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Levels of human immunodeficiency virus type 1 (HIV-1) replication in macrophages determines the severity of murine HIV-1 encephalitis

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The presence of specific neuroinvasive strains and necessity for brain viral replication for disease progression remain controversial issues in neuro-AIDS research. To investigate these questions, the authors injected human monocyte-derived macrophages (MDMs) infected with diverse viral strains were injected into the caudate and putamen of severe combined immunodeficient (SCID) mice. Independent of viral strain, infected MDMs became immunologically activated and elicited profound inflammatory reactions in brain areas most affected in humans. The intensity of neuropathologic changes, including microglial reactions, paralleled levels of viral infection and numbers of infected MDMs. The data suggest that HIV-1–associated neurological disease is related to the level of productive viral infection in activated macrophages. Virus infection, *per se*, may affect the ability of macrophages to respond to immune stimuli by overproduction of proinflammatory factors and neurotoxins, leading to neuronal dysfunction. *Journal of NeuroVirology* (2004) **10(suppl. 1),** 82–90.

Keywords: animal model; encephalitis; HIV-1; viral strain

Introduction

Human immunodeficiency virus (HIV)-1–associated dementia (HAD) is the most frequent central nervous system (CNS) complication seen during advanced immunosuppression in late stage virus infection (Navia *et al*, 1986; Price *et al*, 1988). Although combination highly active antiretroviral therapies (HAARTs) have decreased the overall mortality and prevalence of CNS opportunistic infections, these therapies may be less active in preventing direct HIV-1 effects on the brain. Despite the diminished incidence of HAD to approximately 11% (Maschke *et al*, 2000), the prevalence of HIV encephalopathy is increasing (Neuenburg *et al*, 2002). This could be associated with viral mutations, decreased penetrance of antiretroviral drugs into the brain, and HAART failure (Jellinger *et al*, 2000; Sacktor *et al*, 2001, 2002). Pathologically, HAD is associated with HIV-1 encephalitis (HIVE), characterized by the infiltration and activation of virus-infected mononuclear phagocytes (MPs; microglia, perivascular, and brain macrophages) (Cherner *et al*, 2002; Koenig *et al*, 1986; Wiley *et al*, 1986). The formation of multinucleated giant cells (MGCs), astrogliosis, microglial nodules, myelin pallor, blood-brain barrier (BBB) damage, and neuronal injury/death are all prominent pathological features of disease.

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During HIVE, productive infection occurs almost exclusively in brain MPs and MGCs. HIV-1 infection primes the MPs, which upon activation, secrete a variety of neurotoxins, including, but not limited to, arachidonic acid metabolites, platelet activating factor, cytokines (tumor necrosis factor [TNF]-α and interleukin-1β [IL-1β]), quinolinate, nitric oxide (NO), superoxide anions, and viral proteins (gp120, gp41, and Tat) (Adamson *et al*, 1996; Brenneman *et al*, 1988; Gelbard *et al*, 1994; Genis *et al*, 1992; New *et al*, 1997; Nottet *et al*, 1995). These neurotoxins are responsible for neuronal dysfunction and subsequent clinical manifestations of disease. Nonetheless, a paradox exists between the relatively small numbers of productively infected MPs and degree of clinical and neurological deficits.

A number of host factors may play important roles in individual susceptibility to HIV-1 CNS disease. Recently, homozygosity for the monocyte chemoattractant protein (MCP)-1 2578G allele was associated with accelerated disease progression and a 4.5-fold increased risk for HAD (Gonzalez *et al*, 2002). Cellular transport proteins (P-glycoprotein [P-gp], multidrug resistant-associated proteins [MRPs]) are important in regulating intracellular concentrations of antiretroviral drugs (protease inhibitors) (Fellay *et al*, 2002). Allelic variants of P-gp/MRPs and inhibition (or induction) are critical in active drug delivery across the BBB (Letendre *et al*, 2002). Currently, the impact of HAART on viral genotype and phenotype found in the brain and the relationship to clinical disease remains uncertain. The role of antiviral T-cell responses in the brain during HIVE is also unclear (Poluektova *et al*, 2001).

Previous studies demonstrated that brain-derived viruses are macrophage-tropic (M-tropic) and principally use CCR5 for virus entry (Gabuzda *et al*, 1998; Ghorpade *et al*, 1998b). A recent study indicated the presence of HIV-1 variants with increased CCR5 affinity and reduced dependence on CCR5 and CD4 in the brains of some HIV-1–infected patients with CNS disease. These results suggested that R5 variants with increased CCR5 affinity may represent a pathogenic viral phenotype contributing to HIV-1 neurodegenerative manifestations (Gorry *et al*, 2002). Although HIVE is likely a macrophage-mediated disease, the relationship between the virus and clinical manifestations of HAD is not fully understood. Because the levels of virus in brain do not always correlate with the degree of neurological impairment (Johnson, 1995), it was suggested that qualitative differences between viral strains might be important in development of dementia (Power *et al*, 2002). One possibility is that specific strains of HIV-1 are more neurovirulent than others. To test this hypothesis, we studied the 'neurotoxic' features of divergent HIV-1 primary isolates in a previously developed nontransgenic murine animal model system for HAD that exhibits the hallmarks of HIVE, severe combined immunodeficiency (SCID) mouse model of HIVE (Persidsky *et al*, 1996; Tyor *et al*, 1993). Neuroinflammatory responses (Persidsky *et al*, 1997), neuronal dysfunction, and behavioral abnormalities (Zink *et al*, 2002) seen in HAD were reproduced in this animal model. Our results demonstrated that neuropathological changes were more related to the ability of the viral strain to replicate in MDMs rather than tissue sources of a specific HIV-1.

Results

Productive viral infection of MDMs correlates with secretion of proinflammatory cytokines secreted

To assess the relationship between the level of viral replication and proinflammatory cytokine secretion, human monocyte-derived macrophages (MDMs) were infected with a diverse panel of viral strains (Table 1). The infectious dose was normalized to 1.4×10^6 cpm reverse transcriptase (RT) activity/10^6 cells as previously described (Ghorpade *et al*, 1998a). Viral replication in MDMs was assessed on days 5 and 11 after viral inoculation. Both HIV-1_{ADA}– and HIV-1_{DJV}–infected MDMs showed high levels of RT activity in culture fluids on day 5, whereas the other isolates demonstrated lower levels of virus production (Figure 1A). By day 11, RT activity increased in MDMs infected with HIV-1_{ADA}, HIV-1_{BAL}, HIV-1_{DJV}, and HIV-$1_{JR\text{-}FL}$ (Figure 1B), but was limited in cells infected with the isolates. The ability of the MDM to respond to activation stimuli was next evaluated. MDM were activated by

Table 1 HIV-1 isolates

HIV-1 isolate	*Source*	*Reference*	*RT cpm/ml × 10^5 (peak day)**
HIV-1_{DJV}	Brain tissue	Heinzinger *et al*, 1995	51.4
HIV-$1_{JR\text{-}FL}$	Brain tissue	Koyanagi *et al*, 1987; Yoshimura *et al*, 1991	19.4
HIV-1_{BAL}	Human infant lung	Gartner *et al*, 1986	53.5
HIV-1_{SF162}	CSF	Cheng-Mayer *et al*, 1988	43.6
HIV-1_{ADA}	PBMCs	Gendelman *et al*, 1988	38.5
HIV-$1_{89.6}$	PBMCs	Collman *et al*, 1992	29.1

CSF, cerebrospinal fluid; PBMCs, peripheral blood mononuclear cells.
*Ghorpade *et al*, 1998a.

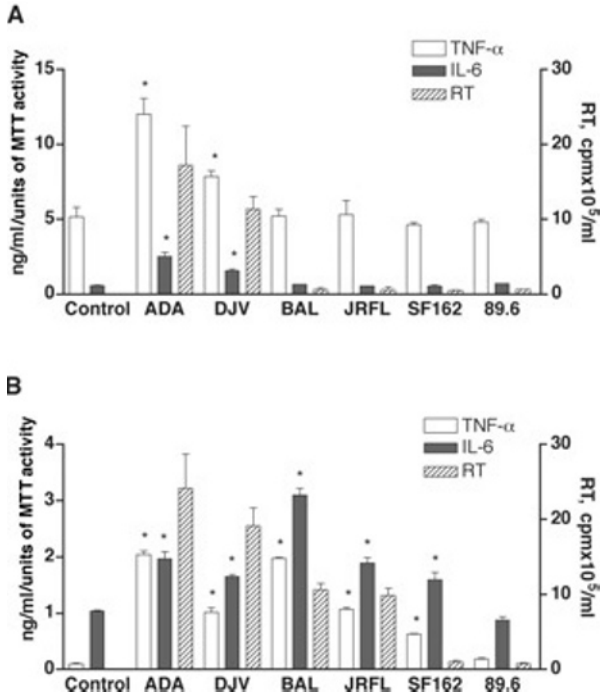

Figure 1 Viral replication and cytokine production by virus-infected and activated MDMs 5 days (**A**) and 11 days post infection (**B**). Adherent monocytes were cultured on 96-well plates at a density of 10^5 cells/well for 7 days. MDMs were infected with a panel of viral strains (Table 1) at equivalent RT levels (2.0×10^6 cpm/ml/10^6 cells). Replicate MDMs were stimulated with LPS (1 μg/ml) 5 and 11 days post infection, and culture fluids from control (uninfected) and HIV-infected cells with/without stimulation were collected 24 h later. The proinflammatory cytokines were assayed by ELISA. The levels of cytokines and RT activity were normalized to cell numbers by measuring cell viability at the end of the sample collection by the MTT assay. Data presented (mean ± SEM) are from one of two independent experiments denote statistically significant difference ($p < .01$) as compared to stimulated uninfected cells.

lipopolysaccharide (LPS; 1 μg/ml) at days 5 and 11 after infection, and production of TNF-α, interleukin (IL)-1β, and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA). At day 5, significant up-regulation of TNF-α and IL-6 production was seen in MDMs infected with HIV-1_{ADA} (1.7 and 1.4 times versus controls, $P < .01$) and HIV-1_{DJV} (5.0 and 3.5 times versus controls, $P < .01$), whereas levels from MDMs infected with the other lower replication isolates did not differ from control (uninfected) cells (Figure 1A). The level of IL-1β production in response to LPS was significantly higher in MDMs infected with HIV-1_{ADA} and HIV-1_{DJV} (6 and 5 times, $P < .01$) as compared to control cells, but overall levels of IL-1β were lower as compared to TNF-α or IL-6 (data not shown). At day 11, LPS activation of MDMs resulted in a 10- to 20-fold increase of TNF-α from cells infected with HIV-1_{ADA}, HIV-1_{BAL}, HIV-1_{DJV}, and HIV-$1_{JR\text{-}FL}$ as compared to uninfected MDMs. Likewise, LPS-stimulated MDMs infected with these same isolates secreted 1.5 to 3.0 times more IL-6 (Figure 1B) than did uninfected MDMs. MDMs infected with the same viruses produced 2 to 7 times more IL-1β than uninfected MDMs (data not shown). The increase in TNF-α production was 2 to 6 times in MDMs infected with HIV-1_{SF162} and HIV-$1_{89.6}$ as compared to control MDMs at day 11. There was a correlation between RT levels and cytokine responses (for TNF-α, $r = .61$, $P < .05$; and for IL-6, $r = .67$, $P < .02$). We conclude that viral infection increases the magnitude of inflammatory responses in MDMs after immune stimulation, which is proportional to the level of productive HIV-1 infection. These data confirm and extend our previous observations (Nottet *et al*, 1995).

Neuropathological analysis of SCID mice with HIVE

MDMs were infected with each of the viral strains (Table 1) as described above. Cells were counted and equal numbers of control uninfected or infected MDMs (3×10^5 in 5 μl) were stereotactically inoculated into mouse brains 6 days after infection with HIV-1_{ADA}, or 9 days after infection with the other strains. Mice were sacrificed after 1 week, and mouse brains were either formalin-fixed and paraffin-embedded for immunohistochemical analyses, or frozen at −70°C for RT-PCR (polymerase chain reaction) determinations.

Seven days after cell injection, HIV-1–infected MDMs (60 to 136 cells per 5-μm section) was found principally in the putamen and globus pallidus and, to a lesser degree, in the cortex (Table 2; Figure 2A, B, I, and J). Similar numbers of infected cells per section were found in the mouse brains (HIV-1_{ADA}, 61 ± 14.1; HIV-$1_{89.6}$, 78 ± 18.0; HIV-1_{DJV}, 92.6 ± 15.2; HIV-$1_{JR\text{-}FL}$, 88 ± 13.9; and HIV-1_{BAL}, 84 ± 27.7) (Table 2). Although HIV-1_{SF162} (115.6 ± 16.3) showed the highest numbers of infected MDMs, these differences were not statistically significant between any of the groups ($P > .36$). MDMs were commonly detected around microvessels (reflective of human HIVE) ~800 to 1200 μm from the injection site (Figure 2J). Dependent upon the viral strain, 34% to 89% of the detected MDMs expressed HIV-1 p24 antigen (Figure 2C, D, K, and L; Table 2). The greatest numbers of HIV-1 p24 stained cells were observed for HIV-1_{BAL} (89.7 ± 2.6), whereas HIV-$1_{89.6}$–infected cells showed the least (34 ± 4.3) (Table 2). Expression of p24 antigen was significantly higher in MDMs infected with HIV-1_{BAL} and lower in cells infected with HIV-$1_{89.6}$ as compared to other viral isolates ($P < .03$). HLA-DR antigen was expressed nearly equally in the infected and uninfected human MDMs (80% to 90%), indicating a state of MDM immune activation (Table 2).

Signs of reactive astrogliosis (accumulation and hypertrophy of astrocytes, increased immunostaining for glial fibrillary acidic protein [GFAP]) were observed in and around the site of MDM injection. The astrocyte reaction was more prevalent in mouse brains containing HIV-1–infected MDMs as compared to uninfected cells (800 to 1100 μm versus 400 μm, respectively) (Figure 2E–H, M–P). In order

Table 2 Neuropathological analysis of SCID mice with HIVE

	Control	*HIV-1$_{ADA}$*	*HIV-1$_{JR-FL}$*	*HIV-1$_{BAL}$*	*HIV-1$_{DJV}$*	*HIV-1$_{89.6}$*	*HIV-1$_{SF162}$*
MDM/section	64 ± 9.7	61.0 ± 14.1	88.0 ± 13.9	84 ± 27.7	92.6 ± 15.2	78.2 ± 18.0	115.6 ± 16.3
% HLA-DR	79.8 ± 5.4	83.0 ± 4.5	88.2 ± 3.3	75.9 ± 5.1	83.6 ± 1.6	80.1 ± 4.6	90.1 ± 2.1
% HIV-1 p24	0	62.2 ± 6.2	55.8 ± 7.6	89.7 ± 2.6	51.4 ± 2.3	34 ± 4.3	52.1 ± 6.4
Astrogliosis*	5.56 ± 1.31	18.65 ± 3.35	18.44 ± 6.11	16.16 ± 3.60	20.52 ± 9.97	8.50 ± 2.02	18.09 ± 5.67
Microgliosis**	4.69 ± 0.80	29.34 ± 8.85	17.07 ± 2.31	38.60 ± 10.79	34.34 ± 4.94	5.37 ± 1.07	17.32 ± 1.47

Note. Results were obtained in two separate experiments from two monocyte donors. Five-micrometer serial paraffin sections were used to assay number of MDM, astrocyte reaction, HLA-DR antigen per total MDMs, and HIV-1 p24 protein expression per total MDMs.
*Astrocyte reaction was calculated as area occupied by GFAP-reactive astrocytes divided by zone of occupied by CD68-positive MDMs using the computer image analysis.
**Microglial reaction was measured as area occupied by lectin-stained microglia divided by area occupied by MDMs by computer image analysis.

to quantitatively assess astrogliosis, we adjusted the astrocyte reaction for the amount of human cells by dividing the area occupied by GFAP-reactive astrocytes by the zone occupied by CD68-positive MDMs using the computer image analysis system (as described in Materials and Methods). A significant 3- to 4-fold increase in astrogliosis was found in mouse brains inoculated with HIV-1$_{ADA}$–, HIV-1$_{BAL}$–, HIV-1$_{DJV}$–, HIV-1$_{JR-FL}$–, and HIV-1$_{SF162}$–infected MDMs as compared to control ($P < .03$, Table 2). Although an augmented astrocyte reaction was found in mouse brains that received HIV-1$_{89.6}$–infected MDMs as compared to uninfected MDMs, it was neither significantly different from control ($P > .3$) nor from the other HIV-1 strains ($P > .1$). The astrocyte reaction correlated with numbers of MDMs found

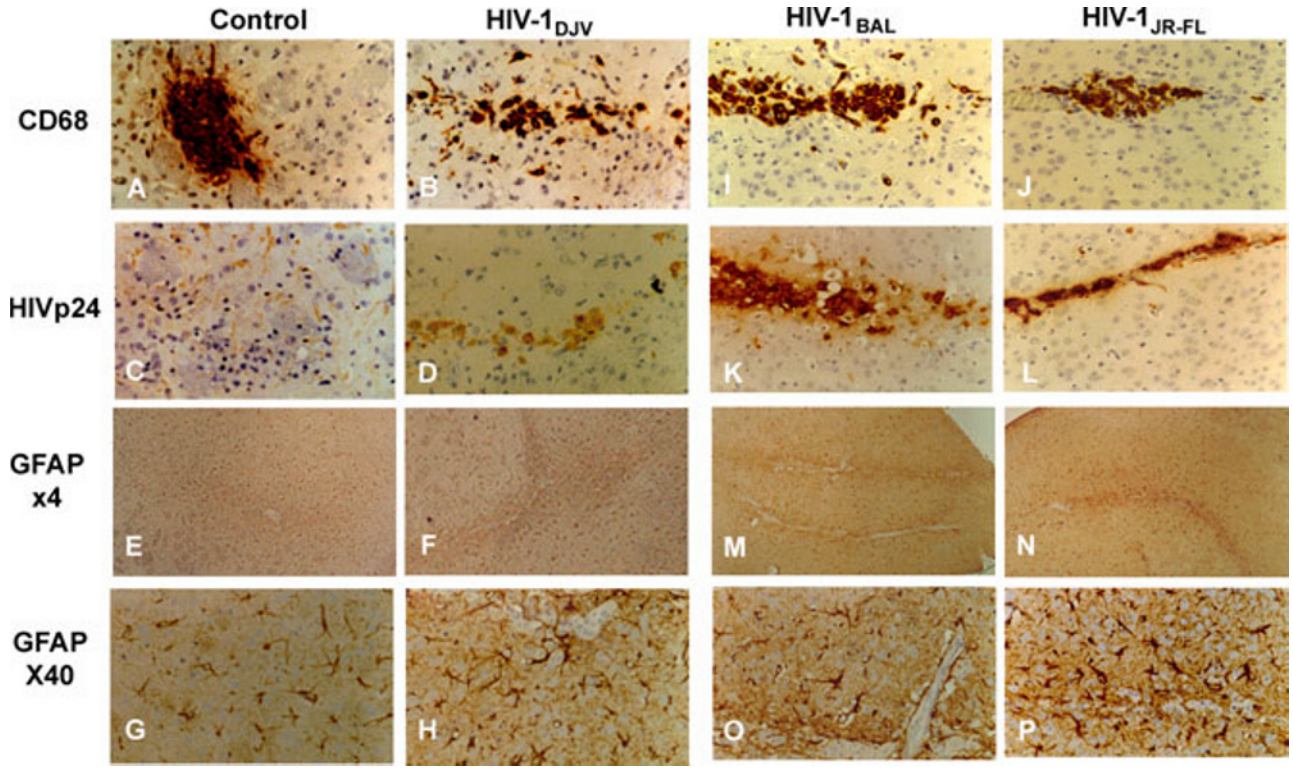

Figure 2 Morphological features of HIVE in SCID mouse brain (7 days after inoculation of human cells). Uninfected and infected vimentin-immunoreactive cells are widely distributed in putamen (**A**, **B**, **I**, **J**). Most virus-infected cells express HIV-1 p24 antigen (**D**, **K**, **L**) whereas uninfected show no immunoreactivity (**C**). Several HIV-1 p24-immunoreactive cells are located around microvessels (**L**). A pronounced and widely spread astrogliosis, as shown by GFAP immunostaining, surrounds HIV-1–infected cells (**F**, **H**, **M**, **N**, **O**, **P**), whereas only a moderate astrocyte reaction is found around uninfected cells (**E**, **G**). **A**, **C**, **E**, and **G** represent serial sections of mouse brain inoculated with uninfected MDMs. **B**, **D**, **F**, and **H** show serial sections of mouse brain inoculated with HIV-1$_{DJV}$–infected MDMs. **I**, **K**, **M**, and **O** are serial sections of mouse brain inoculated with HIV-1$_{BAL}$–infected MDMs. **J**, **L**, **N**, and **P** are serial sections of mouse brain inoculated with HIV-1$_{JR-FL}$–infected MDMs. **A** to **P** are serial coronal sections immunostained with antibodies to CD68 (**A**, **B**, **I**, **J**), HIV-1 p24 antigen (**C**, **D**, **K**, **L**), and GFAP (**E** to **H**, **M** to **P**). Tissue sections were counterstained with Mayer's hematoxylin. Original magnification, **A**, **B**, **I**, **J**, **C**, **D**, **K**, **L**, **G**, **H**, **O**, and **P**: ×200; **E**, **F**, **M**, and **N**: ×40.

in a given area, not with HIV-1 p24–positive MDMs where a trend existed but failed to reach statistical significance ($r = .74$, $P = .056$).

HIV-1–infected MDMs elicited a marked microglial response (by *Griffonia simplicifolia* lectin staining) around the injection site (data not shown). As in our previous experiments (Persidsky *et al*, 1996), uninfected MDMs induced microglial accumulation only in the immediate vicinity of the injection site. Microglial reaction was measured by image analysis as the percent area occupied by lectin-stained microglia divided by percent area occupied by CD68-positive MDMs. A statistically significant 3- to 8-fold increase in microglial reaction was detected in mouse brains injected with MDMs infected with HIV-1_{ADA}, HIV-1_{BAL}, HIV-1_{DJV}, HIV-$1_{JR\text{-}FL}$, and HIV-1_{SF162} as compared to control ($P < .03$; Table 2). Although HIV-1_{SF162}– and HIV-$1_{JR\text{-}FL}$– infected MDMs produced less intense microglial response as compared to the HIV-1_{ADA}–, HIV-1_{BAL}–, HIV-1_{DJV}–infected cells, these differences were not statistically significant. Microglia in brain tissue that received HIV-$1_{89.6}$–infected MDMs were significantly lower as compared to the other viral strains ($P < .02$), but were no different from uninfected controls ($P > .6$). A correlation and significant relationship was found between microgliosis (adjusted for amount of MDMs present in the given brain area) and level of infection (percentage of p24-positive cells) ($r = .83$, $P < .02$). There was a significant association between astrogliosis and microglial reaction ($r = .77$, $P < .05$).

Replicate mice inoculated with the same viral strains were sacrificed and RNA was extracted from the injected and contralateral (noninjected) hemispheres. RT-PCR was used to assess expression of mouse cellular and inflammatory factors as described previously (Persidsky *et al*, 1997). The expression of mouse GFAP was significantly higher in brains injected with HIV-1–infected MDMs than uninfected controls, supporting the findings by immunocytochemical tests ($P < .05$). Mouse TNF-α was significantly increased in mouse brains containing MDMs infected with HIV-1_{BAL}, HIV-1_{DJV}, and HIV-$1_{JR\text{-}FL}$, but not HIV-1_{SF162} or HIV-$1_{89.6}$ ($P < .05$). Although mouse brains inoculated with HIV-1–infected cells showed more mouse IL-6 than those with uninfected cells, the differences were not significant. This data suggested that the level of murine microglial activation was dependent upon strain replication dynamics in MDMs.

Discussion

This study examined whether qualitative and quantitative differences in MDM infection by divergent HIV-1 isolates correlate with the status of immune-activation in infected MPs and together influence the neuroinflammatory responses in the CNS. Our data support the concept that differences in productive infection (RT activity) correlate with the levels of immune-activation of infected MPs (TNF-α and IL-6 production) *in vitro*. Neuropathological analysis demonstrated that the level of microgliosis better correlated with percent of HIV-1 p24–positive MDMs than absolute numbers of MDMs in HIVE mouse brains. These results imply that the mere presence of MDMs in the CNS was necessary but not sufficient to modulate neuroinflammatory changes. Taken together, we propose that there may be a threshold of productive infection levels necessary to induce changes in the neuroinflammatory responses infected MDMs and that there is a direct correlation between level of MP immune activation and disease.

This study examined divergent HIV-1 isolates to determine the role of viral infection of MDMs as it may influence HIV-1 neuropathogenesis. The hypothesis is that neurotropism may be defined by the manner in which viral strains replicate in brain MPs and influence neurotoxic secretory activities that lead to damage or destruction of neurons. Based on this idea, the isolation of a particular viral strain from brain tissue may not necessarily mean that a specific virus is neurovirulent but simply neuroinvasive.

In attempts to decipher possible relationships between specific HIV-1 isolates and the pathogenesis of HAD, neuropathological alterations were examined in brain tissue of SCID mice after injection with MDMs infected with different strains of HIV-1. The results indicate that regardless of the viral isolate, HIV-1–infected human monocytes can persist and cause damage to the SCID mouse brain. Neuropathological abnormalities seen in the mice with HIVE were proportional to the number of MDMs in the brain and the ability of a given viral isolate to replicate in MDMs. Both appeared to be factors for inducing neuropathological changes as well as immune responses, but at different levels.

Although the initial study of Glass *et al* (1995) demonstrated that accumulation of brain macrophages appears to be a better correlate of dementia than levels of viral replication, subsequent reports (Cinque *et al*, 1998; Everall *et al*, 1999; McArthur *et al*, 1997) emphasized the importance of both activation/accumulation of MP and brain viral load for HAD and HIVE. Our *in vitro* data on cytokine secretion by virus-infected MDMs and quantitative neuropathological analysis of brain tissue of SCID mice inoculated with MDMs infected with different HIV-1 isolates may provide an explanation of how a given strain affects MDM neurotoxin production, astrogliosis, microglial reaction, and neuronal damage. Based on these data, we would predict that productive HIV-1 infection enhances secretion of TNF-α, a mediator for disease, in activated MDMs as compared to uninfected cells. Levels of productive infection correlated with TNF-α secretion and microglial reactions in this report ($r = .83$, $P < .02$). Microgliosis (adjusted for amount of MDMs present in the given

brain area) correlated with level of infection (percentage of p24-positive cells, $r = .83$, $P < .02$). Although there was a strong association between astroglial and microglial reactions ($r = .77$, $P < .05$), astrogliosis showed a clear trend but failed to reach statistical significance when compared with the numbers of HIV-1–positive cells ($r = .74$, $P = .056$). Based upon results of this study, we propose that there exists a threshold for levels of productive infection to elicit inflammatory responses upon exposure to secondary immune activation. This work underscores the importance of both the activated macrophage and productive viral infection for disease. Thus, neurovirulence may reflect viral abilities to induce macrophage secretory immune responses leading to neuronal injury and cognitive dysfunction.

Materials and methods

HIV-1 isolates

HIV-1_{BAL}, HIV-$1_{JR\text{-}FL}$, HIV-1_{SF162}, and HIV-$1_{89.6}$ were obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases (Ghorpade *et al*, 1998a). HIV-1_{DJV} was isolated by cocultivation of monocytes and brain tissue of a patient who died of HAD. HIV-1_{ADA} was isolated from the peripheral blood mononuclear cells (PBMCs) of an infected patient with Kaposi's sarcoma (Table 1).

Isolation and propagation of primary human monocytes

PBMCs were obtained by leukopheresis from individuals who were HIV-1, -2 and hepatitis B seronegative. Monocytes were isolated from PBMCs by countercurrent centrifugal elutriation. The isolated cells were >98% pure as determined by CD68 immunohistochemical assays, Wright staining, granular peroxidase, and nonspecific esterase. Monocytes were cultured in Dulbecco's modified Eagle's medium (DMEM), (Sigma, St. Louis, MO), supplemented with 10% heat-inactivated pooled human sera, 1000 U/ml highly purified recombinant human macrophage colony-stimulating factor (MCSF) (a generous gift from Genetics Institute, Cambridge, MA), 1% glutamine (Life Technologies, Grand Island, NY), 50 μg/ml gentamicin (Sigma), and 10 μg/ml ciprofloxacin (Sigma). All reagents used for tissue culture were screened prior to use and found to be free of endotoxin (<10 pg/ml; Associates of Cape Cod., Woods Hole, MA) and mycoplasma contamination (Gen-probe II; Gen-probe, San Diego, CA). Monocytes were cultured in the presence of MCSF 7 days before infection (Ghorpade *et al*, 1998a).

HIV-1 infection of MDMs

After 7 days in suspension, MDMs were infected with each of the HIV-1 isolates described. All viral inoculate were standardized for infection based on RT activity (1.4×10^6 cpm/10^6 cells) (Ghorpade *et al*, 1998a). Culture medium was half-exchanged every 3 days. The levels of infection were determined by measuring RT activity in culture supernatants or by immunostaining with HIV-1 p24 monoclonal antibody (mAb; Dako, Carpinteria, CA) on cytospin preparations.

TNF-α, IL-1β, and IL-6 detection

TNF-α, IL-1β, and IL-6 production was assayed by the Quantikine ELISA kits (R&D Systems, Minneapolis, MN) per manufacturer's instructions. Cells were stimulated with either LPS (1 μg/ml) obtained from *Escherichia coli* serotype 0111:B4 (Sigma) for 2 h, and washed 3 times with medium. Conditioned medium from unstimulated or LPS-stimulated cells was collected at 24 h and ELISA detected levels of cytokines. The levels of TNF-α, IL-1β, and IL-6 were normalized to cell numbers by measurement of cell viability determined by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. The extent of MTT conversion to formazan by mitochondrial dehydrogenase, indicating cell viability, was determined by measuring optical density at 490 nm using a microplate reader (Persidsky *et al*, 1999). The normalized values of cytokines per 10^5 cells were determined. These were used in statistical analysis by two-tailed Student's *t* test. Experiments were repeated 3 times with cells derived from three different donors. LPS-treated cells served to mimic the immune activation of macrophages that would occur in HAD.

Animals and intracerebral injections

Three-week-old SCID mice (male C.B-17/IcrCrl-*scid*BR) were purchased from Charles River Laboratories, Wilmington, MA. The animals were maintained in sterile microisolator cages under pathogen-free conditions in the Laboratory of Animal Medicine at the University of Nebraska Medical Center in accordance with ethical guidelines for care of laboratory animals set forth by the National Institutes of Health. Monocytes for mouse injections were cultured in suspension in 250-ml Teflon flasks (1.5×10^8 cells per flask) and infected with the HIV-1 isolates described above. Seven days after virus infection, the MDMs were centrifuged (1200 rpm for 10 min at 4°C) and suspended in serum-free DMEM (Sigma). Cytospins of both infected and uninfected cells were prepared. These were fixed in ice-cold acetone/methanol for 10 min and the percentage of HIV-1–infected cells was determined by immunostaining with HIV-1 p24 mAb. Prior to injections, mice were anesthetized with intraperitoneal ketamine/xylazine, and secured in a stereotaxic apparatus (Stoetling, Wood Dale, IL) designed specifically for mice (Persidsky *et al*, 1996). The animal's head was

secured with earbars and mouthpiece. Animals were inoculated into the putamen with 15 μl of serum-free DMEM containing 3×10^5 HIV-infected or uninfected cells. The mice were sacrificed after 1 week. Nine or 10 mice were inoculated with MDMs infected with each viral isolate or uninfected cells in two separate experiments.

Histopathology and immunohistochemistry

Brain tissue was collected at necropsy. Tissue for histochemical analyses was fixed in 4% phosphate-buffered paraformaldehyde and paraffin-embedded; while that for PCR was snap frozen in liquid nitrogen. After fixation, brain tissue was divided into five coronal blocks (approximately 1 mm) and paraffin-embedded. Ten sections (5 μm each) were cut from each block and stained with hematoxylin and eosin, or immunocytochemically labeled with Ab to GFAP and CD68 to identify the site of inoculation. When the inoculation site was established (usually in block 3), 45 extra serial sections were cut and stained with antibodies to HIV-1 p24 antigen, HLA-DR, and vimentin. Two adjacent blocks were also sectioned and stained in the same manner. This approach made it possible to analyze morphological alterations at specific distances from the site of injection of human MDMs.

The sections (5 μm) were incubated with primary antibody for 1 h at room temperature in a humidified chamber. Human MDMs were identified with CD68 KP-1 mAb (1:100 dilution; Dako) or vimentin mAb (1:25 dilution; Boehringer Mannheim). Activated MDMs were detected using mAb to human HLA-DR (1:25 dilution; Boehringer Mannheim). Mouse microglia were identified with biotinylated *G. simplicifolia* lectin-isolectin B4 (Vector Laboratories, Burlingame, CA; at 1:100 dilution). Polyclonal antibody against GFAP (1:1000 dilution; Dako) served for the identification of mouse astrocytes. The presence of viral proteins was shown with HIV-1 p24 mAb (1:10 dilution; Dako). Sections incubated with mouse immunoglobulin G as primary antibody or secondary antibody alone served as negative controls. After the incubation period, primary antibody was washed off and tissue incubated for 1 h with biotinylated secondary antibodies. After incubation with secondary antibodies the avidin-biotin immunoperoxidase (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) and then the chromogen, 3,3′-diaminobenzidine was added. The sections were counterstained with Mayer's hematoxylin. Immunostained tissues were analyzed with a Nikon Microphot-FXA microscope.

RNA isolation and RT-PCR

Mouse brain tissue was homogenized in TRIzol (1 ml per 10–100 mg of tissue) with a tissue homogenizer (Power Gene 125, Fisher Scientific, St. Louis, MO). RNA was extracted using chloroform (1/5 the volume of TRIzol) and precipitated with isopropanol (1/2 volume of TRIzol). The RNA pellet was then washed with 75% ethanol (equal volume as TRIzol) and dissolved in RNase-free water. The extracted RNA was treated with DNase (RNase-free RQ1 DNase; 1000 U/ml; from Promega) and then adjusted to 0.2 μg/μl. RNA samples were analyzed by PCR performed without the addition of reverse transcriptase using primers to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control assessing contaminating cellular DNA.

RNA was extracted from the whole injected and noninjected hemispheres of each mouse. RNA-PCR assays were performed to assess the levels of viral and inflammatory products in brain tissue injected with HIV-1–infected and uninfected monocytes. In the process, RNA was reverse transcribed using specific sense and antisense primers (Persidsky *et al*, 1997) and the cDNA was then amplified during 28 cycles of PCR. Coupled RT/PCR-amplified products were transferred to nitrocellulose membrane by Southern blot and hybridized with ^{32}P-labeled oligonucleotide probes. Hybridized products were visualized and quantified on a phosphorimager SF (Molecular Dynamics, Sunnyvale, CA) as described previously (Persidsky *et al*, 1997).

Computer image and statistical analyses

Reactive astrocytosis and microglial reactions were quantified as area occupied by GFAP-positive astrocytes or lectin-positive microglia divided by zone occupied by CD68-positive MDMs on serial coronal paraffin sections of brains injected with HIV-1–infected MDMs or uninfected MDMs for 1 week. Image analysis was performed as previously described (Persidsky *et al*, 1996) with a cooled CCD camera (Photometrics, Tucson, AZ) mounted on a Nikon Microphot-FXA. Digital images were analyzed with the Oncor Image V1.6 (Oncor, Gaithersburg, MD) computer image system. The scanned zone covered 1000 μm medially and laterally from the needle track on coronal sections. GFAP-immunostained (astrogliosis) or lectin-stained areas (microglial reaction) were expressed as a percentage of the total brain area assayed. Two-tailed unpaired *t* tests, two-way analysis of variance (ANOVA), and linear correlations were performed using InStat 1.12 software.

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