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Characterization of cultured microglia that can be infected by HIV-1

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Parenchymal microglia are targets of HIV infection. We, as well as others, have used *in vitro* microglia culture systems to study the tropism and replication of HIV. Characterization of perivascular and parenchymal microglia surface markers *in vivo*, *in vitro*, and *ex vivo*, has led to the understanding that these cell populations are different, and data from both the HIV and SIV models support the hypothesis that they may play different roles in infection of the CNS. We determined that human adult parenchymal microglia cultured from temporal lobe tissue for use in HIV replication studies, were CD11c$^+$, CD45$^+$, CD68$^+$, CD14$^-$ when cultured with standard serum/cytokine-supplemented media. To determine the influence of serum and cytokines on HIV replication in microglia, we designed a new protocol for culturing microglia, and compared the results obtained with this protocol with the standard approach previously described. Microglia cultured in the presence of a 'feeder' layer of glial cells and in the absence of serum and cytokines expressed the same surface markers as pure microglia (>95%) cultured in supplemented media. However, pure microglia cultured in the absence of both serum/cytokines supplements and other glial cells, did not have characteristic microglial morphology and did not support HIV replication to as high a level. Lastly, we determined that unlike monocytes, *ex vivo* parenchymal microglia were capable of supporting HIV replication. *Journal of NeuroVirology* (2000) **6,** S53–S60.

Keywords: microglia; HIV-1 infection; glia; microglial activation

Introduction

Microglia and/or monocyte-derived macrophages (MDM) are the principal targets of HIV and SIV infection in the brain, as has been extensively documented by both immunohistochemical and *in situ* hybridization studies (Gabuzda *et al*, 1986; Lackner *et al*, 1991; Wiley *et al*, 1986; Lane *et al*, 1996b). The neuroglial cells, namely astrocytes and oligodendrocytes, appear to be infected much less commonly and infection is much more restrictive (Saito *et al*, 1994), with little production of structural proteins, perhaps because differences in their cellular milieu alter the effects of some of the regulatory HIV genes such as *rev* (Shahabuddin *et al*, 1992). Neurons are seldom infected, if at all (Bagasra *et al*, 1996). Many investigators have thus concentrated on the *in vivo* and *in vitro* study of microglia and their infection by HIV.

Hickey and others have divided microglia or CNS macrophages into several subtypes, based on surface markers, anatomic localization, and morphological features (Lassmann *et al*, 1993; Graeber *et al*, 1989; Streit and Graeber, 1993; Williams and Hickey, 1996). It is now widely believed that parenchymal microglia arise from bone marrow derived cells migrating into the CNS during fetal life (Rezaie, 1999), and based on studies with rodents, parenchymal microglia are thought to have a low turnover or replacement rate (Hickey *et al*, 1992). These microglia are therefore only infrequently exchanged with peripheral blood monocytes. Perivascular cells expressing macrophage markers have a much higher turnover rate, are frequently replaced

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by blood monocytes, and may only rarely penetrate the brain parenchyma. Leptomeningeal and choroid plexus cells with macrophage and microglial surface markers are also probably not exchanged with parenchymal cells, but may also arise directly from circulating monocytes (Lassmann *et al*, 1993).

The microglial or perivascular macrophage subset that is infected *in vivo* by either HIV-1 or simian immunodeficiency virus (SIV), a non-human primate relative, has been the subject of some discussion (Lackner, 1996). Neuropathological data from the SIV system indicate that perivascular cells with myelogenous surface markers are the initial targets of SIV infections in the brain (Lane *et al*, 1996a,b). Parenchymal microglia are infected at later points in the course of SIV infection, either by: (a) trafficking T-cells (b) trafficking monocytes; (c) free virus crossing the CSF-parenchymal barrier, or (d) by direct contact with infected cells or virus residing in the Virchow-Robin space. Parenchymal microglial foot processes are in contact with the CNS boundary of the Virchow-Robin space (Gehrmann *et al*, 1995).

Investigators have used primary microglia, oligodendrocytes, and astrocytes, as well as a number of immortalized neural lines in order to study neurotropism of HIV and SIV, whose well-documented restrictions demand the use of primate cells. Many of the studies undertaken with human tissues have used cells of fetal origin. However, procedures for the culture of human tissue obtained from temporal lobe resections performed for the removal of epileptic foci were described carefully by Antel and collaborators (Yong and Antel, 1992), and adapted for the study of HIV infection by Dubois-Dalcq and her laboratory (Jordan *et al*, 1991; Sharpless *et al*, 1992). We have also adopted this methodology for studying tropism and replication of HIV in microglial tissue (Albright *et al*, 1996, 1999; Shieh *et al*, 1998; Strizki *et al*, 1996).

Immunohistochemical studies have identified some of the surface markers associated with microglial tissue obtained at the time of temporal lobectomy. This includes Becher and Antel's study of human adult microglia immediately *ex vivo* which found that they expressed low levels of CD45, were negative for CD14, and were positive for CD11c (Becher and Antel, 1996). This pattern is similar to that of parenchymal microglia studied *in situ*, and distinguishes them from perivascular cells and MDM, which are both $CD14^+$ (Heidenreich, 1999; Ziegler-Heitbrock and Ulevitch, 1993). However, one group has reported that microglia become $CD14^+$ after *in vitro* culture (Becher and Antel, 1996). Similar reports of $CD14^-$ microglia were published by Dick *et al* (1997), who studied *ex vivo* microglia purified from tissue obtained during diagnostic stereotactic biopsy. Interestingly, these microglia isolated from HIV positive patients undergoing sterotactic surgery showed higher levels of CD4, which the authors interpreted as evidence of chronic activation (Dick *et al*, 1997).

We have analyzed the human adult microglia cultured in our laboratory to determine the expression pattern of monocyte/macrophage surface markers in the cells we have used to study HIV replication. We then determined what effect culturing microglia, with or without serum supplements or glial 'feeder' cells, had on HIV infectability of microglia. Lastly, we compared HIV infection of *ex vivo* isolated parenchymal microglia with monocytes and MDM.

Results

Microglia are $CD11c^+$, $CD45^+$, $CD68^+$, $CD14^-$

We examined the expression pattern of the well-characterized surface markers CD11c, CD14, CD45, and CD68 using indirect immunofluorescence (see Figure 1 and next section for quantification). Only dark-green fluorescence (from fluorescein-labeled probes) was considered positive in the immunofluorescence experiments on microglia. Non-specific signals, such as yellowish-green fluorescence

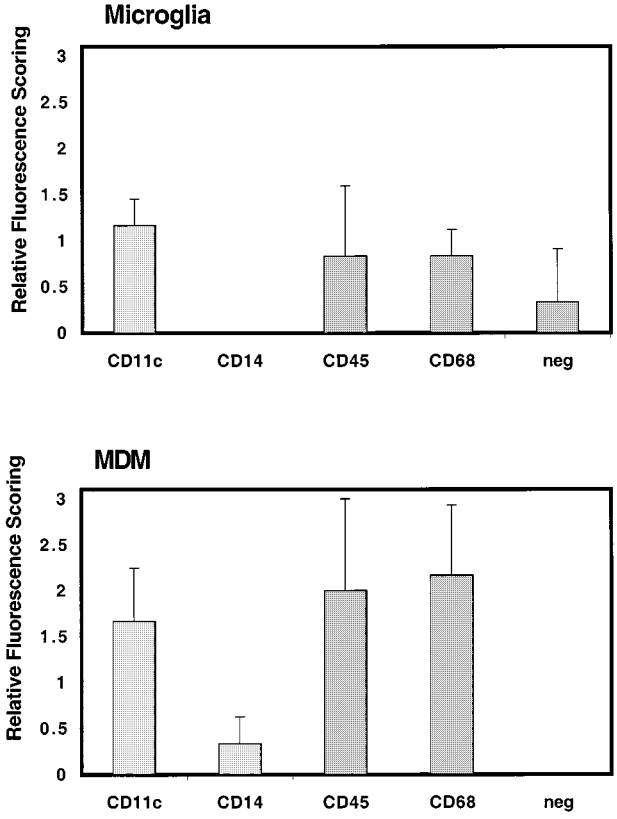

Figure 1 Quantification of indirect immunofluorescence of microglia and MDM. An observer who was unaware of the experimental scheme used a scale ranging from zero (negative) to four (very positive) to score the whole cell, dark-green staining. The graph demonstrates the average and standard deviations from three separate experiments with either microglia or MDM.

were ignored. In three separate experiments, we noted clear staining with CD11c, CD45, and CD68 (data not shown), which is consistent with previously published reports (Becher and Antel, 1996; Williams *et al*, 1992). We did not see evidence of CD14 expression, even in microglia that had been cultured for up to 7–10 days, a period of time when others have reported CD14 positive microglia (Becher and Antel, 1996). As a control, all antibodies were tested on MDM, and as expected they were positive for CD11c, CD45, and CD68 antigens (not shown), although CD14 positive cells were only identified in one of three experiments. However, similarly cultured microglia can respond to lipopolysaccharide (unpublished results) indicating that they have a functional receptor, which could be CD14 expressed at levels too low to detect by immunofluorescence techniques.

Quantification of indirect immunofluorescence on microglia

To quantify the immunofluorescence studies, one observer scored the cells in Lab-Tek chamber slides without any previous knowledge of the experimental scheme (except for the negative control well). Using a scale from zero (negative) to four (strongly positive), CD11c staining on microglia yielded an average score of 1.17 with a standard deviation of 0.29 (1.17 ± 0.29). CD68 (0.83 ± 0.29), and CD45 (0.83 ± 0.76) were also positive in 3/3 and 2/3 experiments respectively (Figure 1). Dark green signal (fluorescein) was not detected in the CD14 wells in any of the three experiments on microglia.

MDM were used as a positive control and analyzed in the same experiments. Staining with CD11c, CD45, and CD68 was more intense, as reflected in Figure 1. CD14 expression was noted in 1/3 experiments, which could be due to our use of 7–10 day old MDM (Ulvestad *et al*, 1994). Additionally, background yellowish-green fluorescence was much less prominent with MDM. The degree of cell surface staining in MDM was less prominent than has generally been reported. This may be due to culturing the MDM in media that was designed for microglia, which we did in order to make meaningful comparisons between the two cell types.

Culture of microglia with glial cells

Since there is a concern that *in vitro* culture of microglia in supplemented media activates the microglia and alters the expression of markers such as CD11c and CD14, we prepared one set of

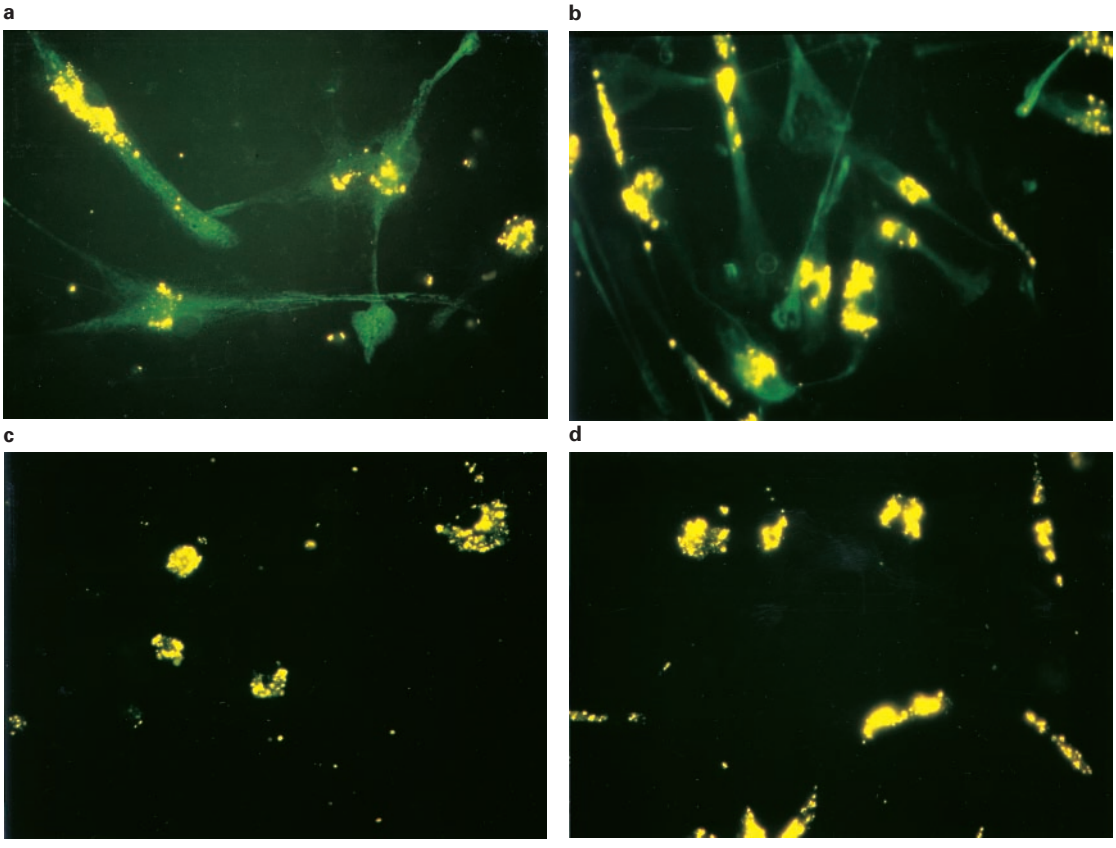

Figure 2 Microglia cultured with other glial cells, but not FCS/GCTS, demonstrate similar morphology to the similar microglial cultures, which had received 3 days of FCS/GCTS treatment. (**A**) Microglia grown without FCS/GCTS and (**B**) after 3 days of treatment with FCS/GCTS stain positive with a mixture of the two antibodies against CD11c/CD68. These same microglia grown without FCS/GCTS (**C**) or with FCS/GCTS for 3 days (**D**) were negative for CD14 staining.

mixed glial cultures that included microglia in medium without fetal calf serum (FCS) or giant cell tumor supernatant (GCTS) (Figure 2A,C). In a second set, mixed glia were cultured without serum for 7 days, then supplemented with FCS and GCTS for 3 days (Figure 2B,D). There were no gross morphological changes between microglia in these two sets of cultures. Immunofluorescence with antibodies against CD11c and CD68 was used to supplement the morphological observations. As shown in Figure 2A,B, there was no difference in this combination staining. Also shown in Figure 2C,D, are the results of CD14 staining, which was negative under all culture conditions.

In contrast, purified microglia appeared to require supplementary serum in order to grow well.

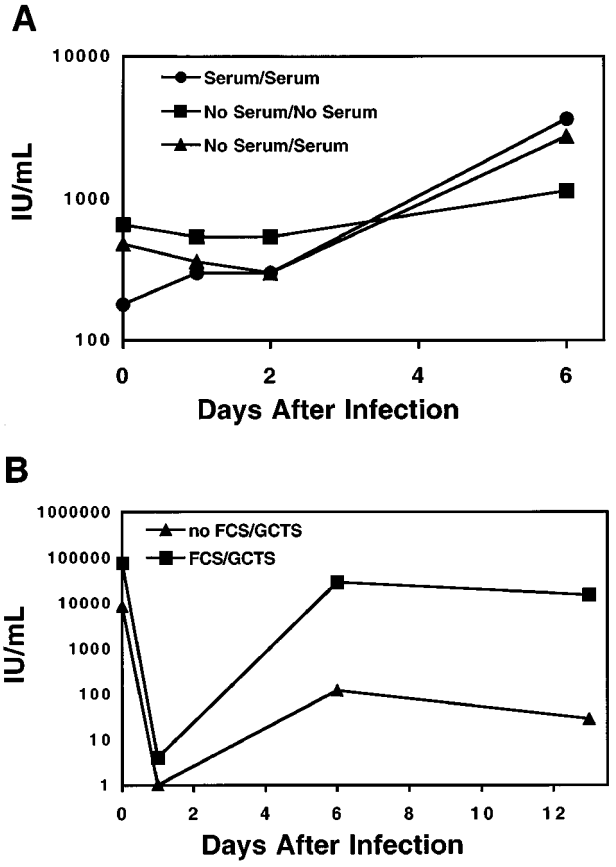

Figure 3 (**A**) Microglia cultured in the absence of FCS/GCTS or other glial cells can be infected with HIV-$1_{DS\text{-}br}$. Microglia isolated and cultured without FCS/GCTS were exposed to HIV-$1_{DS\text{-}br}$ and the supernatant assayed, on U373-MAGI-CCR5 with β-gal assay, for the presence of infectious virus release at regular intervals. Microglia treated with FCS/GCTS produced the most HIV, whereas microglia cultured without FCS/GCTS replicated virus to lower levels. Microglia treated with FCS/GCTS for 3 days showed intermediate virus production. (**B**) The same experiment was performed except microglia were used that were initially cultured in mixed glial cultures with no FCS/GCTS for 4 weeks, and then either stimulated with FCS/GCTS for 5 days or continued to be cultured without FCS or GCTS.

Cultures consisting of >95% pure microglia had a typical morphology only when FCS/GCTS were included in the culture media. Those microglia cultured in serum-free media were rounded, and did not stain as clearly with the vital stains diI-Ac-LDL or Hoechst.

CD14-negative microglia are infectable

We then determined that the CD14-negative microglia grown without serum or other glial cells, were able to be infected by HIV-$1_{DS\text{-}br}$ (Figure 3A). These microglia, cultured only in serum-free medium, could replicate virus, but the level was considerably lower than with cultures supplemented with FCS/GCTS. As the microglia were exposed to FCS/GCTS earlier and longer in culture, so did the ability to produce virus (Figure 3A). Microglia that were determined to be $CD11c^+$, $CD45^+$, $CD68^+$, $CD14^-$ were able to be productively infected by several strains of microglia-tropic HIV isolates (RC-br and BORI-P20) (not shown).

Microglia that were cultured in mixed glial cultures for 4 weeks in DMEM without FCS/GCTS were examined for their ability to sustain HIV replication. While these microglia were infected and released infectious virus, they were only able to produce very low amounts of infectious HIV (IU/mL) as determined by the U373-MAGI assay (Figure 3B). Alternatively, microglia that were cultured for 4 weeks in DMEM without FCS/GCTS, but then incubated with FCS/GCTS for 5 days prior to infection, were able to sustain a very productive infection (Figure 3B).

Ex vivo *parenchymal microglia can support a productive HIV-1 infection*

Lastly, to ensure that *ex vivo* microglia were infectable with HIV-1, we exposed cells to the R5-tropic isolate HIV-1_{BaL} immediately after the microglia were isolated from brain tissue. As a control we infected *ex vivo* blood monocytes that had been isolated with similar procedures (see Materials and methods). As shown in Figure 4A (Day 0), *ex vivo*-infected microglia replicated virus well, whereas monocytes were highly resistant as expected (Rich *et al*, 1992; Sonza *et al*, 1996). After 7 days in culture (Figure 4B), the now differentiated MDM replicated virus at levels equivalent to those obtained from microglia cultured for the same time period. This is consistent with data indicating equivalent expression of CCR5 between recently harvested microglia and 6–7-day-old MDM (Albright *et al*, 1999; Lee *et al*, 1999)

Discussion

In these experiments we have characterized the expression of several MDM surface markers in adult microglia that were harvested from tissue obtained

during temporal lobectomy. Besides providing a comparison between MDM and microglia cultured under similar conditions, we undertook these experiments to determine whether any differentiation marker is associated with the infectability of cultured microglia.

In general we found a similar cell surface immunofluorescence staining pattern between microglia and MDM; both cell types expressed the differentiation markers $CD11c^+$, $CD45^+$ and $CD68^+$, although overall the MDM stained more intensely. Moreover, we found that the microglia were negative for expression of the CD14 antigen, one of the components of the lipopolysaccharide receptor, although with these staining conditions MDM were positive in only one of three experiments. Previous reports have differentiated perivascular microglia from parenchymal cells through the former's expression of CD14 (Becher and Antel, 1996; Dick *et al*, 1997). However, this pattern has not been consistent, and clearly the results are influenced by source of the tissue, the culture conditions, and possibly by the source species, since some experiments have used simian brain. Notably, multinucleated giant cells found in brain parenchyma have been found to be $CD14^+$ (Dick *et al*, 1997). It is unknown whether these are cells that were originally blood-derived MDM or infected perivascular cells that have trafficked to the parenchyma; or alternatively whether they are infected parenchymal microglia that were infected and then subsequently increased expression of CD14.

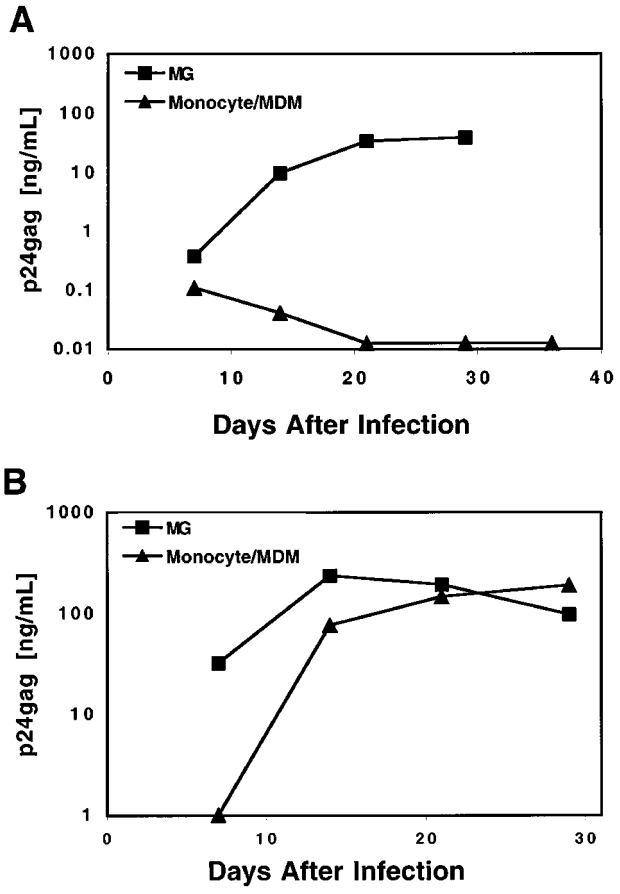

Figure 4 Infection of microglia and monocyte/MDM at different days after isolation: Microglia (MG) and MDM were infected with HIV-1_{BaL} either (**A**) on the day of isolation from brain tissue or blood (*ex vivo*) or (**B**) after 7 days of culture. The culture medium was replaced at regular intervals and assayed for $p24^{gag}$ antigen concentration. Microglia were easily infected immediately after isolation, whereas monocytes required differentiation in culture. Microglia and monocyte/MDM cultured for 7 days were able to support viral replication. This graph is representative of three separate experiments.

Overall, we agree with those investigators who have indicated that no single surface marker can definitively identify these overlapping populations, especially in the context of HIV infection (Brinkmann *et al*, 1992, 1993; Dickson and Lee, 1996; Theele and Streit, 1993; Watkins *et al*, 1990). As to the phenotype of those microglia obtained from temporal lobectomies, based on their origin and on the absence of CD14 expression we would conclude that they are most likely to be parenchymal, as previously hypothesized (Williams *et al*, 1992).

We also compared the morphology of microglia cultured with or without serum and cytokine supplements, and with or without a 'feeder' layer consisting of other glial cell types. Microglia grown in the mixed glial cultures for 4 weeks without FCS or GCTS, had characteristic *in vitro* microglial morphology, were stained with vital dyes, and could be infected by HIV-1. In contrast, microglia that were >95 % pure required the FCS and GCTS supplements in order to maintain a healthy appearance.

Microglia that had been cultured without serum or feeder cells were still infectable, although to a lesser extent than microglia cultured initially without supplements or feeder cells, but then treated with serum for 3 days and then infected (Figure 3). Microglia that had been cultured continually in supplements (FCS/GCTS) yielded the highest concentration of infectious virus. Thus, other glial cells provide a milieu that is conducive to long-term microglial culture, and that supports HIV replication, although for full expression of replication potential the microglia needed growth supplementation. The exact component responsible for the increased viral replication has not yet identified.

Since there is only relatively limited literature with respect to comparison between microglia grown with or without feeder cells and the effect this would have on HIV infection, we feel that our results, while largely preliminary, deserve further consideration. Additionally, our laboratory has previously found that *ex vivo* microglia express the same chemokine receptors on their surface as microglia cultured *in vitro* for $7-10$ days. We conclude that, at least for those cell surface proteins, the *in vitro* culture of microglia does not necessarily change the overall phenotype.

Lastly, we performed an *in vitro* infection of human adult microglia with minimal manipulation of the cells after temporal lobe resection. In contrast to monocytes, which must undergo differentiation before they are infectable, microglia supported HIV replication from their initial isolation. This is at least partly due to the presence of the chemokine receptor CCR5 on the surface of *ex vivo* microglia, and supports the hypothesis that parenchymal microglia are probably infectable *in situ*, and that they may represent a pool of cells that is highly susceptible to HIV infection. It is also indirect evidence that the cells isolated from the temporal lobe tissue are indeed microglial in origin, and not contaminating blood monocytes.

In summary, we have used this system for analysis of viral replication in microglial cells. While no model can reproduce the conditions in the brain completely, this model, in combination with other similar systems, can provide valuable information regarding the parameters of HIV infection in the brain.

Materials and methods

Isolation of microglia and culture in microglia media with fetal calf serum (FCS) and giant cell tumor supernatant (GCTS)

Microglia were isolated from fresh human brain tissue and cultured for 4–7 days in Dulbecco's Minimal Essential Media (DMEM), which was supplemented with 5% FCS (Atlanta Biologicals), 5% GCTS (Fisher), 4.5 g/l glucose, 50 μg/ml gentamicin and 1 mM sodium pyruvate (microglia media) (Strizki *et al*, 1996; Yong and Antel, 1992). To plate microglia for experiments, cultures were washed three times with PBS and incubated with 0.25% trypsin diluted in PBS. When 90–95% of the adherent microglia were detached, the microglia were centrifuged for 10 min at $1750 \times g$ on a Beckman tabletop centrifuge. Microglia were then plated at $3-4 \times 10^4$ microglia per well in 8-well Lab-Tek Permanox chamber slides (Nalge Nunc International, Naperville, IL, USA), and cultured for 1–4 days in microglia media. The purity of the microglia in the cultures was determined to be greater than 95% using diI-Ac-LDL (Biomedical Technologies Inc., Stoughton, MA, USA) as previously described (Strizki *et al*, 1996).

Isolation of microglia and culture without FCS or GCTS

Microglia were isolated as above, except FCS was omitted from all steps of the isolation protocol and the microglia were cultured in DMEM supplemented with 4.5 g/l glucose, 50 μg/ml gentamicin, and 1 mM sodium pyruvate. For comparison studies between microglia cultured with or without FCS/GCTS, two sets of microglia were cultured without serum for 4–7 days and then plated in chamber slides (as described above). One set was maintained in culture without FCS/GCTS for 3 days, while the second set of microglia was cultured with FCS/GCTS for 3 days. At the end of these 3 days, immunofluorescence was performed on both sets of microglia.

Mixed glial cultures

To culture microglia, astrocytes, and oligodendrocytes together, the purification protocol was modified such that the remaining intact samples of brain tissue were treated with fresh 0.25% trypsin for a second 30 min incubation at 37°C (Strizki *et al*, 1996; and unpublished results). The Percoll gradient step was omitted and the trypsin-treated cells were centrifuged for 10 min at $1750 \times g$, suspended in microglia media without FCS plated in plastic T-75 flasks (Costar), cultured for 3–7 days, and then removed and plated as previously described.

Indirect immunofluorescence antibody staining of microglia and MDM

Microglia cultured at a density of 3×10^4 cells per well of 8-well Lab-Tek chamber slides were washed twice in PBS, fixed in 1% paraformaldehyde for 60 min, washed twice in PBS, and incubated with blocking solution for 60 min. All steps were performed at room temperature (Shieh *et al*, 1998). Primary antibodies directed against macrophage-specific surface markers were diluted as per manufacturer's instructions (DAKO, Carpinteria, CA, USA): CD11c@1 : 25, CD14@1 : 10, CD45@1 : 50 CD68@1 : 100, W632 (anti human Class-I)@1 : 10, and 10 μg/ml of negative control antibody in PBS with 0.1% BSA, 0.02% sodium azide, and 8% goat serum (blocking solution). This was added to the cultures for 45–60 min and then washed three times in PBS. The secondary antibody: anti-mouse IgG (Fab specific)-FITC conjugate/F(ab')$_2$ fragment of goat antibody adsorbed with human serum; was diluted 1 : 100 in blocking solution and incubated with microglia for 45–60 min. The cells were then washed three times in PBS. A Leitz Aristoplan microscope was used to observe the immunofluorescence. The Leitz I3 Ploemopak fluorescent vertical illuminator with a 515 nm long pass suppression filter was used to observe fluoroscein positive microglia instead of using the more conventional L3 illuminator, which has a 525/20 nm band pass suppression filter and is not able to distinguish background autofluorescence from fluoroscein signal. Photographs were taken with a Wild Leitz MPS46 Photoautomat camera, Kodak Ektachrome slide film (ASA400) at an exposure of 20 s for all images, such that the intensity of staining could be compared among different photographs. Three separate experiments were scored by an observer, who was unaware of the experimental scheme, using a scale ranging from minus (negative) to four pluses (very positive) based on whole cell

staining observed on the I3 illuminator, as dark green staining (fluoroscein). Non-specific background staining which appeared as a bright yellowish-green color of autofluorescence was generally confined to perinuclear patches. The scorer was only told which well contained no antibody (background). These three experiments were averaged, the standard deviations determined, and both graphed.

HIV-$1_{DS\text{-}br}$ infection of microglia cultured with or without FCS/GCTS

Microglia were cultured at a density of 3×10^4 cells per well of a 96-well plate (Costar) or 8-well Lab-Tek chamber slides and infected with 10 ng ($p24^{gag}$) per well with the HIVD isolate HIV-$1_{DS\text{-}br}$ (Popovic and Gartner, 1987) for 2 h, washed extensively, and cultured in appropriate media. Supernatant samples were obtained at days 0, 1, 2, 6, and 13 post-infection, frozen, and assayed together for the presence of infectious virus on the indicator cell lines, U373-MAGI-CCR5 and U373-MAGI-CXCR4, with the parental U373-MAGI as a negative control cell line (Vodicka *et al*, 1997). Cultures of MDM were used as a positive control for infection.

Infection of microglia and monocyte/MDM on different days after isolation

For monocyte isolation, whole blood was centrifuged over a Ficoll gradient ($1200 \times g$ for 20 min). The mononuclear cell fraction was collected and run through a Percoll gradient with identical characteristics to the gradient used to isolate microglia. This ensured that cell isolation techniques for microglia and monocyte/MDM were similar. Microglia or monocytes were then plated for 2 h at 37°C in T-75 tissue culture flasks, the non-adherent cells were washed off, and the adherent population was then removed with 0.02% EDTA in Hank's balanced salt solution (Versene), and plated in 24-well plates at a density of 2.5×10^5 cells per well. We performed infections: (a) immediately after isolation, by infecting microglia or monocytes 1 h after plating; or (b) after the cells were cultured for 7 days in microglia media. The cells were inoculated with 10 ng ($p24^{gag}$) of HIV-1_{BaL} (AIDS Reagent Program) for 1 h at 37°C, the virus washed off, and the media replaced. Culture supernatants were assayed for $p24^{gag}$ antigen concentration (NEN/DuPont) and replaced at regular intervals.

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