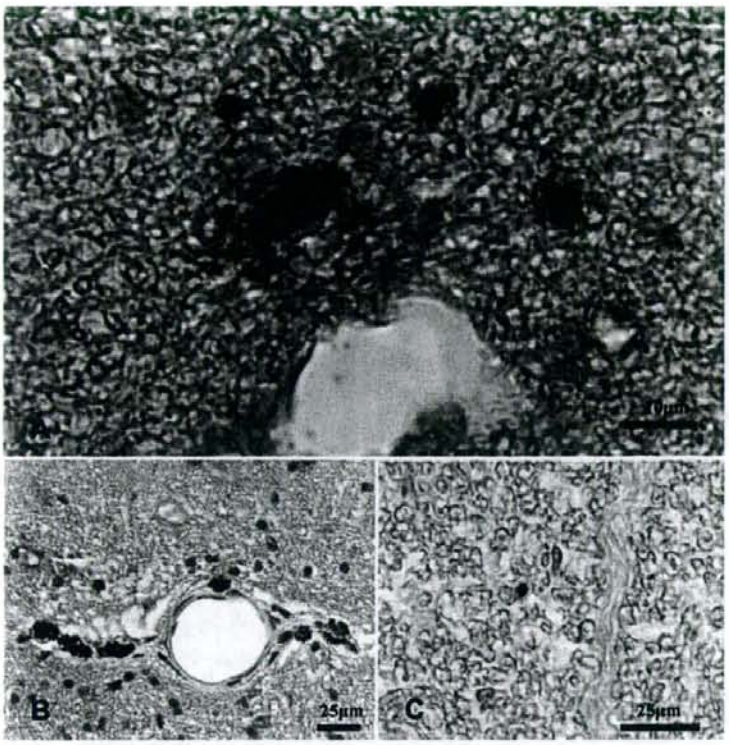

Fig. 2 Expression of a simian immunodeficiency virus (SIV) envelope protein by the immunostaining of SIVenvgp160/gp32 in macaque #531. SIV envelope protein is demonstrated in an inflammatory lesion in the frontal white matter (A), some perivascular macrophages in the basal ganglia (B), and a few in the spinal cord (C).

(Figs 2A,4B), and seemed to be microglia/macrophages and MNGCs based on their morphology. Some perivascular macrophages were also positive in basal ganglia (Fig. 2B). We also detected a few positive cells in the cerebellum and the spinal cord (Fig. 2C) as well as meningeal mononuclear cells. In another macaque, #626, SIVenvgp160/gp32 positive cells were limited to a few perivascular and meningeal mononuclear cells.

The lymph nodes of two virus-infected animals showed hyperplasia of follicles and their germinal centers showed irregular shapes. Decrease of CD3+ T-cells in the paracortical region was not evident.

All control macaques showed no abnormality in both brains and lymph nodes.

The expressions of TNF-α and IL-1β in inflammatory lesions

Since macaque #531 showed typical inflammatory lesions with MNGCs, we further examined expression of proinflammatory cytokines by immunohistochemistry. IL-1β-positive cells showed intracytoplasmic labeling. Positive cells were detected only in inflammatory lesions with MNGCs of the frontal white matter, that is to say, we could not detect IL-1β-positive cells in the parenchyma of basal ganglia as well as in the spinal cord. In order to investigate the relation between expression of IL-1β and virus infection, we performed double-label immunohistochemistry for IL-1β and SIVenvgp160/gp32. Interestingly, the IL-1β positive cells were found around the SIVenvgp160/gp32-positive cells, but not SIVenvgp160/gp32-positive cells (Fig. 3). The brain parenchyma of macaque #626 did not showed any IL-1β-positive cells.

TNF-α was also labeled as cytoplasmic staining. Positive cells were detected in some mononuclear cells of inflammatory lesions in the frontal white matter and basal ganglia, as well as a few perivascular macrophages. We could not detect TNF-α-positive cells in the spinal cord. TNF-α-positive cells seemed to be SIVenvgp160/gp32-negative cells in comparison with distribution of SIVenvgp160/gp32-positive cells stained using a serial section (Fig. 4). In macaque #626, a few TNF-α-positive cells were detected in perivascular and meningeal mononuclear cells.

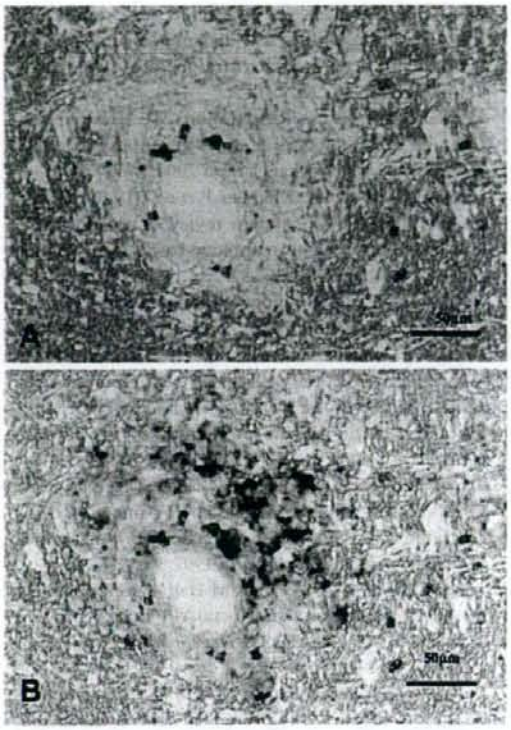

Fig. 3 Expression of IL-1β and SIVenvgp160/gp32 in an inflammatory lesion with microglial nodule with multinucleated giant cells (MNGCs). (A) IL-1β-positive cells are detected only in an inflammatory lesion with MNGCs seen in the frontal white matter of macaque #531. (B) IL-1β positive cells were found around the SIVenvgp160/gp32-positive cells but not SIVenvgp160/gp32-positive cells demonstrated by double-labeling immunohistochemistry performed using the same section of (A). A: anti-IL-1β; B: double-label immunohistochemistry for IL-1β (red) and SIVenvgp160/ gp32 (dark blue).

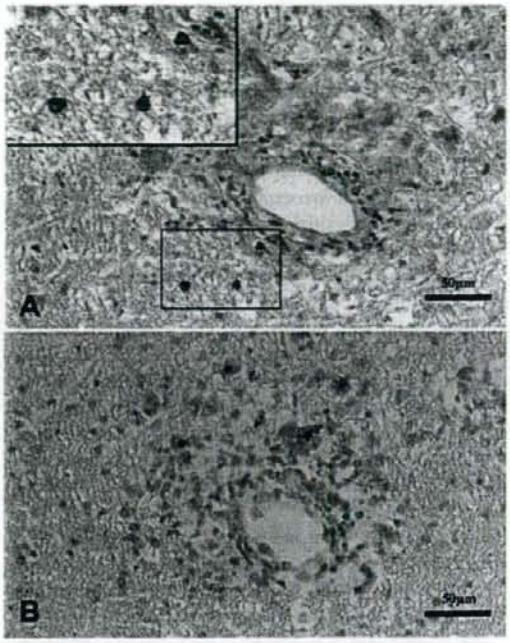

Fig. 4 Expression of TNF-α and SIVenvgp160/gp32 in an inflammatory lesion with microglial nodule with multinucleated giant cells (MNGCs). (A): TNF-α-positive cells are detected in mononuclear cells of an inflammatory lesion with MNGCs seen in the frontal white matter of macaque #531. (B): Distribution of SIVenvgp160/gp32-positive cells differs from that of TNF-α-positive cells demonstrated by SIVenvgp160/gp32 immunohistochemistry of serial section. A: anti-TNF-α, B: anti-SIVenvgp160/gp32.

DISCUSSION

Cytokines such as TNF-α and IL-1β may have toxic effects on CNS cells and have been postulated to contribute to the pathogenesis of the neurological complications of human immunodeficiency virus (HIV) infection.[22] However many of such studies were done by in vitro experiments; exposure of macrophages and microglia to either gp120 or Tat resulted in up-regulation of TNF-α expression,[23,24] and exposure of microglia to gp120 resulted in the production of IL-1β.[25,26] In contrast, there are only a few reports which demonstrated proinflammatory cytokines in the AIDS brain tissues directly in vivo. Tyor *et al.*[11] reported that there were significant increases in IL-1β and TNF-α in HIV-positive patients compared with HIV-negative brains, but no correlation was found between levels of cytokines and the presence or absence of CNS disease among HIV-positive individuals. In addition, in vivo expression of these cytokines in the microenvironment of HIV encephalitis, microglial nodules with MNGCs, was not demonstrated in their study. Zhao *et al.*[27] reported that IL-1β was expressed at high levels in areas of microglial nodules in HIV encephalitis. Because some MNGCs were positive for IL-1β in their report, they suggested that IL-1β was induced by HIV-1 infection.

In our present study, expression of IL-1β and TNF-α were detected in the inflammatory lesions with MNGCs, and these positive cells were found to be negative for SIVenvgp160/gp32. There were a few TNF-α positive cells and almost no IL-1β positive cells in the area other than inflammatory lesions including microglial nodules. Our findings indicate that virus-infected microglia/macrophages do not always express IL-1β and TNF-α. The

findings seen in macaque #531 might indicate a limited role of IL-1β and TNF-α in the very early stage of ADC. In order to understand a precise role of proinflammatory cytokines in ADC, further studies are required focusing on origin or nature of the cells expressing proinflammatory cytokines.

The differences between previous reports[22,27] and our present data about in vivo expression of cytokines might be explained by complexities of brain pathology in patients who have died from terminal AIDS. Human autopsies were usually performed in the advanced stages of AIDS. In such conditions, the brains may contain a variety of pathologic conditions other than HIV encephalitis such as diffuse poliodystrophy, another pathologic event of ADC, many kinds of opportunistic infections and tumors, and/or effects of anti-viral agents. Our macaque #531 with typical pathologic findings of SIV encephalitis was not in the stage of AIDS, and opportunistic diseases or diffuse poliodystrophy were not observed. We can also exclude the effects of chemotherapy.

In the present study, macaque #531 with typical SIV encephalitis did not show obvious neurological symptoms or behavior abnormality. This reminded us of a previous report in which the brains of asymptomatic HIV-1-positive individuals who died accidentally revealed HIV-1 infection and inflammatory response in the cerebral white matter.[28] These observations indicate that histopathologic findings of HIV encephalitis might be subclinical in many individuals infected with HIV-1. Another macaque (#626) did not show microglial nodules with MNGCs. The plasma viral load of this animal was much lower than that of macaque #531. This suggested that presence or absence of HIV encephalitis might simply depend on the value of plasma viral load.

Our macaque infected with SIV239env/MERT induced typical microglial nodules with MNGCs as a model of HIV encephalitis, and this macaque model may be useful for the better understanding of HIV encephalitis pathogenesis.

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