independently, and the cell tropism of inoculated viruses may be involved in selection of the types of pathology.[20] Further analyses of the frontal cortex of these macaques with advanced AIDS demonstrated astrocytic degeneration and diffuse activation of microglia, suggesting roles of these cells in pathogenesis of DPD.[21]

Regarding pathogenesis of ADC, it has been discussed that proinflammatory cytokines such as TNF-α and IL-1β may have toxic effects on CNS cells, inducing neuronal cell death.[22,23] Many studies suggest that IL-1β and TNF-α are released by microglia and macrophages and play a role in CNS injury.[24,25] On the other hand, infection of astrocytes may also occur with limited virus replication,[26] and astrocytes could cause CNS injury by secreting these cytokines.[27-30] However, in vivo expression of these cytokines in the microenvironment of HIVE, microglial nodules with MNGCs, are not yet clarified. Our previous study suggested that virus-infected microglia/macrophages do not always express IL-1β and TNF-α in the macaque model.[31]

In order to clarify roles of these cytokines in pathogenesis of ADC, we examined human autopsy brains from HIV-1-infected individuals focusing on types of cells expressing IL-1β and TNF-α in the microenvironment of HIVE.

MATERIALS AND METHODS

Autopsy cases

Among all autopsy cases of the Neurological Institute, Vienna University since 1983, there were altogether 429 who died from AIDS. The records of these cases were screened and the cases with opportunistic infection, neoplasm, and massive cerebrovascular sickness in the brain were excluded. Finally, 11 cases with HIVE in the brain were selected.

Histopathological examination

Paraffin serial sections of the frontal lobe and pons were processed for further histopathological examination. An analysis for degrees of HIVE was done using HE and KB stainings. We used the EnVision system (Dako, Carpinteria, CA, US) for immunohistochemistry. The following antibodies were used as first antibodies: mouse anti-human (TNF-α; 1:400; Abcam, Cambridge, MA, US), rabbit polyclonal antibody TNF-α (1:200; Abcam), and rabbit polyclonal antibody IL-1β (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, US) were used to detect the respective cytokines. We also performed GFAP, ubiquitin carboxyl-terminal esterase L (UCHL)-1, CD45 RB, and CD20 immunohistochemical staining for routine cell characterization. To identify activated microglia, we used a mouse monoclonal antibody to human macrophage CD68 (KP1, 1:50; Dako, Glostrup, Denmark) and a rabbit anti-ionized calcium-binding adaptor molecule 1 antibody (Iba1; 1:500; Wako Chemicals, Osaka, Japan). For HIV-infected cells, we used an antibody HIVp24 (1:10, DaKoCytomation, Glostrup, Denmark). Antigen retrieval was performed by pretreatment in 0.067% protease (Sigma-Aldrich, St Louis, MO, US) in PBS for 3–5 min for immunohistochemistry of CD68, CD3, HIVp24, TNF-α, and IL-1β. Immunoreactivity was visualized using either diaminobenzidine/peroxidase or 3-amino-9-ethylcarbazole (Dako) substrate-chromogen system (Dako). Light counterstaining was done with hematoxylin. For the TNF-α and IL-1β immunostaining, tonsil sections with chronic inflammation were used as a positive control.

Double-label immunohistochemistry

To determine the phenotype of virus-infected cells, we performed a double-label immunohistochemistry for HIVp24 and either Iba1 or GFAP. We first performed immunohistochemistry for Iba1 or GFAP using avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, US) and then for HIVp24 using the EnVision system (Dako). The double-labeling was done using Vector blue/alkaline phosphatase followed by the 3-amino-9-ethylcarbazole substrate-chromogen system (Dako; red)/peroxidase.

We performed a double-label immunohistochemistry for TNF-α and either GFAP, CD68, UCHL-1, or CD20 to determine the phenotype of the TNF-α positive cells. We first performed immunohistochemistry for either GFAP, Iba1, UCHL-1, or CD20 using avidin-biotin-peroxidase complex (Vector) and then followed with TNF-α using the EnVision system (Dako). The double-labeling was done using Vector blue/alkaline phosphatase followed by the 3-amino-9-ethylcarbazole substrate-chromogen system (Dako; red)/peroxidase. To determine the phenotype of the IL-1β positive cells, we performed a double-label immunohistochemistry for CD68 or UCHL-1 and IL-1β using the same method. A double-label immunohistochemistry was performed for GFAP and IL-1β by first performing immunohistochemistry for GFAP using the EnVision system (Dako), followed by immunohistochemistry using IL-1β avidin-biotin-peroxidase complex (Vector). The double-labeling was performed using diaminobenzidine/peroxidase followed by Vector blue/alkaline phosphatase.

We performed a double-label immunohistochemistry for IL-1β and HIVp24 to examine the expressions of IL-1β correlating with the HIVp24-positive cells. This entailed performing immunohistochemistry for IL-1β using the avidin-biotin-peroxidase complex (Vector), followed by

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immunohistochemistry for HIVp24 using the EnVision system (Dako). The double-labeling was performed using Vector blue/alkaline phosphatase followed by diaminobenzidine/peroxidase. To examine the expressions of TNF-α correlating with the HIVp24-positive cells, we performed double-label immunohistochemistry for TNF-α and HIVp24 by first performing immunohistochemistry for HIVp24 using the EnVision system (Dako) and then for TNF-α using avidin-biotin-peroxidase complex (Vector). The double-labeling was performed using 3-amino-9-ethylcarbazole substrate-chromogen system (Dako; red)/peroxidase followed by the Vector blue/alkaline phosphatase.

Semiquantitative analysis

We performed semiquantitative assessments for routine histopathological changes as follows: 3; severe, 2; moderate, 1; mild, 0; none. We also semiquantitatively estimated numbers of cells positive for the antibodies to HIVp24, CD68, TNF-α, and IL-1β as following: 3; many, 2; moderate, 1; a few, 0; none.

RESULTS

Routine histopathological changes

Among 11 cases of the frontal white matter and pons from HIV-1 infected patients, all cases showed the typical pathology of HIVE such as glial nodules with multinucleated giant cells and mononuclear cell infiltration. A varying degree of white matter changes such as loosening of myelin staining, vacuolar changes, or astrocytic gliosis were also observed in all cases. HIVp24-positive cells were detected in all cases (Fig. 1). Semiquantitative judgments of histopathological findings of the frontal lobe and pons are summarized in Tables 1 and 2.

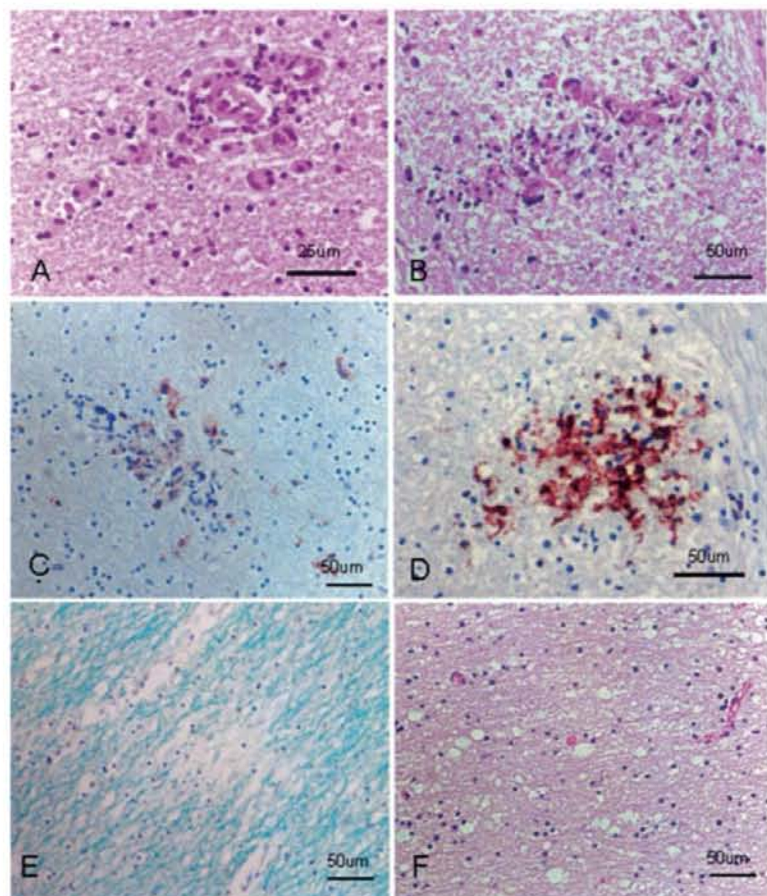

Fig. 1 Pathologic changes of HIV encephalitis. Glial nodules with multinucleated giant cells in the frontal white matter (A) as well as pons (B). HIVp24-positive cells were detected in the glial nodules seen in the frontal white matter (C), as well as pons (D). A varying degree of white matter changes such as loosening of myelin staining (E), vacuolar changes (F) seen in the frontal white matter. A–D, #32–92; E–F, #98–02; A, C, E–F, frontal white matter; B, D, pons; A, B, F, HE staining; C, D, anti-HIVp24; E, KB staining.

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Table 1 Routine histopathological changes

Patient no.	Frontal white mater					Pons		
	Microglial nodule	Myelin pallor	Vacuolation	Gliosis	Microglial nodule	Myelin pallor	Vacuolation	Gliosis
32–92	3	3	2	3	1	1	1	2
50–95	3	3	2	3	3	2	2	3
98–02	3	3	3	3	3	3	3	3
137–99	2	1	1	1	1	1	1	0
10–87	2	1	2	3	2	2	2	3
133–00	2	2	2	1	1	1	1	0
82–91	2	1	1	3	1	1	1	2
206–86	2	2	2	3	3	3	3	3
45–96	1	1	1	1	2	1	1	2
61–93	1	1	1	2	1	1	1	0
118–91	1	1	1	1	0	1	1	2

3, marked; 2, moderate; 1, slight; 0, none.

Table 2 Expression of proinflammatory cytokines TNF-α and IL-1β in HIV encephalitis

Patient No.	HIVP24		IL-1β of frontal white matter				TNF-α of frontal white matter			
	Frontal white mater	Pons	MNGC	Microglia	Macrophage	Astrocyte	MNGC	Microglia	Macrophage	Astrocyte
32–92	3	3	3	2	3	3	0	3	3	3
50–95	3	1	3	2	3	3	0	3	3	3
98–02	2	2	0	3	2	0	0	3	3	0
137–99	2	1	0	1	2	0	0	2	2	0
10–87	2	3	0	0	1	0	0	0	0	0
133–00	1	1	1	1	1	0	0	1	1	0
82–91	1	2	0	1	1	0	0	1	1	0
206–86	1	2	0	1	1	0	0	0	0	0
45–96	1	2	0	1	1	0	0	1	1	0
61–93	1	1	0	1	1	0	0	0	0	0
118–91	0	3	0	1	1	0	0	0	0	0

3, many; 2, moderate; 1, a few; 0: none; MNGC, multinucleated giant cells.

Detection of HIVp24 in microglial cells and macrophages as well as MNGCs, but not in astrocytes

To determine which cell types are infected with HIV-1, we performed double-label immunohistochemistry. The HIVp24 staining was detected in infiltrating macrophages, MNGCs, macrophages and microglia of glial nodules, as well as scattered activated microglia, but not in astrocytes in the frontal white matter (Fig. 2).

The expressions of IL-1β by macrophages, microglial cells, and astrocytes from HIVE

IL-1β-positive cells were shown as intracytoplasmic labeling. Among 11 cases, IL-1β was detected in the frontal white matter of all cases, and in the frontal cortex of one case. Most of the positive cells had morphological features of perivascular macrophages and multinucleated giant cells in the glial nodules (Fig. 3A,D). On the other hand, in two cases of 32–92 and 50–95, some IL-1β positive cells showed morphological features of astrocytes in the white matter (Fig. 3B,C). In order to search expressions of IL-1β, we compared IL-1β and CD68, Iba1, UCHL-1 and GFAP and HIVp24 in serial sections. IL-1β-positive cells had the same shape and distribution as Iba1- and CD68-positive cells, although CD68-positive cells were more numerous (Fig. 3D–H). The double-label immunohistochemistry demonstrated that IL-1β was detected in CD68-positive macrophages/microglia of most cases (Fig. 3I), and in GFAP-positive astrocytes of only two cases, 32–92 and 50–95 (Fig. 3J), and not in UCHL-1-positive cells (Fig. 3K).

The expressions of TNF-α by macrophages, microglial cells, and astrocytes from HIVE

TNF-α-positive cells were shown as intracytoplasmic labeling. Among 11 cases, TNF-α was detected in seven cases in the frontal white matter. Positive cells were detected in some mononuclear cells of inflammatory lesions in the frontal white matter, as well as a few perivascular macrophages, and some TNF-α-positive cells showed morphological features of astrocytes in the white matter (Fig. 4A,B). The double-label immunohistochemistry demonstrated that the TNF-α-positive cells were restricted to CD68-positive macrophages/microglia in most cases (Fig. 4C),

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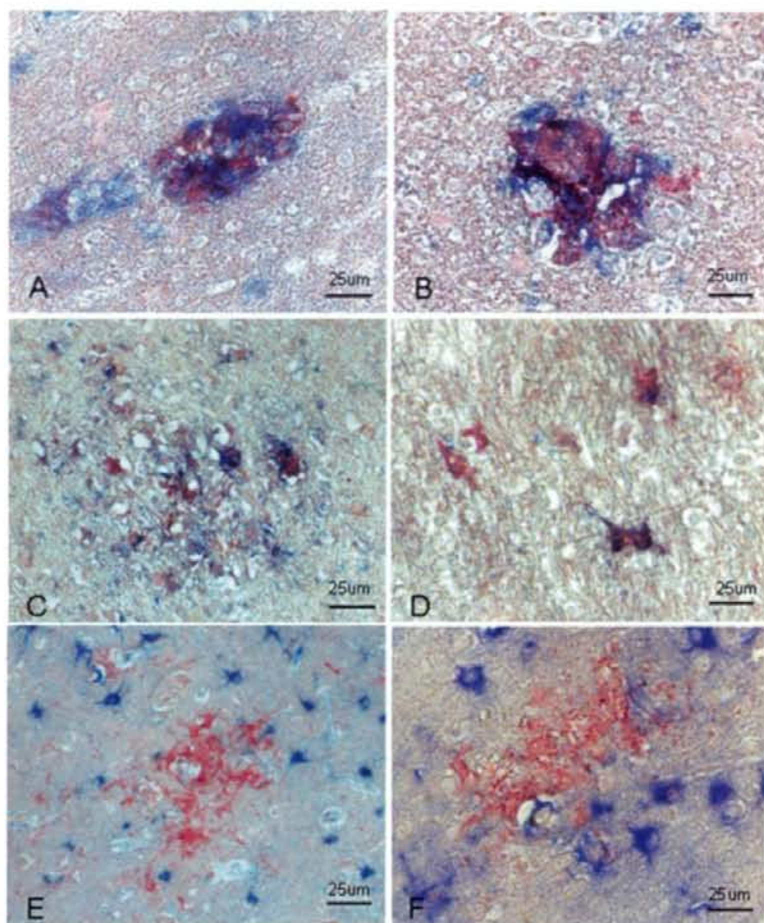

Fig. 2 Double-label immunohistochemistry for HIVp24 and anti-ionized calcium-binding adaptor molecule 1 antibody (Iba1) or GFAP in HIV encephalitis. HIVp24 staining was detected in infiltrating macrophages (A), multinucleated giant cells (B), macrophages and microglia of glial nodules (C), as well as scattered activated microglia (D), but not in astrocytes (E, F) in the frontal white matter. A, B, F, #50–95; C–E, #32–92. A–D, double-label of HIVp24 (red) and Iba1 (blue); E–F, double-label of HIVp24 (red) and GFAP (blue).

and in some GFAP-positive astrocytes of only two cases, 32–92 and 50–95 (Fig. 4D), and not in UCHL-1-positive cells (Fig. 4E) and the CD20-positive cells (Fig. 4F).

Some HIVp24-positive cells express IL-1β but not TNF-α

In order to investigate the relationship between expression of IL-1β or TNF-α and virus infection, we performed double-label immunohistochemistry for IL-1β or TNF-α and HIVp24. Interestingly, some IL-1β-positive cells were HIVp24-positive multinucleated giant cells in the glial nodules. Several other IL-1β-positive cells were also found around the HIVp24-positive cells, but they were negative for HIVp24 (Fig. 5A–C). Although the TNF-α-positive cells were found around the HIVp24-positive cells, they were negative for HIVp24 (Fig. 5D,E).

DISCUSSION

The cytokines such as TNF-α and IL-1β stimulate macrophages and endothelial cells to express chemokines such as monocyte chemoattractant protein-1 (MCP-1) and adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) that attract leucocytes into the peripheral site of injury or infection.[32–37] In HIV-1 infection, it has been considered that HIV-infected macrophages and microglia cells directly produce such cytokines and may have toxic effects on CNS cells and induce neuronal death, which has been discussed as the pathogenesis of ADC.[22,24,29,38,39] Several in vitro studies have demonstrated that exposure of macrophages and microglia to either gp120 or Tat resulted in up-regulation of TNF-α expression[40,41] and exposure of microglia to gp120 resulted in the production of IL-1β.[42,43] A number of hypotheses concerning the roles

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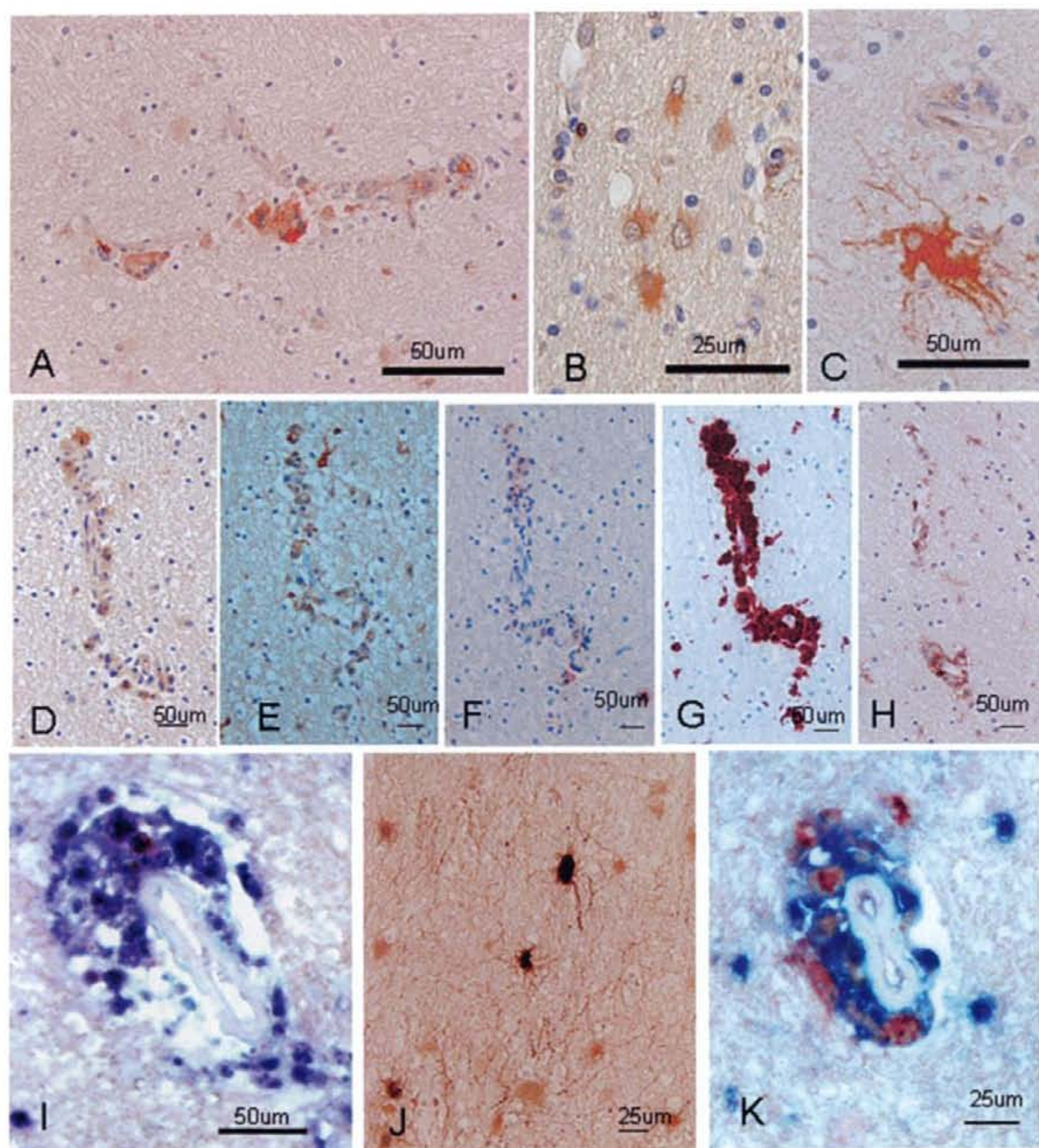

Fig. 3 Expression of IL-1β in the frontal white matter of HIV encephalitis. IL-1β immunoreactivety was detected in perivascular macrophages and multinucleated giant cells in the glial nodules (A), and mild expression of IL-1β was also detected in some reactive astrocytes (B) and rarely in bizarre astrocytes (C). In serial sections (D–H), IL-1β-positive cells had the same shape and distribution as anti-ionized calcium-binding adaptor molecule 1 antibody (Iba1) and HIVp24-positive cells. Double-labelled immunohistochemistry (I–K), IL-1β-positive cells were co-localized in CD68-positive macrophages/microglia (I) and GFAP-positive astrocytes (J), but not in UCHL-1-positive cells (K). A, D–K, #32–92; B,C, #50–95. A–D, anti-IL-1β; E, anti-Iba1; F, anti-HIVp24; G, anti-CD68; H, anti-UCHL-1; I, double-label of IL-1β (red) and CD68 (blue); J, double-label of IL-1β (dark blue) and GFAP (brown); K, double-label of IL-1β (red) and UCHL-1 (blue).

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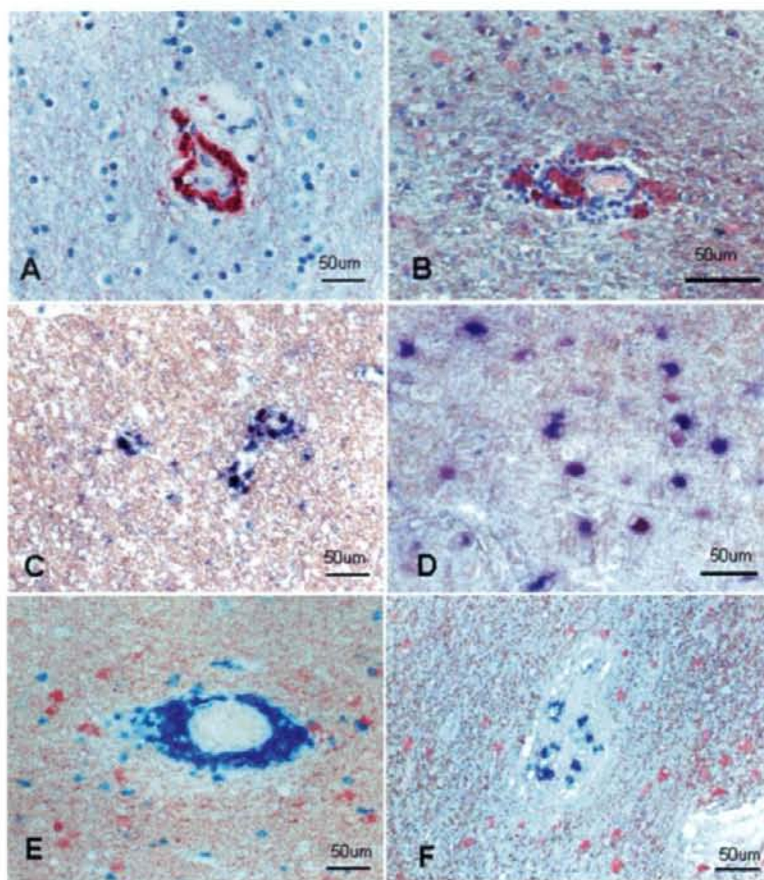

Fig. 4 Expression of TNF-α in the frontal white matter of HIV encephalitis. TNF-α immunoreactivity was detected in perivascular macrophages (A, B). Mild expression of TNF-α by astrocytes was detected in surrounding areas of inflammatory lesions (B). Double-labelled immunohistochemistry, TNF-α was detected in some CD68-positive perivascular macrophages/microglia (C) and some GFAP-positive reactive astrocytes (D), but TNF-α was not detected in UCHL-1-positive cells (E) as well as CD20-positive cells (F). A, C, #137–99; B, D–F, #50–95. A, B, anti-TNF-α; C, double-label TNF-α (red) and CD68 (blue); D, double-label TNF-α (red) and GFAP (blue); E, double-label TNF-α (red) and UCHL-1 (blue); F, double-label TNF-α (red) and CD20 (blue).

of these cytokines are based on these in vitro experiments. However, little is known about in vivo expression of these cytokines in the microenvironment of HIVE, microglial nodules with MNGCs, and roles of these cytokines with relation to HIV infection are not yet clarified.

In the present study, we examined human autopsy brains from HIV-1 infected individuals and explored whether expression of cytokines such as IL-1β and TNF-α can be detected in macrophages/microglia or astrocytes in HIVE, and whether expression of these cytokines is related to viral infection. Interestingly, IL-1β was detected in the frontal white matter of all 11 cases where microglial nodules were observed to varying degrees. TNF-α was detected in seven cases, and we could not detect expression of TNF-α in four cases. IL-1β-positive cells were mostly restricted to CD68-positive macrophages/microglia and some of them were HIVp24-positive multinucleated giant cells in the glial nodules. On the other hand, TNF-α positive cells were also restricted to mainly CD68-positive macrophages/microglia; however, we could not detect TNF-α expression in the HIVp24-positive cells. These findings suggest that IL-1β is induced by HIV-1 infection and may be one of the important factors for induction of HIVE.

Barak *et al.*[44] reported that intra-cerebroventricular administration of HIV-1, gp120 induced the expression of IL-1β, but not TNF-α mRNA in the rat hypothalamus, 3 h after injection. Zhao *et al.*[45] reported that IL-1β was expressed at high levels in areas of microglial nodules and some MNGCs were positive for IL-1β in HIVE and they suggested that IL-1β was induced by HIV-1 infection. We confirmed that the HIV-1 infection of macrophages/microglia induced the expression of IL-1β, but not TNF-α for the first time in the brain lesions of HIV-1 encephalitis.

There are some in vitro studies suggesting that infection of astrocytes may also occur with limited virus replication,[26] and astrocytes could cause CNS injury by secreting cytokines.[27–30] In the present study, we detected mild

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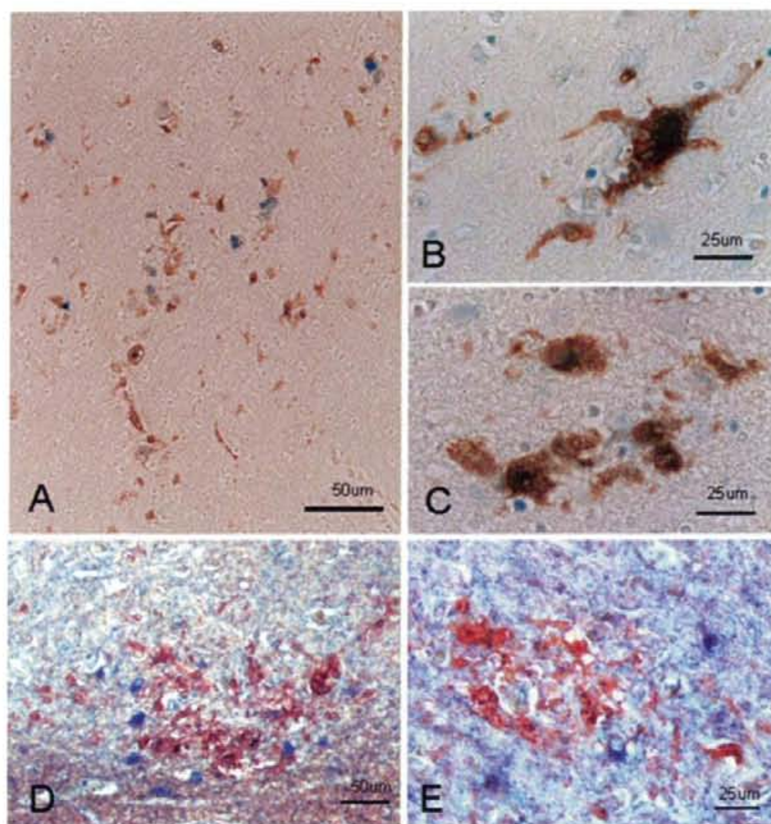

Fig. 5 Double-label immunohistochemistry for HIVp24 and IL-1β or TNF-α in HIV encephalitis. IL-1β positive cells were found around the HIVp24-positive cells and some of them were also positive for HIVp24 (A). Some HIVp24-positive multinucleated giant cells were positive for IL-1β in the glial nodules (B, C). TNF-α-positive cells were found around the HIVp24-positive cells, but they were negative for HIVp24 (D, E). A–C, #32–92; D–E, #5095. A–C, double-label HIVp24 (brown) and IL-1β (blue); D–E, double-label HIVp24 (red) and TNF-α (blue).

expression of IL-1β or TNF-α by astrocytes in only two cases with severe HIVE. Our results are similar to the report of Wesselingh *et al.*[25] in which IL-1β-positive cells were restricted to CD68-positive macrophages/microglia and rare GFAP-positive astrocytes. These findings suggest that the cells of macrophages/microglia lineage are main cells to release cytokines such as IL-1β and TNF-α in the lesions of HIV-1 encephalitis. Recent studies reported that latent or low-level infection of astrocytes may occur in HIV-1 infected brains.[46] Cytokine-expressing astrocytes seen in the present study might be infected with HIV-1 in a latent manner, although our double immunohistochemistry could not detect HIVp24 in these astrocytes.

On the other hand, in our present study, IL-1β was detected also in many HIVp24-negative cells, and most TNF-α-expressing cells were negative for HIVp24. Expression of these cytokines on HIVp24-negative cells is consistent with our previous study on the macaque model,[31] and suggests that non-infected microglia/macrophages as well as astrocytes might have a role in the pathogenesis of ADC. Interestingly it has been proposed that uninfected macrophages and microglia also release these neurotoxic cytokines, thus explaining why apoptotic neurons do not co-localize with infected macrophages and microglia.[22,47,48] Our previous studies on the frontal cortex pathology also suggested that cortical degeneration and presence of virus-infected cells are independent of each other in the SIV model[21] as well as in human autopsy brains.[49]

In conclusion, our present study demonstrated that HIV-1-infected macrophages/microglia express IL-1β but not TNF-α in HIVE inflammatory lesions. In addition, many non-infected macrophages/microglia as well as some astrocytes express IL-1β and TNF-α, which may contribute to the pathogenesis of ADC.

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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

Hui Qin Xing,[1] Takashi Moritoyo,[1] Kazuyasu Mori,[2,3] Chie Sugimoto,[2,3] Fumiko Ono[4] and Shuji Izumo[1]

[1]Division of Molecular Pathology, Center for Chronic Viral Diseases, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, [2]AIDS Research Center, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, [3]Tsukuba Primate Research Center, National Institute of Biomedical Innovation, and [4]Corporation for Production and Research of Laboratory Primates, Ibaraki, Japan

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

Correspondence: Hui Qin Xing, MD, PhD, Division of Molecular Pathology, Center for Chronic Viral Diseases, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan. Email: xhqw63@hotmail.com

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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

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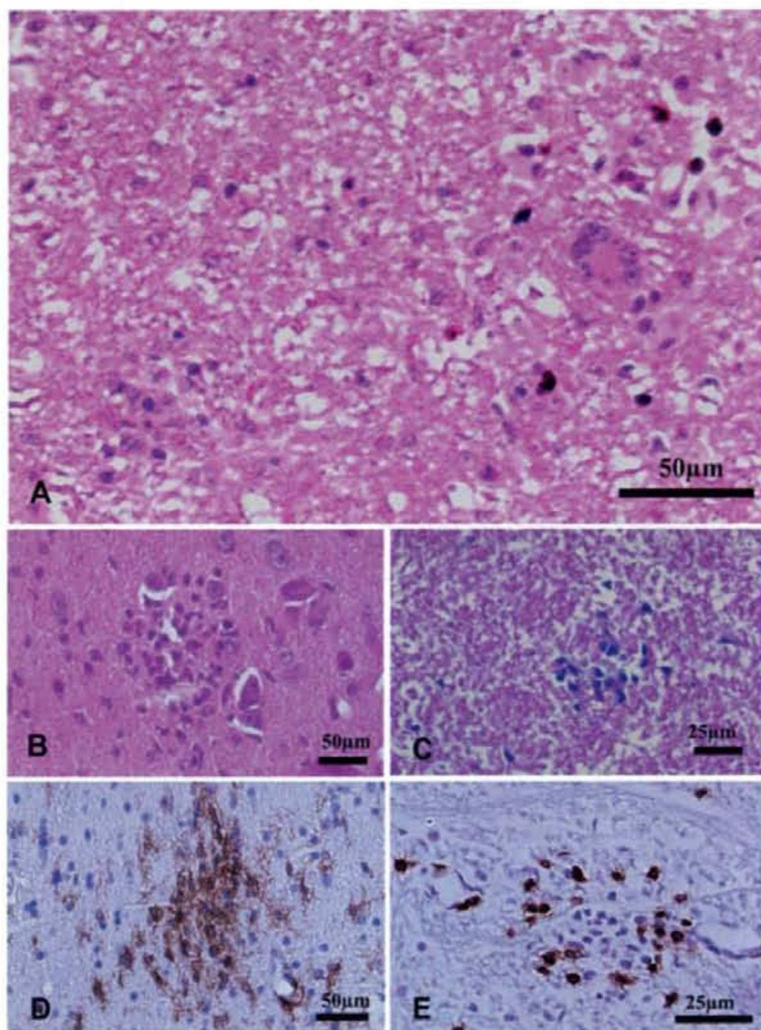

Fig. 1 Histopathological findings of the brain in macaque #531. A typical inflammatory lesion with microglial nodule with multinucleated giant cells (MNGCs) in the frontal white matter (A). Small microglial nodules seen in the basal ganglia (B), and the spinal cord (C). Microglial nodules were mainly 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

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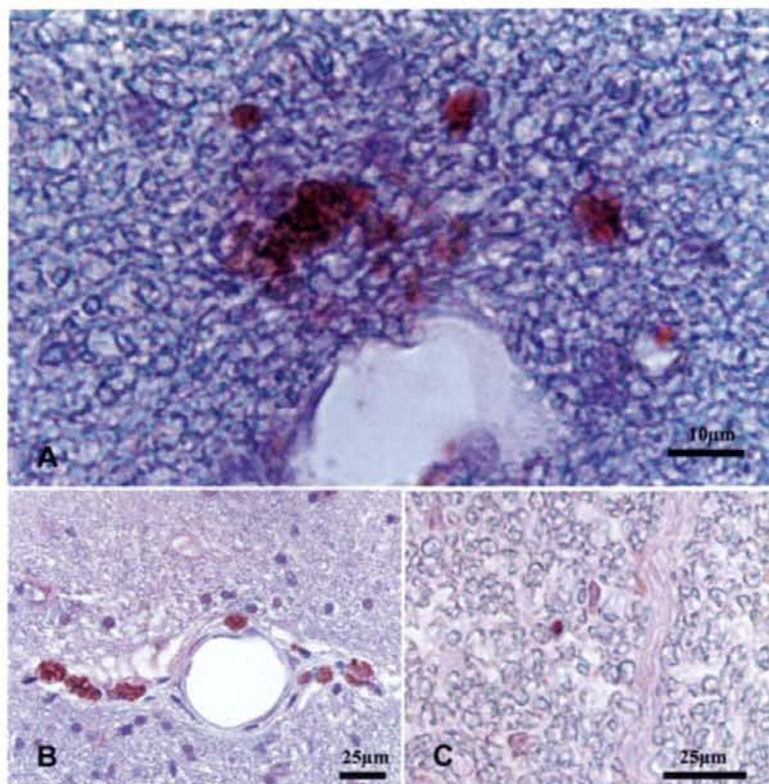

Fig. 2 Expression of a simian immunodeficiency virus (SIV) envelope protein by the immunostaining of SIVenvgp160/gp32 in macaque #531. SIV envelope protein is demonstrated in an inflammatory lesion in the frontal white matter (A), some perivascular macrophages in the basal ganglia (B), and a few in the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

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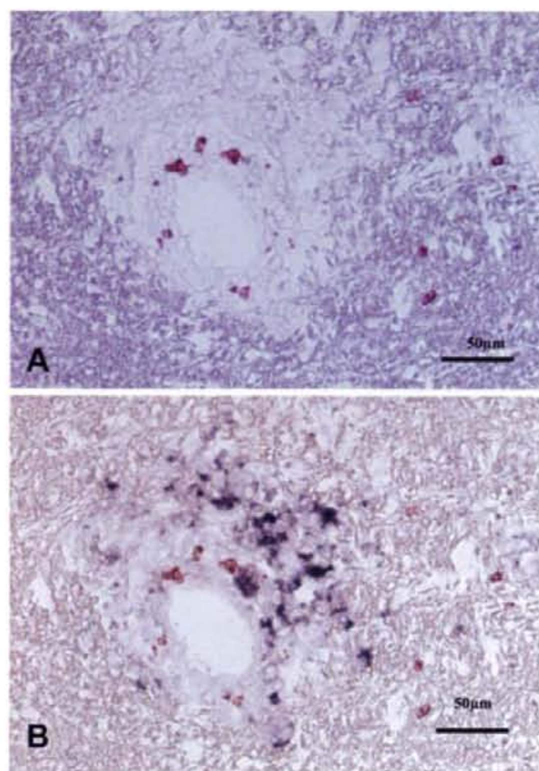

Fig. 3 Expression of IL-1β and SIVenvgp160/gp32 in an inflammatory lesion with microglial nodule with multinucleated giant cells (MNGCs). (A) IL-1β-positive cells are detected only in an inflammatory lesion with MNGCs seen in the frontal white matter of macaque #531. (B) IL-1β positive cells were found 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).

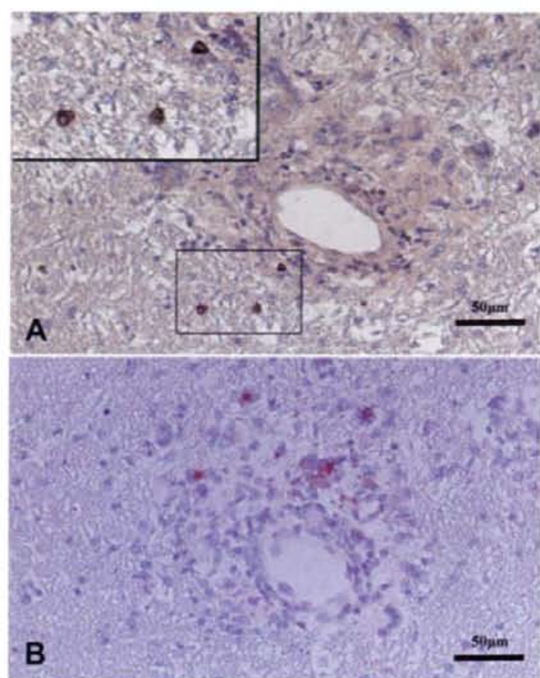

Fig. 4 Expression of TNF-α and SIVenvgp160/gp32 in an inflammatory lesion with microglial nodule with multinucleated giant cells (MNGCs). (A): TNF-α-positive cells are detected in mononuclear cells of an inflammatory lesion with MNGCs seen in the frontal white matter of macaque #531. (B): Distribution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

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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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