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Human microglial cell isolation from adult autopsy brain: Brain pH, regional variation, and infection with human immunodeficiency virus type 1

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Microglia are the main source of productive infection by human immunodeficiency virus type 1 (HIV-1) in the central nervous system (CNS). Infection of microglia is difficult to study because nonhuman microglia are not infected by HIV-1, adult human microglia from surgically removed brain tissues are scarce, and fetal human microglial cells differ from adult cells in potentially important ways. Adult autopsies are a potential source of brain tissue for HIV-1 research, but the technique of isolating and infecting cells postmortem is not completely standardized. The authors determined optimal conditions for isolating and infecting adult microglial cells using 18 adult autopsy brain specimens from HIV-1–infected and noninfected decedents. The yield of mixed glial cells overall was on average 0.5×10^6 cells per gram of wet tissue. There was no correlation between the number of microglia isolated and the postmortem interval (PMI), HIV seropositivity, age, or gender. Brain pH accounted for about 41% of yield variability; a pH of less than 6.0 generally was not compatible with adequate cell recovery. The highest microglial cell yields were derived from anterior brain sectors (frontal lobe and temporal lobe) versus occipital lobe and cerebellum. A PMI of up to 25.5 h produced excellent cell yields in frontal lobe samples with high brain pH. HIV-1 infection of frontal lobe microglia was 100% successful using both CXCR4- and CCR5-tropic strains of HIV-1. With proper selection of cases and brain region, autopsy brain specimens are a dependable source of viable microglial cells to study CNS HIV-1 infection. *Journal of NeuroVirology* (2003) **9,** 346–357.

Keywords: HIV-1 infection; microglia; postmortem interval; tissue pH

Introduction

Microglial cells are the main reservoir of productive infection by human immunodeficiency virus type 1 (HIV-1) in the brain (Bagasra *et al*, 1996; Fischer-Smith *et al*, 2001; Porwit *et al*, 1989; Watkins *et al*, 1990). HIV-1 infection of microglia is considered to be critical for HIV-associated dementia (HAD) (Glass *et al*, 1995; Williams and Hickey, 2002) and is a prominent feature of the encephalitis caused by HIV brain infection observed at autopsy (HIVE) (Budka *et al*, 1991). An autopsy diagnosis of HIVE is associated strongly with HAD (Wiley and Achim, 1994), and preliminary data from six autopsies has suggested that brain HIV RNA concentration is positively correlated with HAD (McClernon *et al*, 2001). Other central nervous system (CNS) cell types, including astrocytes, endothelial cells, and possibly neurons, could also be infected with HIV-1 or involved with HAD, but macrophages and microglia are considered to be the predominant reservoir of infection in the brain (Bagasra *et al*, 1996) and can lead to HAD. Dementia and milder neurocognitive

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deficits are believed to arise from microglial cell secretion of neurotoxins and proteases, and/or shedding of neurotoxic virus proteins such as gp120, Tat, and Nef (Gelman *et al*, 1997; Giulian *et al*, 1990; Nath and Geiger, 1998).

Potential sources of human brain tissue used for microglia isolation and culture include human temporal lobes surgically resected to treat epilepsy, fetal brain tissue, and autopsy brain specimens (De Groot *et al*, 2000; Gonzalez-Scarano *et al*, 1997; Kim, 1985; Kim *et al*, 1983; Williams *et al*, 1992; Yong and Antel, 1997). Microglia isolated from surgically resected temporal lobe are difficult to obtain, and it is not known if cells from an epileptic focus of brain are comparable to normal tissue. Fetal human brain tissue is a plentiful source of microglia, but there is some evidence that these cells may be functionally different from adult microglia. For example, fetal microglia express higher levels of adhesion molecules than do adult microglia, and many surface molecules expressed on fetal microglia are down-regulated as they develop into adult resting cells (Rezaie *et al*, 1997; Rezaie and Male, 1999). Fetal astrocytes and/or their secreted products are required for fetal microglia cell differentiation (Liu *et al*, 1994; Rezaie *et al*, 2002), factors that must be taken into account when using cultured fetal cells as a source of microglia. Autopsy brain specimens are a potential source of human cells for research, but concerns about long postmortem intervals (PMIs) and the possibility of low yields of viable cells have tended to limit their widespread use for research (Yong and Antel, 1997). Having more tissue available from a postmortem specimen can compensate for a lower cell yield at autopsy, but the potential importance of the PMI in autopsy brain cell isolation remains unclear. There is some indication that the correlation between glial cell yields and PMI is not strong (De Groot *et al*, 2001), but the precise conditions that produce good glial cell yields at autopsy remain to be elucidated. In addition, the infectivity of HIV-1 for microglia derived from postmortem brains needs to be established in order to use this resource in neuro–acquired immunodeficiency syndrome (neuroAIDS) research.

The objective of this research was to refine the technique of microglial cell isolation at autopsy as it applies to studies of brain HIV-1 infection, and define the conditions that lead to successful *in vitro* HIV-1 infection. Here, data from 18 autopsy brains show that postmortem brain cell isolates were phenotypically and functionally microglial cells and were infected consistently with HIV-1. Two newly recognized factors were correlated strongly with microglial cell yields: brain pH and the region of brain that was sampled. In contrast, postmortem delay was not a critical factor within the first 24 h of death. Measuring brain tissue pH at the time of death serves as a rapid screen for potential microglial cell viability. Specimens that have a poor likelihood of yielding useful material can be quickly excluded from consideration, leading to improved overall success rates.

Results

Cell yields of glial cells and purified microglia

To test the feasibility of using adult brain specimens at autopsy as a reliable source of microglia, autopsy cases from 12 HIV-positive and 6 HIV-negative individuals were used. Table 1 shows the characteristics of these cases. The method of Yong and Antel (1997) was used to isolate glial cells. Table 1 shows

Table 1 Characteristics of HIV-positive and uninfected cases used in this study

HIV status	*PMI (hours)*	*Age*	*Gender*	*Neuropathologic findings*[◦]	*Neurocognitive diagnosis*
Negative	10	72	Male	Moderate cerebral atrophy (congestive heart failure)	Not tested
Negative	20	69	Male	Mild cerebral atrophy (cardiac arrest)	Not tested
Negative	4	59	Male	Alzheimer type II gliosis (cirrhosis)	Not tested
Negative	24	34	Male	Normal (Hodgkin's/cardiac arrest)	Not tested
Negative	6	54	Female	Old necrosis and gliosis in cerebellum (heart disease)	Not tested
Negative	22.5	51	Female	Normal (necrotizing cellulitis)	Not tested
Positive	24	43	Male	Normal	NPI-O[a]
Positive	5	46	Female	Normal	MCMD[b]
Positive	11.5	37	Male	Normal	MCMD[b]
Positive	5	53	Male	Alzheimer type II gliosis and hydrocephalus ex vacuo	NPI-O[a]
Positive	25.5	41	Male	Normal	Not tested
Positive	2.75	43	Male	Alzheimer type II gliosis	Subsyndromic
Positive	13.5	36	Male	Progressive multifocal leukoencephalopathy	NPI-O[a]
Positive	15	49	Male	Alzheimer type II gliosis	NPI-O[a]
Positive	13.6	32	Male	HIV encephalitis	HAD[c]
Positive	11	31	Male	Normal	MCMD[b]
Positive	5.3	56	Male	Mild atherosclerosis	HAD[c]
Positive	27.3	42	Male	Normal	MCMD[b]

[◦]Cause of death for HIV-negative cases is shown in parentheses.
[a]Neuropsychologically impaired due to cause other than HIV-associated dementia.
[b]Minor cognitive/motor disorder.
[c]HIV-associated dementia.

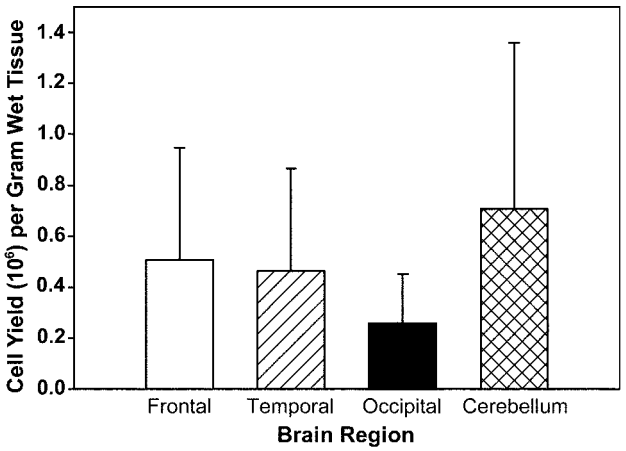

Figure 1 Viable glial cell yields vary with different brain region. Yields from occipital lobe were consistently lower than from the other regions. Cell yields are calculated as the number of total viable cells (including astrocytes, oligodendrocytes, and microglia) per gram of wet tissue. There was a wide standard deviation and the difference between the groups was just out of range for being statistically significant (ANOVA; $P = .06$).

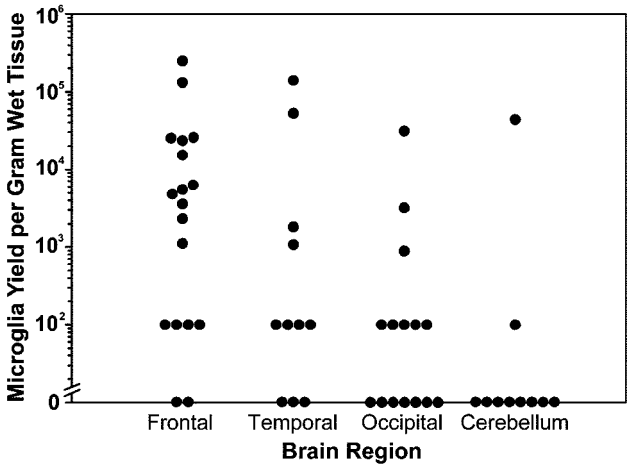

Figure 2 Viable microglial cell yields from frontal lobe were more consistent than from other regions, although the average yield for both frontal lobe ($n = 18$) and temporal lobe ($n = 11$) was approximately $0.5 \circ 10^5$ microglia. Cerebellum ($n = 10$) yielded the highest average number of glial cells obtained initially, but the yield of viable microglia was dramatically reduced compared to frontal lobe and temporal lobe. Average microglial cell yields from the occipital lobe ($n = 15$) were the lowest.

the National NeuroAIDS Tissue Consortium (NNTC) neurocognitive classifications for the HIV-positive cases, which demonstrate that several degrees of neurocognitive impairment were represented in this series. We successfully isolated frontal lobe microglia from every group listed.

Differences in the number of viable glial cells obtained from frontal lobes, temporal lobes, occipital lobes, and cerebellum are shown in Figure 1. The gross cell isolates obtained after Percoll separation contain microglia, astrocytes, and oligodendrocytes prior to placing them in culture. Cell viability was checked using trypan blue dye exclusion. Freshly isolated cells were composed of 10% to 40% glial fibrillary acid protein (GFAP)-positive cells (astrocytes), 13% to 30% CD11b/c-positive cells (microglia), and 33% to 66% 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase)-positive cells (oligodendrocytes). There was wide variation in yields, and so differences between brain region was statistically marginal (analysis of variance [ANOVA]: $F = 2.78$; $P = .06$). Per gram of wet tissue, frontal and temporal lobes averaged $0.5 \circ 10^6$ viable cells, cerebellum yield averaged $0.7 \circ 10^6$ cells, and occipital lobe yield averaged $0.3 \circ 10^6$ cells. Adherent microglial cells were separated from astrocytes by differential trypsin treatment and enumerated, and yields for each region are shown in Figure 2. The number of viable microglial cells depended on the brain region; highest yields were from the frontal lobe and ranged up to $2.5 \circ 10^5$ cells per gram of wet tissue. Yields from temporal lobe averaged $1.4 \circ 10^5$ cells per gram of wet tissue, and yields from occipital lobe and cerebellum were the lowest. All 18 cases were used in the analysis of frontal lobe, but fewer cases were used for the other regions as it became apparent that they yielded fewer viable microglia (see Figure 2).

Factors that influence microglial cell yields from autopsy brains

To investigate the factors that may predict microglial cell yield from a tissue specimen, we examined several factors as shown in Table 1. The percentage of viable microglia obtained from total viable cells is shown in Figure 3 as it relates to PMI, age, gender, and the HIV status of the patient. There was no correlation between the percentage of microglia cultured and the PMI (Figure 3**A**). Autopsy material up to 25.5 h postmortem often produced substantial yields of microglial cells. There was no apparent linkage between microglial cell yield and age, gender, or HIV status (Figure 3**B**, **C**, **D**). Because the postmortem delay in the first 24 h was not critical to microglial cell yields, we pursued the hypothesis that antemortem factors might be important. Specifically, it is known that terminal hypoxia, acidosis, and low brain pH lead to postmortem instability of brain mRNAs and proteins (Bahn *et al*, 2001; Johnston *et al*, 1997; Kingsbury *et al*, 1995; Yates *et al*, 1990). A low brain tissue pH is associated with an agonal death versus sudden death, and brain lactic acid concentration correlates strongly with low pH (Yates *et al*, 1990). Brain tissue pH is stable after death, and is not related to PMI or the length of time tissue is stored frozen (Harrison *et al*, 1995). We performed brain pH measurements, and found that high pH was associated strongly with increased recovery of frontal lobe microglia ($r = .64$, $P = .004$) (Figure 4**B**). Figure 4**A** shows that frontal lobe tissue pH below 6.0 sharply reduced the chances of having acceptable cell yields. A brain pH above 6.25 almost always produced microglial cell yields that were in the useful range. No apparent covariation between brain pH and PMI was

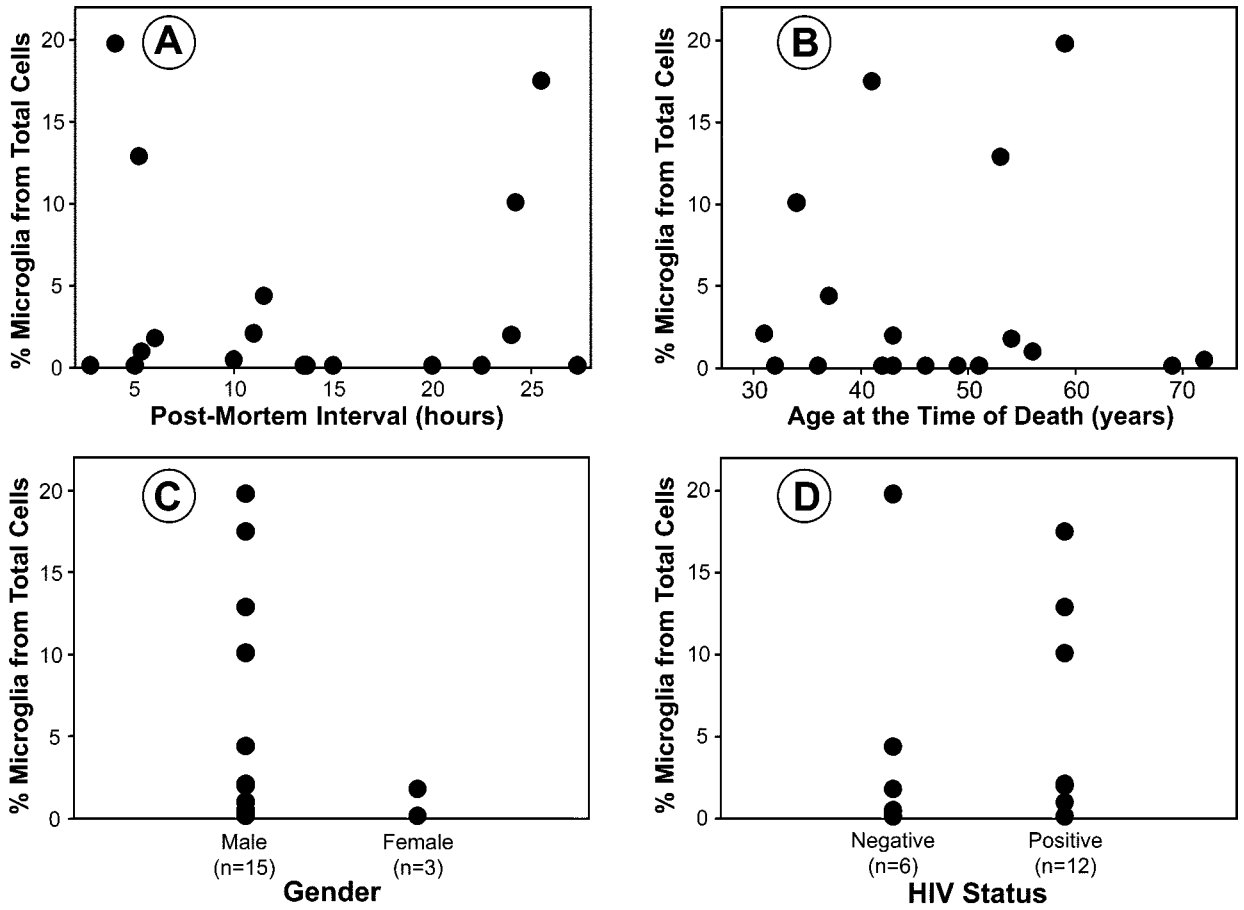

Figure 3 The percentage of viable frontal lobe microglia obtained is not dependent upon PMI (**A**), age at the time of death (**B**), or HIV status (**D**). There were too few female cases to exclude gender (**C**). These cases are described in Table 1.

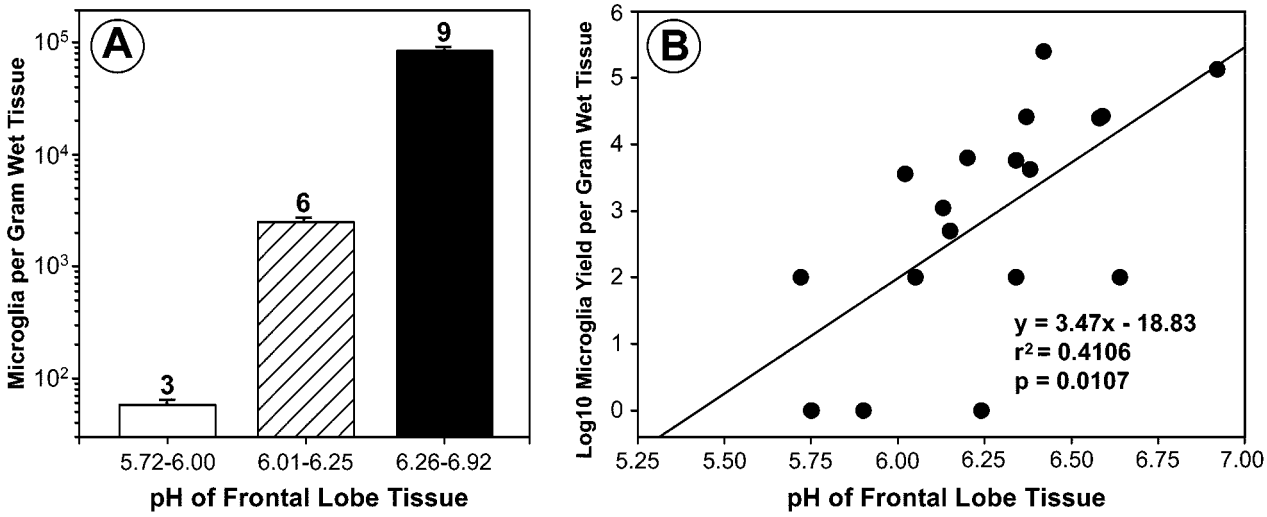

Figure 4 Higher tissue pH increases microglia cell yield from frontal lobe. The effect of pH was examined on previously frozen post-mortem frontal lobe tissue. The number of cases for each pH range is shown above the bar in (**A**). A tissue pH of 6.0 or below resulted in a reduction of microglia compared to a tissue pH of 6.01 to 6.25. If the tissue pH was 6.26 or higher, the average microglia yield was increased 27-fold over the pH 6.01 to 6.25 group. Linear regression analysis using the "least squares" method (**B**) revealed a trend that a higher pH resulted in better recovery of frontal lobe microglia and was independent of the PMI (see Figure 3**A**).

evident. For example, a very brief PMI (2.75 h) was associated with a low microglial cell yield when the decedent had lactic acidosis prior to death and a brain pH of 6.05. In reviewing the autopsy reports, it was evident that decedents with the lowest brain pH values tended to have pulmonary dysfunction or hypoxemia, which causes acidosis.

Purified microglial cells exhibited appropriate phenotypes, engaged in phagocytosis, and were activated by endotoxin

To verify that cells isolated from autopsy brain tissue were phenotypically consistent with microglia, cells were grown for up to 4 weeks in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF), which changed the shape of the cells from amoeboid to ramified (Figure 5**A**). The addition of GM-CSF to the culture media from the outset increases the proliferation rate of microglia over monocyte-derived blood macrophages (Ganter *et al*, 1992; Guillemin *et al*, 1997; Lee *et al*, 1994). Cells were stained for complement receptors CD11b/c, as well as various leukocyte markers, including CD68, CD14, and HLA-DR. Figure 5**B** shows CD11b/c-positive microglia with a GFAP-positive astrocyte (*arrow*) in a mixed culture prior to trypsinization. Following differential trypsinization to separate microglia and astrocytes, microglial cultures were greater than 95% pure as judged by CD11b/c immunostaining. Microglial cell isolates also stained positively for CD68, HLA-DR, and CD14 after 1 to 4 weeks in culture with added growth factors, although staining was less strong than with antibodies to CD11b/c. Freshly purified microglial cultures (after 1 day in culture) were immunostained using only CD11b and CD11c antibodies.

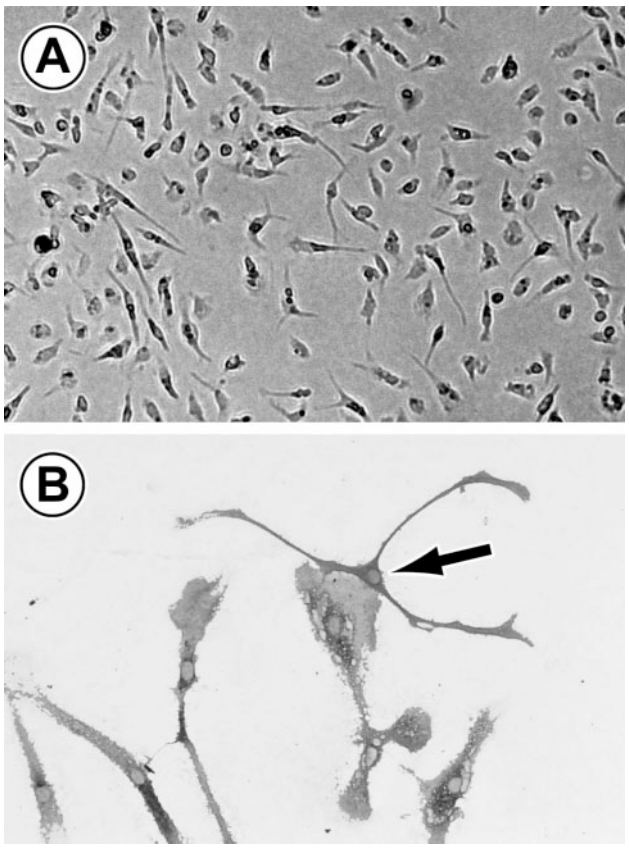

Figure 5 Cellular morphology and immunocytochemistry. To verify that cells isolated from autopsy brain tissue were microglia, cells were grown for up to 4 weeks in the presence of GM-CSF, which changed the shape of the cells from amoeboid to ramified (**A**). (**B**) CD11b/c-positive microglia with a GFAP-positive astrocyte (*arrow*) in a mixed culture prior to trypsinization.

Microglia from postmortem human brain exhibit phagocytic activity using the latex bead method (Oda and Maeda, 1986). Figure 6 illustrates a microglial cell that has engulfed latex beads (Figure 6**A**) along with separate staining for the cell nucleus (Figure 6**B**). Nonphagocytic Vero cells did not phagocytose the beads (data not shown). Viable microglia should also secrete cytokines upon stimulation with lipopolysaccharide (LPS), so we stimulated microglia in culture and measured the supernatants for secreted interleukin (IL)-6 and tumor necrosis factor (TNF)-μ. A representative experiment is shown in Figure 7. IL-6 and TNF-μ were found in supernatants from cells stimulated with LPS only, and secretion was enhanced by the addition of GM-CSF. Untreated cells did not secrete detectable TNF-μ and IL-6. In the GM-CSF–only group, TNF-μ was not detected and a small amount of IL-6 was usually present.

Purified microglial cells from both HIV-seronegative and -seropositive cases were infectable with HIV-1 in vitro

Figure 8 illustrates that primary microglial cell cultures from human autopsy brain can be infected consistently with HIV-1 *in vitro*. A "feeder" layer of other glial cells types was not necessary to establish or maintain the infection. We were able to infect viable microglia with HIV-1 isolate JR-CSF in 11 out of 11 cases, regardless of brain region. Seven of the 11 cases were from HIV-seropositive individuals. Secreted HIV p24 was never detected in "control" cultures of microglia obtained from HIV-seropositive cases; p24 was detected in tandem wells that had been infected *in vitro* with HIV. JR-CSF envelope protein binds preferentially to the CCR5 coreceptor for cell entry, and because coreceptor usage is important for strain variation, we also examined HIV-1 strain IIIB, in which the envelope protein binds preferentially to the CXCR4 coreceptor. Five cases were infected with JR-CSF and IIIB separately. JR-CSF produced more HIV-1 core protein p24 than IIIB in two of the cases (Figure 8**A**); in two other cases, HIV IIIB infection produced more p24 than for JR-CSF (Figure 8**B**). In one case, p24 levels in the JR-CSF culture were initially highest, but by day 21, the IIIB culture had become higher (not shown). Cultured microglia secreted p24 for up to 11 weeks, the longest time cells were cultured. Thus, HIV-1 varied from

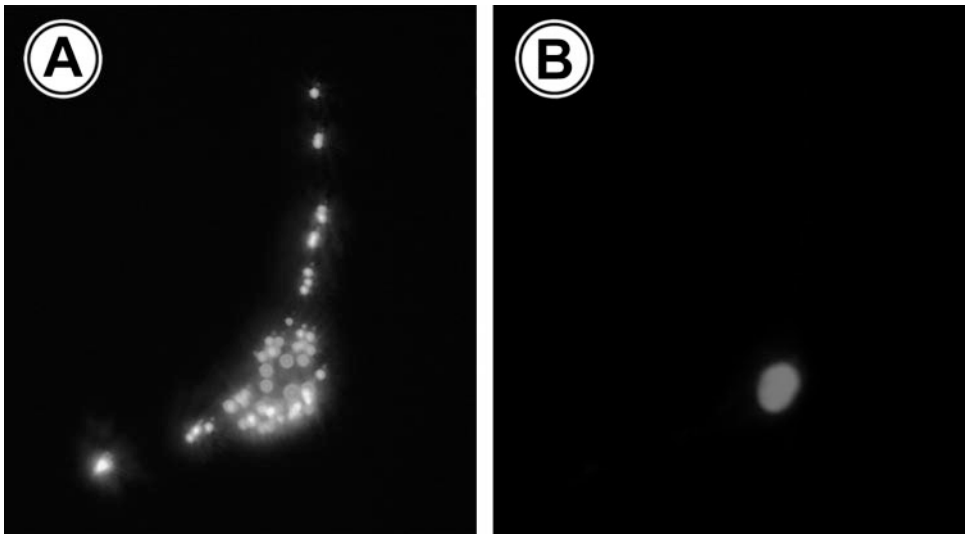

Figure 6 Phagocytic activity. Microglia from postmortem human brain exhibit phagocytic activity using the latex bead method. (**A**) A microglial cell that has engulfed latex beads. (**B**) A propidium iodide nuclear counterstain for the microglial cell shown in **A**. Nonphagocytic Vero cells treated with an identical mixture of beads and incubated in the same way as the microglial cultures. These cells did not phagocytose the fluorescent beads (data not shown).

subject to subject with respect to strain susceptibility that relates primarily to coreceptor utilization. Regardless of strain preference, HIV-1 infection was uniformly persistent in all the cultures.

Discussion

This work establishes the feasibility of using human autopsy specimens to obtain primary microglial cell cultures for HIV-1 research. Having new sources of human brain cells is important to the field, because alternate sources of human brain cells are not available to many investigators. Fetal human tissue is a dependable source of cells, but there are ethical considerations associated with using fetal tissue that can restrict the availability of that resource (Rojansky and Schenker, 1993). The use of fetal microglia as a model for adult CNS infection also can present scientific problems because the pathology of HIV disease in the CNS of children is not identical with that of adults (Epstein and Gelbard, 1999; Mintz, 1994; Sanchez-Ramon *et al*, 2002). For example, children are not susceptible to vacuolar myelopathy (Dickson *et al*, 1993). Fetal cells may not be equivalent to adult cells because stages of maturation may be important in HIV-1 infection (Lee *et al*, 1993). Fetal microglia use CXCR3 and CCR5 as coreceptors for HIV-1 (He *et al*, 1997), whereas the preference for CCR3 coreceptor utilization by HIV in adult temporal lobe microglia is not observed (Shieh *et al*, 1998). Many studies using fetal tissue rely on morphology and CD68 staining as the sole means of comparison between adult microglial cells and differentiated fetal microglia, which may not be adequate in describing differences between the two. Microglia from adult brains obtained at autopsy may produce a more homogeneous population of mononuclear phagocytes relative to fetal microglia, which are a heterogeneous and less differentiated population of cells (Rezaie *et al*, 1997).

Surgically resected temporal lobe tissues are also used to culture human microglia, but not all investigators have access to this type of material. There is concern that microglia from epileptic brains are not normal, and there is some evidence to support this. Cystatin C, a lysosomal cathepsin inhibitor, is expressed mainly in microglia of normal brain. In a rat model system of temporal lobe epilepsy induced by amygdala stimulation, cystatin C immunostaining was increased and correlated with the severity of neuronal damage (Lukasiuk *et al*, 2002). Seizures also affect cytokine secretion, which alters microglia proliferation (Jankowsky and Patterson, 2001). Cell yields from surgical specimens are reported to be higher than for autopsy brain, but larger amounts of starting tissue can be obtained at autopsy compared to surgical tissue.

Murine macrophages and microglia cannot be directly infected with HIV-1 unless transgenic or SCID mouse systems are used (Persidsky *et al*, 1995). Although neuropathology in these systems has been examined (Zink *et al*, 2002), it is difficult to study neurocognitive functions that contribute to HAD. In addition, rodent microglia differ from human microglia in important physiologic ways, such as their expression of nitric oxide in response to inflammatory stimuli and their proliferation to macrophage colony-stimulating factor (Walker *et al*, 1995). Nonhuman primates are required for the simian immunodeficiency virus model, and their high cost precludes them from use by many researchers.

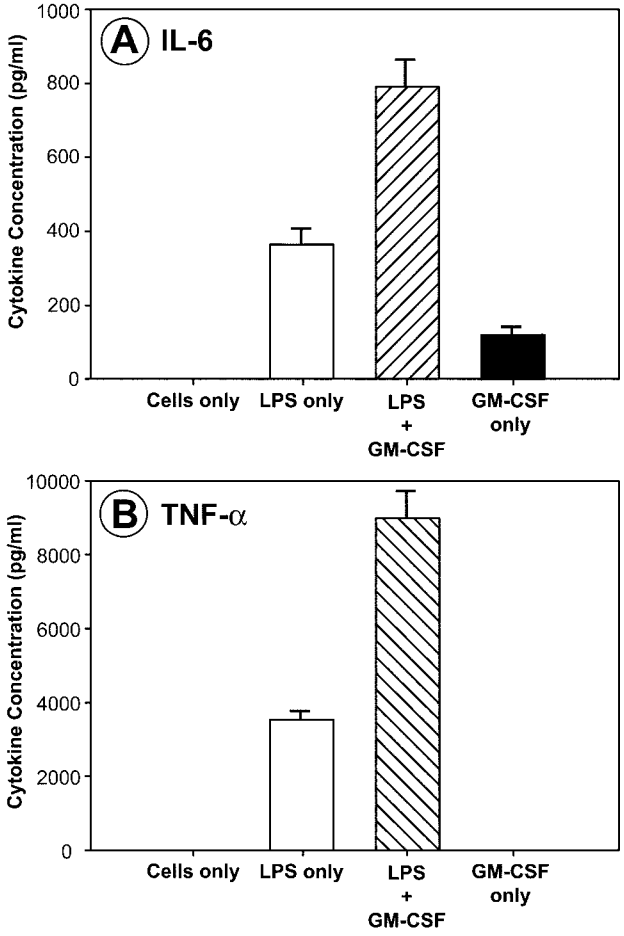

Figure 7 Cytokine secretion by cultured microglial cells. A representative experiment showing microglial secretion of the cytokines IL-6 and TNF-μ following stimulation with LPS, a well-known activator of macrophages and microglia. (**A**) IL-6 secretion was measured in cytokine supernatants by ELISA after 24 h. (**B**) TNF-μ secretion measured in the same cultures by ELISA after 40 h. The basal level of secretion of TNF-μ and IL-6 in the presence of GM-CSF was examined because microglia are routinely cultured in media containing this growth factor. Cells were stimulated in triplicate wells.

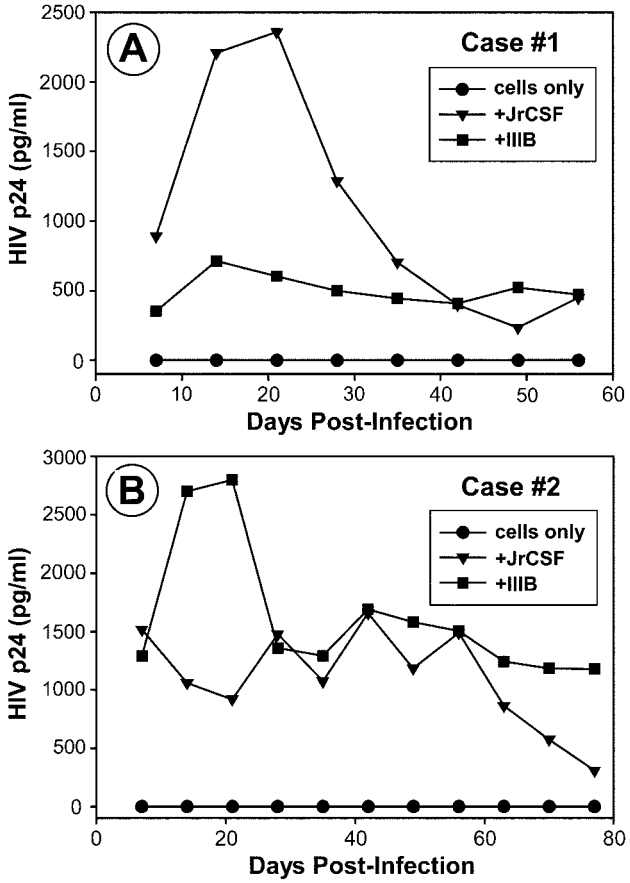

Figure 8 Microglia from human autopsy brain can be infected with HIV-1. Microglial cells were exposed to isolates of HIV-1 overnight. Free virus was washed away and cells were fed in Feeding Medium. Culture supernatants were collected every week and replaced with fresh Feeding Medium. A total of 11 JR-CSF infections were done that resulted in a productive infection (as measured by p24 ELISA) in all 11 cases. A total of five IIIB infections were done, and all five resulted in a productive infection. (**A**) A representative infection in which the R5-tropic (JR-CSF) strain resulted in higher p24 secretion than with the IIIB strain. (**B**) A representative culture in which the X4-tropic (IIIB) yielded higher p24 secretion. A productive infection was obtained for a number of weeks with both strains. Microglia from all lobes tested (frontal, temporal, and occipital) were infectable with HIV-1; cerebellum was not done due to a lack of viable cells. Case 1 was a 53-year-old HIV-positive male and case 2 was a 34-year-old HIV-negative male (see Table 1).

Human brains are a desirable choice for studying HIV-1 infection of CNS cells *in vitro*. We have refined the technique of isolating human microglia obtained at autopsy, and establish that these cells can be consistently infected with HIV-1. Good yields of viable mixed glial cells, including astrocytes and oligodendrocytes, also can be obtained from autopsy brain specimens. Contamination of cultured cells with microorganisms was negligible if due attention was paid to using sanitary techniques in the autopsy suite. Using repetitive sterile rinses of whole and minced brain tissue, and including antibiotics and antifungals agents in the isolation procedure, minimized biological contamination. Under these conditions, a "rapid" autopsy protocol was not required to achieve consistent results. Indeed, there was little or no apparent correlation between the postmortem time, age, gender, or HIV status of the patient and the number of viable microglial cells recovered. The yield of microglia from HIV-negative individuals was equivalent to that of end-stage AIDS patients. The lack of correlation in microglial yields with PMI, age, or Alzheimer's disease was described previously (De Groot *et al*, 2001). Indeed, viable microglial cells could be isolated from autopsy brains more than 24 h postmortem. It is worth noting that for cases that were analyzed more than 15 h postmortem, the body always was refrigerated prior to autopsy.

In addition to finding that the PMI within 24 h of death was not correlated with microglial cell yield, we have demonstrated for the first time that brain pH and brain region together can explain a large part

of the variability between cases. Frontal lobe always was the most suitable region for microglial cell isolation, and temporal lobe also produced acceptable cell yields. More posterior sectors of the brain (i.e., cerebellum and occipital lobe) produced relatively poor microglial cell yields. We suspect that the difference in results due to region is probably associated with the environment of the cells postmortem (versus intrinsic differences in microglial cells between regions). When the decedent lies in the supine position after death, posterior sectors are exposed to blood pooling due to gravity; draining of blood from the anterior sectors decreases blood exposure. What precisely happens when the posterior microglia are exposed to blood pooling is not clear. A lower pH does not entirely explain the phenomenon, because brain pH of posterior sectors in a few cases tested was above 6.4 (data not shown). Whatever the mechanism for regional variation is, it is clear that occipital lobe and cerebellum do not produce consistently good results and should be avoided.

We have explained a very substantial proportion of the variability of microglial cell yields at autopsy by measuring the brain pH. Acidosis and low pH had a striking negative influence. Our findings are analogous to results that indicate that postmortem brain mRNA and protein stability are both decreased when the brain pH is low (Harrison *et al*, 1995; Johnston *et al*, 1997; Kingsbury *et al*, 1995). Low brain pH strongly correlates with increased brain lactic acid concentration (Yates *et al*, 1990); these changes are believed to be established antemortem during an agonal death, during which respiratory or metabolic acidosis is likely to have occurred. In contrast, a sudden death is associated with higher brain pH and more stable postmortem brain mRNA and protein products (Harrison *et al*, 1995; Johnston *et al*, 1997). That principle seems also to apply to microglial cell viability at autopsy. In practice, measuring the brain pH level prior to attempting cell isolation serves as an effective screen. A brain pH less than 6.0 had almost no chance of producing viable microglia, whereas a pH greater than 6.25 consistently predicted an acceptable frontal lobe yield. In contrast, the PMI provided very little predictive information regarding microglial cell yield.

Primary microglia isolated from four regions (frontal lobe, temporal lobe, occipital lobe, and cerebellum) all expressed CD11b/c. Frontal lobe cells stained positively with antibodies to CD14, CD68, CD45, and HLA-DR, characteristic of microglial cells, although the staining was less intense than with CD11b/c. There was no observed difference in the phenotype of microglial cells from control cases or HIV-positive cases. The aim of this study was not to elucidate the phenotypic profile of adult microglia in HIV disease, but it is notable that distinction between parenchymal microglia and perivascular macrophages in the CNS (Williams and Hickey, 2002) may be important in the neuropathogenesis of HAD. It is suggested that most productive HIV-1 infection in the brain occurs in the perivascular cells (Fischer-Smith *et al*, 2001); we did not distinguish between these two cell types in our isolates.

A final important finding is that these microglial cell isolates are infectable with HIV-1 and useful for neuroAIDS research. We were successful at infecting all (100%) isolates with HIV JR-CSF or IIIB. HIV JR-CSF is a CNS-derived clone from a patient with AIDS dementia (Boyd *et al*, 1993; Cann *et al*, 1990; Koyanagi *et al*, 1987) and utilizes the CCR5 coreceptor with CD4 for virus entry; HIV IIIB is a blood-derived prototypic laboratory isolate (Collman *et al*, 1989; Popovic *et al*, 1984) that utilizes the CXCR4 coreceptor. Both strains of virus can replicate within microglia, although they exhibit variable tropism using infant and temporal lobe microglia (Ioannidis *et al*, 1995; Strizki *et al*, 1996). R5-tropic isolates are associated with most primary CNS infections (Strizki *et al*, 1996; Watkins *et al*, 1990). In this limited survey of *in vitro* infections, it was difficult to establish the HIV-1 strain preference of the cells from every donor. Of the 11 cases in which we attempted to infect microglial cell cultures, 7 specimens were from HIV-positive people with AIDS. In those patients, it is possible that some of the productive *in vitro* infections could have arisen due to activation of latent provirus. Two factors argue against that possibility. First, negative-control cultures (microglia only, no virus) that were run in parallel never produced any detectable p24. The lowest calibration standard for detection in the p24 enzyme-linked immunosorbent assay (ELISA) assay was 7.8 pg/ml. Second, in 4 cases we attempted but failed to isolate any native HIV-1 using a coculture containing microglia and phytohemagglutinin-stimulated peripheral blood mononuclear cells as donor cells. No HIV p24 could be detected after 6 weeks in culture. In one attempt, purified CD4-positive donor cells were used, again with negative results. The viral burden in the cerebrospinal fluid of these four cases was never high (ranging from 446 to 5968 HIV RNA copies/ml), so it remains possible that native HIV-1 provirus could be an important factor when brain HIV loads of the donor are high. A productive infection by HIV is not required to induce the inflammatory cytokines TNF-μ and IL-1 in microglia (Merrill *et al*, 1992), and it is possible that these cytokines could enhance the recovery of "latent" virus from microglia isolated from HIV-seropositive brain tissue. We will explore this possibility by identifying the genotype of the virus we detected in *in vitro* infections where the microglia were isolated from HIV-seropositive individuals.

Factors that might contribute to HIV infectivity of postmortem brain samples include donor gene variability, passage history of the viral isolates, the stage of cellular maturation (in the case of fetal cells), or the state of activation of the microglia (Albright *et al*, 2000; Ghorpade *et al*, 1998; Shieh *et al*, 1998; Strizki *et al*, 1996).

Materials and methods

Brain tissue specimens
Human postmortem brain tissues from HIV-positive cases were obtained as part of the NNTC's Texas Repository for AIDS Neuropathogenesis Research autopsy protocol. Postmortem brain tissues from HIV-negative patients without neurodegenerative diseases were obtained from the Department of Pathology Autopsy Services at The University of Texas Medical Branch. Cases used in this study are outlined in Table 1. Cortex and subcortical frontal lobe tissue (coronal slices 1 and 2; (Morgello *et al*, 2001)) were obtained on all cases, and temporal lobe, occipital lobe, and cerebellum tissue were obtained from cases selected at random to determine regional variability of cell recovery.

Isolation and culture of microglial cells
Microglia were isolated using the method of Yong and Antel (1997). All tissue was handled using sterile technique and appropriate biohazard containment measures. Tissue was collected, weighed, and washed extensively with Dulbecco's phosphate-buffered saline (D-PBS) containing 20 μg/ml gentamicin, and 1○ pen-strep-fungizone solution (100 U/ml penicillin, 10 μg/ml streptomycin sulfate, 25 μg/ml amphotericin B) (all Invitrogen GIBCO Life Technologies, Rockville, MD). Surface blood vessels were removed, and tissue was minced and washed twice with D-PBS (containing antibiotics/antimycotic) by centrifugation at 300 ○ g for 7 min each. Minced brain tissue was then treated in a final volume of 125 ml D-PBS per 50 g tissue containing trypsin (final concentration 0.25%; Invitrogen/GIBCO) and 4000 Kunitz units DNase I (Sigma, St. Louis, MO). The trypsin/tissue mixture was stirred continuously at 37°C. Trypsin digestion was stopped after 1 h upon the addition of fetal calf serum (FCS) (2% final volume, Sigma). Trypsinized tissue was passed through a 20-mesh followed by a 100-mesh cell sieve and washed. Percoll (Amersham Biosciences, Piscataway, NJ) was then added to the cells at a final concentration of 30%, and centrifuged in a swinging bucket rotor at 15,000 ○ *g* for 30 min at 24°C. After centrifugation, the middle cell layer was collected. After several washes in D-PBS, the resultant cell pellet was resuspended in 2 to 5 ml feeding medium (minimal essential medium [MEM] with Earle's salts, 5% final FCS, 1 mg/ml final D-glucose, 2 mM final L-glutamine, 1 mM final MEM sodium pyruvate, 20 μg/ml final gentamicin, and 1○ pen-strep-fungizone solution; all Invitrogen/GIBCO) and the total number of viable cells was counted by trypan blue dye exclusion using a hemacytometer. Cells were seeded in T25 tissue culture flasks at no more than 10 ○ 10^6 cells per flask in a total volume of 5 ml, and incubated at 37°C in 5% CO_2.

The next day, nonadherent cells were removed and cytospins of these cells were made. Adherent astrocytes and microglia were separated by trypsinization using warm 0.05% trypsin/0.2 mM EDTA treatment for 15 min. Astrocytes and some microglia floated off the flask and were collected and cultured. Remaining adherent cells were microglia, and >95% stained with antibodies to CD11b/c. Microglia were removed by scraping or treatment with 0.25% trypsin/1 mM EDTA for 15 min. Microglia were routinely cultured in feeding medium containing 5% ORIGEN giant cell tumor supernatant (GCTS) (IGEN International, Gaithersburg, MD) as a source of growth factors.

Immunocytochemistry
Adherent microglial cells and astrocytes were grown in 2-well Lab-Tek II chamber slides (Nalge Nunc International, Naperville, IL) in the presence of 5% GCTS. At various times, the cells on the slides were fixed in 4% paraformaldehyde and blocked in D-PBS containing 1% bovine serum albumin and 0.1% Triton X-100. Slides were incubated with antibodies to either CNPase (clone 11-5B, Chemicon International, Temecula, CA) for oligodendrocytes, CD11b/c (clone OX-42, BD PharMingen, La Jolla, CA) for microglia, or GFAP cocktail (clones 4A11/1B4/2E1, BD PharMingen) for astrocytes. For some cases, microglia slides were also stained for CD68, CD45, HLA-D, or CD14 (all Dako, Carpinteria, CA). Secondary antibody was goat anti-mouse IgG-biotin (Vector Laboratories, Burlingame, CA). Biotinylated antibodies were detected using streptavidin–horseradish peroxidase (HRP) followed by NovaRED substrate (Vector Laboratories). In the case of double labeling for CD11b/c and GFAP, slides were incubated as above and developed with NovaRED followed by incubation with goat anti-mouse immunoglobulin G (IgG)-biotin and streptavidin-HRP. The second color was developed with 3,3′-diaminobenzidine (DAB) substrate (Vector). Methyl green was used as a nuclear counterstain. Slides were mounted with Vectamount (Vector).

Phagocytic activity
The latex bead method of Oda and Maeda (1986) was used to visualize phagocytic activity of cultured microglia. Fluoresbrite YG latex beads (1 μm; Polysciences, Warrington, PA) were added to microglia cells in culture at a 50:1 particle-to-cell ratio and incubated for 90 min at 37°C. Nonphagocytized beads were removed with gentle washing, and the slide was fixed in 4% paraformaldehyde in preparation for counterstaining using Vectashield mounting media with propidium iodide (Vector Laboratories). The nonphagocytic cell line Vero (African green monkey kidney fibroblasts) was used as a control.

Cytokine secretion
Microglia were seeded at 1 ○ 10^5 cells per well in a 96-well plate. Triplicate wells were stimulated with either 10 μg/ml LPS (*Eschericia coli* serotype O55:B5; Sigma), 25 ng/ml recombinant human GM-CSF (R&D

Systems, Minneapolis, MN), or both. Supernatants were collected after 24 and 40 h and stored at ○ 85°C until use. The presence of IL-6 was measured in pooled cell supernatants after 24 h, and TNF-μ was measured in pooled cell supernatants after 40 h using ELISA kits (Endogen, Woburn, MA).

Tissue pH
Frontal lobe brain tissue that had been frozen and stored at ○ 85°C for up to 1 year was used for pH measurements. It has been reported that brain tissue pH is stable after death, and is not related to PMI or the length of time tissue is stored frozen (Harrison *et al*, 1995). Frozen tissue containing white and grey matter (approximately 1 g) was resuspended in 2 ml sterile distilled, deionized water that had a neutral pH. The tissue was homogenized using 0.5-mm silica beads and a Mini-Beadbeater (BioSpec Products, Bartlesville, OK) for 4 10-s pulses at 4800 rpm until a smooth mixture was obtained. The sample was then diluted with 10 volumes (final) of water, and the silica beads sank to the bottom of the tube. The presence of the beads did not alter the pH. The pH of the diluted tissue was measured using an Accumet model AB15 pH meter and microprobe.

Infection with HIV-1
Microglia were seeded at 1 ○ 10^5 cells per well in a 24-well plate, and exposed to R5-tropic (JR-CSF) and X4-tropic (IIIB) isolates of HIV-1 at a multiplicity of infection (MOI) of approximately 1 for 18 h. Both isolates were obtained from Miles Cloyd (The University of Texas Medical Branch, Galveston, TX) and were propagated in VB cells (for JR-CSF) or H9 cells (for IIIB). Wells were then gently washed with D-PBS to flush away free virus and fed with feeding medium containing 5% GCTS. Wells containing microglial cells but no virus were used as negative controls. Supernatants were removed weekly, refed with fresh medium containing growth factors, and stored at ○ 85°C before p24 analysis. A commercially available HIV p24 ELISA kit (Beckman Coulter, Miami, FL) was used according to the manufacturer's directions to assess infectivity. The rest of the conditioned medium is for future research examining the secretion of neurotoxins and activation proteins by microglia.

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