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Low-level HIV replication in mixed glial cultures is associated with alterations in the processing of $p55^{Gag}$ ☆

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Abstract

We report a novel long-lived infection model in human mixed glial cultures (microglia) whereby cells harbor replication-competent HIV-1 for up to 2.5 months after infection; a model that potentially mimics latency within the central nervous system (CNS). Infection of mixed glial cultures in the presence of serum, cytokines, and growth factors (activating conditions) resulted in a robust productive infection of microglial cells as previously described for purified microglia. In contrast, similar mixed glial cells cultured in serum-free medium without cytokines or growth factors (mirroring a nonactivated CNS) supported HIV-1 entry, reverse transcription, integration, and transcription, yet released little or no infectious virus. We found instead that nonactivated mixed glial cells expressed almost 10-fold less Gag protein, but more importantly, analysis of the intracellular Gag products in quiescent cells showed an aberrant p55/p24 Gag processing phenotype that appeared to be due to the premature activity of the viral protease. These results suggest that the cellular environment in nonactivated microglia cells in these mixed glial cultures is not conducive to proper Gag processing and virus release. This long-lived infection model will be useful in identifying factors that are key for viral maturation in cells of the macrophage lineage.
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Keywords: HIV replication; Glial cultures; $p55^{Gag}$; Microglia

Introduction

Highly active anti-retroviral therapy (HAART) has had a dramatic effect on systemic HIV replication with a consequent reduction in the overall morbidity and mortality of infected persons (Gulick et al., 1997; Hammer et al., 1997; Palella et al., 1998). Widespread use of HAART has also reduced the number of new cases of HIV-associated dementia (HAD), an important HIV complication in the central nervous system (CNS). Still, HIV-associated cognitive impairment continues to be a major clinical problem, in part due to the increased life span of HIV-infected individuals, and additionally because of a recently described less severe form of the disease (Dore et al., 1999; Neuenburg et al., 2002; Sacktor et al., 2002).

HAART's efficacy in reducing plasma viral load also facilitated detailed studies of the viral decay kinetics in infected individuals (Ho et al., 1995; Perelson et al., 1996, 1997; Wei et al., 1995); these studies uncovered the existence of multiple pools of infected cells, including some long-lived viral reservoirs. Among these, $CD4^+$ T lymphocytes (T cells), which when activated are responsible for producing the majority of the infectious virus in the bloodstream, can also support a latent or slowly productive infection (Chun et al., 1995). Resting memory $CD4^+$ T cells, responsible for this slow phase of the infection, are estimated to have a half-life of 6–44 months (Pierson et al., 2000), making complete eradication of the virus with current therapies difficult or unlikely (Demoustier et al., 2002). However, this cell type probably does not entirely account for HIV persistence (Chun et al., 1995, 1997a, 1997b; Perelson et al., 1997). Other potential reservoirs include lymph nodes, CNS microglia and macrophages, other tissue macrophages, gut lymphoid tissue, rectal mucosa, the urogenital tract, and HIV held in the follicular dendritic cell (FDC) network (Cavert and Haase, 1998; Cavert et al., 1997; Chun et al., 1997a, 1997b; Crowe and Sonza, 2000; Kedzier-

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ska and Crowe, 2002; Lebargy et al., 1994; McIlroy et al., 1996; Mikovits et al., 1992; Persaud et al., 2000; Pomerantz, 2002; Schrager and D'Souza, 1998; Sierra-Madero et al., 1994; Sonza et al., 2001; van Furth, 1989).

There are known major blocks to HIV replication at two key points in the viral life cycle: after reverse transcription but before integration (pre-integration latency), and after the provirus has been incorporated into the host cell genome (post-integration latency) (Blankson et al., 2000). Pre-integration latency appears to be more common, but is limited by the half-life of the labile pre-integration complex, which is generally measured in days (Pierson et al., 2002). In post-integration latency, which may last for the lifetime of the infected memory T cell, the provirus is integrated into the host cell genome but remains transcriptionally silent (Jordan et al., 1991; Persaud et al., 2000). Based on in vitro models, several mechanisms have been proposed for post-integration latency, including reduced levels of transcriptional activators in resting cells and integration into silent regions of the host genome (Butera, 2000; Jordan et al., 2003; Kutsch et al., 2002; Quivy and Van Lint, 2002). Current models would propose that upon cellular activation, transcription factors that are key to HIV replication are upregulated and viral replication resumes. Nevertheless, there are likely to be important cell type differences in the HIV-specific transcription factors between T lymphocytes and macrophages (Kutsch et al., 2002; Roberts et al., 1997).

Other than their mostly theoretical contribution to viral reservoirs, little is known regarding the specific role of chronically infected macrophages (Butera, 2000; Kutsch et al., 2002; Laurence, 1993; Meltzer et al., 1990; Pierson et al., 2000; Roy and Wainberg, 1988). While recent reports indicate that cellular activation and reactivation of HIV replication can be uncoupled in latently infected T lymphocytes (Kutsch et al., 2002), macrophages have not been studied as intensively. In this regard, microglia and other CNS macrophages may serve as replication competent HIV reservoirs, because some retroviral drugs have poor CNS penetration and a T lymphocyte immune response in the CNS is both limited and to some extent undesirable (De Luca et al., 2002). Additionally, HIV-infected monocytes from outside the CNS may constitutively seed the brain and return to the periphery in the normal process of trafficking of immune cells in and out of the nervous system (Lawson et al., 1992; Vass et al., 1993). Technologies used to study the occurrence of HIV latency in primary resting T cell populations in vitro (Chun et al., 1995) have not been adapted to terminally differentiated tissue macrophages; therefore, there are no commonly used in vitro models to study viral persistence in macrophages.

To develop a model to address some of these questions, we adapted a well-characterized microglial culture system that we have used previously for studies of HIV replication in the CNS (Albright et al., 1999; Strizki et al., 1996). When cultured in a mixed culture system with astrocytes and oligodendroglia, the microglia survived for weeks absent exogenous growth factors or even supplemental fetal calf serum (FCS) (Albright et al., 2000b). Under these medium conditions, which mirror a nonactivated CNS milieu, the cells were easily infected, but had a markedly reduced level of infectious virion production and release that could be restored by adding serum components, growth factors, and cytokines. We compared low-level HIV infection in non-activating culture conditions with replication in activated cultures using assays for reverse transcription, integration, transcription, and protein expression. We conclude there are multiple levels of restriction of viral replication in the nonactivated microglia model with the greatest restriction apparently at a posttranscriptional level. The restriction was also associated with an alteration in the processing of $p55^{Gag}$. Our results may help identify host factors involved in viral persistence in macrophages.

Results

HIV infection in nonactivating and activating medium conditions

We cultured mixed glial cells isolated from fresh human adult brain tissue obtained as a by-product of therapeutic temporal lobe resections as described previously (Yong and Antel, 1992) and in the Materials and methods section. Using a phagocytosis assay, we then determined that phagocytic cells constituted an average of 61.9% (±17.3%) of the cells in the cultures. There were no significant differences in the phagocytic phenotype between mixed glia cultured in non-activating medium and those cultured in 0.5%, 1%, 5%, or 10% fetal calf serum (FCS) as measured by the number of beads ingested per cell (data not shown). Astrocytes and oligodendrocytes were the other major cell types in the mixed glial cultures: in three experiments using histochemical markers, an average of 17.7% of the cells in the cultures were oligodendrocytes and 3.5% were astrocytes (data not shown).

Cells maintained for 7–10 days in either nonactivating or activating medium were infected with R5 HIV isolates that can infect purified microglia as previously shown (Albright et al., 1999, 2000b; Strizki et al., 1996). The culture supernatant was assayed at regular intervals for the production of infectious virions using a MAGI assay (Fig. 1A) and for the presence of $p24^{Gag}$ protein with a standard ELISA (Fig. 1B). As expected (Gartner et al., 1986; Strizki et al., 1996; Watkins et al., 1990), infection of the cells maintained in activating medium was productive, with a rapid rise in $p24^{Gag}$ concentration (to 10–50 ng/ml) by day 5, and a peak 5–10 days after inoculation (Albright et al., 2000a). In contrast, infection of cells maintained in nonactivating medium led to little or no detectable HIV release, or less than 0.5 ng/ml of $p24^{Gag}$. We refer to this as a 'quiescent' infection. MAGI assay indicated that the differences in viral production between the cells maintained in nonactivating

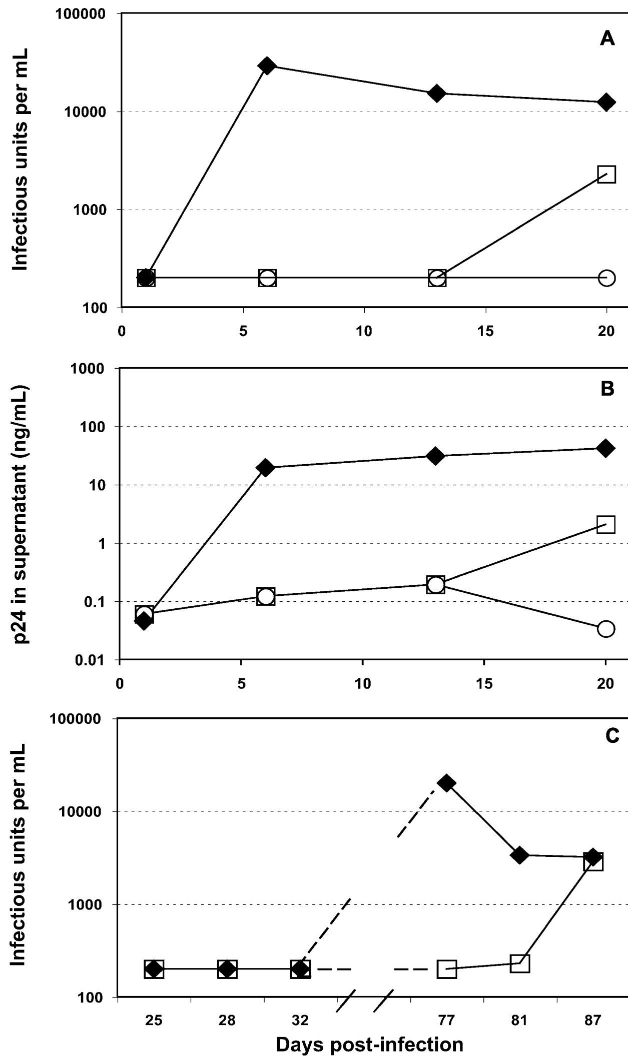

Fig. 1. Addition of activating medium to HIV_{DS-br}-infected mixed glia cultured without FCS/GCTS (nonactivating medium) causes reactivation and release of infectious HIV. Open circles are supernatants from infected mixed glia cultured in nonactivating medium, open squares are cells that had activating medium added at day 13 after infection in nonactivating medium, and black diamonds are from cells maintained in activating medium throughout. HIV expression is measured in IU/ml (A) or $p24^{gag}$ (B). HIV reactivation could be seen after mixed glia were infected with HIV and cultured in nonactivating medium for up to 77 days before the addition of activating medium (C).

medium and the cells maintained in activating medium were statistically significant ($P < 0.0001$; 200 IU/ml was used as the limit of detection). In paired analysis of 146 samples from 39 separate experiments, we determined there was an average of 11.9-fold more infectious virus detected in activating medium when compared to nonactivating medium (data not shown). Using the more sensitive $p24^{Gag}$ ELISA, there was a 14.2-fold greater release of $p24^{Gag}$ in activating medium when compared with nonactivating medium in 17 independent experiments (data not shown). The similarity of the quantitative results obtained with both assays ensured that the differences in the patterns of replication between the two culture conditions were not reflective of altered $p24^{Gag}$/infectious particle ratios or other potential artifacts.

To determine whether the cells already exposed to HIV and cultured in nonactivating medium were still capable of productive viral replication, we added activating medium at selected time points. The addition of serum components and cytokines resulted in a rapid increase in viral production to levels similar to those observed in cells maintained in activating medium since the time of infection. Figs. 1A and B indicate that replacement of the nonactivating medium 13 days after infection quickly converted the low-level infection to a productive infection. To better mirror the long-lived HIV infection of the CNS that occurs during the entire life of HIV-infected patient, an experiment was also performed with cells cultured with nonactivating medium for 77 days (Fig. 1C); there was a rapid increase in viral output after exposing cells to the activating medium.

To ascertain whether a specific pro-inflammatory cytokine (Si et al., 2002) was individually responsible for HIV reactivation, we analyzed the reactivation response to various doses of some of the more prominent components of the FCS and GCTS in the activating medium (Table 1). The combination of 5% FCS and 5% GCTS consistently resulted in the highest levels of replication in comparison to nonactivating medium, 5% FCS alone, or 5% GCTS alone (Fig. 2A), which is not surprising in view of the complexity of the pathways involved in macrophage activation (Winston et al., 1999). Tumor necrosis factor-α (TNF-α), granulocyte macrophage (GM-CSF), macrophage-(M-CSF), and granulocyte-colony stimulating factors (G-CSF) all resulted in significant HIV release when compared to nonactivating medium or control fibroblast growth factor (Fig. 2B). Dose response curves of G-CSF and M-CSF demonstrated that the reactivation effect seen in Fig. 2 was consistent and due to the added factors (data not shown).

Identifying the block to productive HIV infection in resting mixed glia

HIV can attach to DC-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) on the surface of follicular dendritic cells (FDCs) and survive for long

Table 1
Components of culture supplements

Fetal calf serum	Giant cell tumor supernatant
Albumin (2.8 g/dl)	Granulocyte-macrophage colony stimulating factor (GM-CSF)
Bilirubin (0.2 mg/dl)	Granulocyte colony stimulating factor (G-CSF)
IgG (16.0 mg/dl)	Macrophage colony stimulating factor
Insulin (< 5.0 μIU/ml)	Human erythroid enhancing activity
Thyroxine (191.5 nmol/l)	Interleukin-1
Testosterone (< 20.0 ng/dl)	Interleukin-6
17-*b*-Estradiol (2.1 pg/ml)	Plasminogen activator, Collagenase, Prostaglandin E

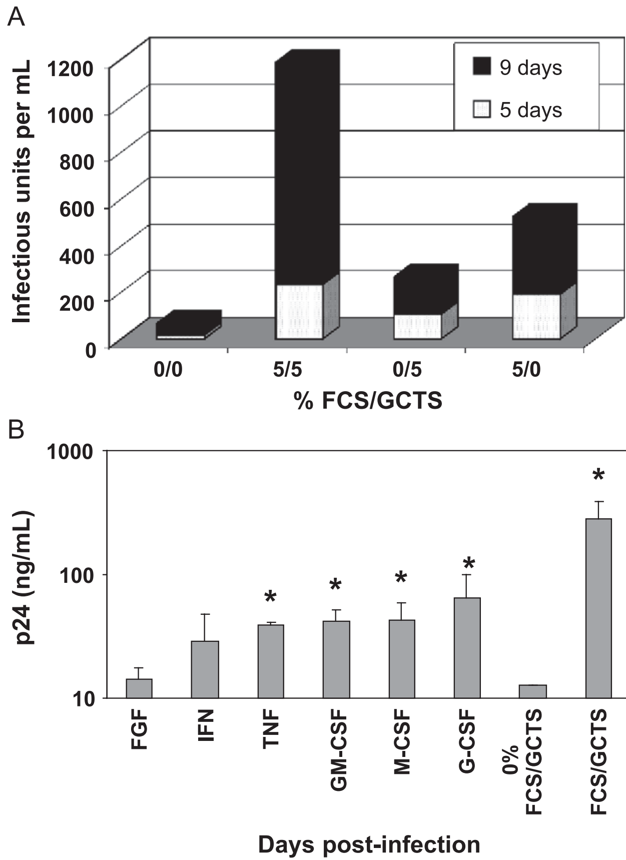

Fig. 2. Characterization of serum and cytokine components reactivating HIV in quiescently infected mixed glial cultures. (A) Cultures maintained in combinations of 0% or 5% FCS/GCTS as noted: 0/0, 5/5, 0/5, 5/0. The supernatant IU/ml was plotted for days 5 and 9 after infection. The combination of 5% FCS/GCTS induced a greater viral burst than either 5% FCS or 5% GCTS alone. (B) Some cytokines individually can reactivate HIV, but not as well as FCS/GCTS. "*" Indicates that there was significantly ($P < 0.05$) more virus as measured by $p24^{gag}$ assay than in the supernatant of the negative control cultures (nonactivating medium).

periods of time (Geijtenbeek et al., 2000; Spiegel et al., 1992), and because microglia expresses DC-SIGN (Shawver et al., 2000, and Maria Chen unpublished), it was theoretically possible that microglia cultured in nonactivating medium incorporated HIV into a compartment where the virus could survive without undergoing reverse transcription. Under such circumstances, inhibition of reverse transcription would alter the reactivation of viral replication when the activating medium was added to quiescently infected cells. Approximately 1 month after infection, the nonactivating medium was replaced with either nonactivating or activating medium, with or without 200 nM efavirenz, a non-nucleoside reverse transcriptase inhibitor. Efavirenz had no effect on HIV reactivation as measured by release of virus, although it inhibited de novo infection in previously uninfected glial cultures (data not shown and Albright et al., 2000a). Experiments with the nucleoside reverse transcriptase inhibitor AZT, antibodies against CXCR4 (12G5), CCR5 (2D7), and CD4 (#21), and a CCR5 small molecule inhibitor (TAKEDA 779) also had no effect on reactivation (data not shown).

These initial results indicated an HIV replication block after reverse transcription, but it was possible that small differences at several stages in the replication cycle could eventually result in inefficient HIV replication. We therefore performed real-time PCR for *gag* DNA to quantitatively detect a major product of reverse transcription and observed slightly higher levels of *gag* copies per cell in activating medium (range 10–30 copies/cell) compared to the levels in nonactivating medium (0–10 copies/cell) (Fig. 3). Throughout the entire time course of infection, several of these time points were significantly different ($P < 0.05$). Focusing on early times points (24–48 h postinfection), which is the estimated average time necessary for one round of replication transcription in macrophages (Schmidtmayerova et al., 1998), we observed only a 2- to 3-fold greater *gag* copies per cell in activating medium compared to nonactivating medium. DNAse treatment of viral stocks reduced the signal due to transfected DNA by 91–95%, making the contribution of this DNA minimal; therefore, the signal seen at day 0 in the nonactivated cultures may be due to intravirion reverse transcription within particles adhered to cells. Previous reports have estimated 10^3–10^4 copies of HIV DNA per 10^6 resting T lymphocytes (Chun et al., 1997a, 1997b), which is considerably lower but is not from a uniform infection (Pierson et al., 2000). With an MOI of 1, we expect a much higher frequency of infection of microglia in this system. Cumulative analysis from four separate experiments showed a similar trend (data not shown). Analysis

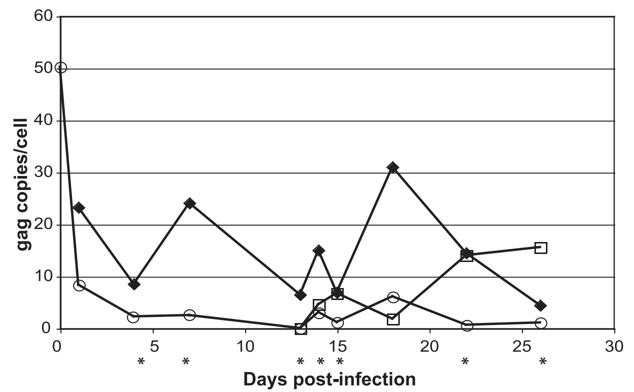

Fig. 3. Reverse transcription proceeds in cells cultured in nonactivating medium at levels similar to productive infection (activating medium). Real-time PCR was performed on DNA samples from various times after infection using HIV-specific primers and primers specific for GAP-DH, a cellular housekeeping gene. Levels of proviral genomes are presented as copies per cell to account for variations due to cell numbers in these primary cultures. Asterisks (*) indicate significant difference between nonactivating and activating medium using the nonparametric Mann–Whitney *U* test for significance between two data sets. Real-time PCR was performed on six sets of experimental samples; four using *gag* primers and two using *ltr-gag* primers. Data shown are from one representative experiment with *gag* primers. Each data point represents at least four PCR evaluations.

following reactivation 13 days postinfection also demonstrated a slight increase in DNA copies per cell (Fig. 3). PCR primers used to detect a later stage of reverse transcription, second-strand transfer, yielded similar results (data not shown). Therefore, we concluded that there was a slight difference in levels of reverse transcripts between cells in activating and nonactivating medium; however, this small difference was not able to account for the greater than 10-fold difference seen in virus release (Fig. 1).

We then looked at viral integration into the host cell genome, a process that has been found to have a predilection for transcriptionally active sites (Schroder et al., 2002). In long-lived T cells, post-integration latency has been proposed to be the most clinically relevant form of long-term latent infection (Cavert and Haase, 1998; Cavert et al., 1997; Perelson et al., 1997; Pierson et al., 2002). We used Alu–LTR PCR as previously described (Sonza et al., 1996) in either nonactivating or activating medium culture conditions at 1, 2, 3, and 5 days after infection (Fig. 4). Integrated genomes were detected as early as 1 day postinoculation in cells from both culture conditions, and through 2, 3, and 5 days postinfection (Fig. 4). To control for the presence of DNA in each sample, GAPDH was used in concurrent PCR assays. From these reverse transcription and integration results, we concluded that HIV integration occurs under both culture conditions.

Most of the current literature suggests that during post-integration latency, there is little or no viral transcription and concomitantly no protein expression (Cavert and Haase, 1998; Cavert et al., 1997). Therefore, we examined RNA production of a late viral transcript using quantitative RT-PCR specific for full-length viral RNA.

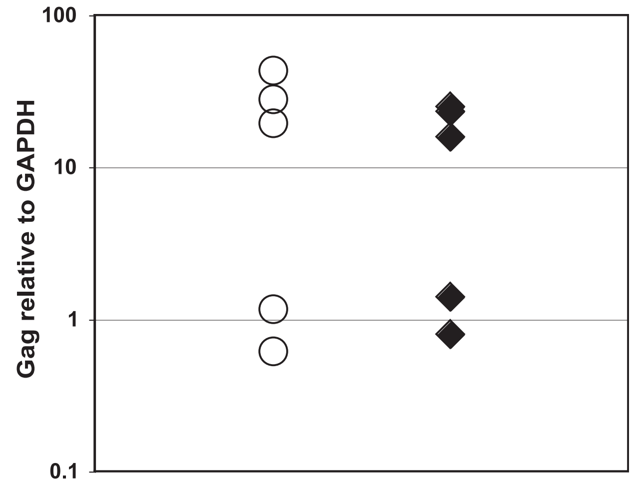

Fig. 5. HIV transcription occurs in activated and nonactivated cultures. Quantitative RT-PCR was performed on RNA isolated from activated and nonactivated mixed glial cultures from four different donors 3–5 days postinfection. To account for differences in cell number, HIV gag values are expressed relative to GAP-DH. (Open circles: nonactivated cultures; black diamonds: activated cultures).

Taking the level of transcription in the activated cultures as 100%, in five separate experiments (four donors), viral RNA was detected at a level of 123.4% (±52.9%) in nonactivated cultures (Fig. 5). No statistically significant difference in the relative ratio of gag/GAP-DH between the two culture conditions was found using ANOVA. We also performed immunofluorescence assays with antibodies against Gag protein as a test of the degree of gene expression in the cells maintained in nonactivating medium. Cells that had the morphological appearance of

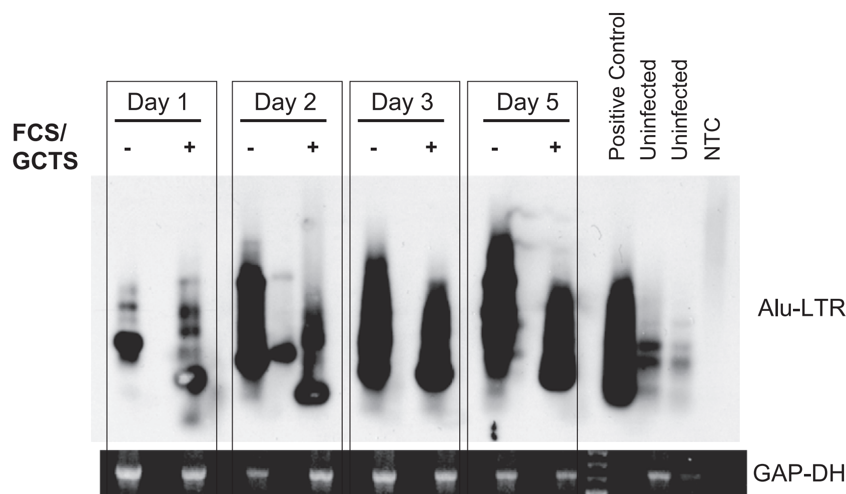

Fig. 4. Mixed glia cultured in nonactivating and activating medium contain integrated provirus. Alu–LTR PCR and Southern blotting with an HIV-1 LTR specific probe was performed on DNA from HIV-1_{YU-2}-infected mixed glia cultured in nonactivating or activating medium at days 1, 2, 3, and 5 postinfection. Integration was detected as early as 1 day (24 h) with signal seen through days 3 and 5 in this representative blot. GAP-DH PCR was performed to control for total DNA in each sample. DNA from uninfected glia from the same experiment, as well as a no template reaction, was used as negative controls, which contains some spillover signal. DNA from infected MAGI-CCR5 cells was used as a positive control. Signal in the center lane of day 2 represents loading error. This figure is representative of a total of five experiments.

microglia and stained positive for the microglial marker di-I-AcLDL (Strizki et al., 1996) were positive for gag expression using a polyclonal antiserum (Fig. 6), but the proportion of cells positive for viral antigen was greater in the cultures maintained in activating medium compared to cultures in nonactivating medium. At 4–8 days post-infection, activating medium cultures had on average 35.1% cells that were Gag positive; nonactivating medium cultures had an average of 6.4% Gag-positive cells (data not shown).

We then performed Western blots with a mouse anti-Gag (see Materials and methods) on cellular lysates from both culture conditions to examine $p55^{Gag}$ proteolytic processing. Surprisingly, using cells from four separate donors, we found that infected mixed glia in nonactivating culture conditions displayed a p24/p55 ratio of greater than 1 in comparison with the activated cultures (Fig. 7), suggesting premature cleavage of $p55^{Gag}$ under nonactivating conditions. In cells infected under nonactivating conditions then reactivated, the phenotype reverted to one where the p55 band predominated (data not shown).

It was possible that premature $p55^{Gag}$ proteolytic cleavage by a cellular protease was responsible for the defect in viral output observed in nonactivated cultures. To test this hypothesis, we infected activated and nonactivated mixed glia in the presence or absence of 5 μM Saquinivir, a specific inhibitor of the HIV-1 protease, and confirmed the

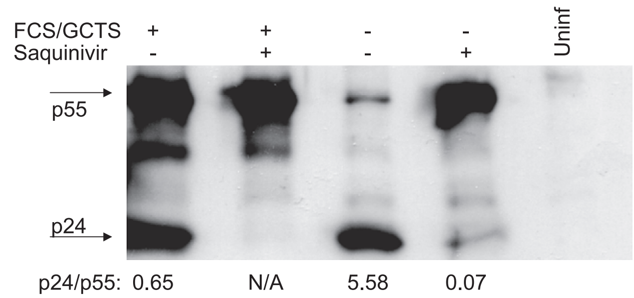

Fig. 7. Western blot performed with an anti-Gag serum demonstrates that the nonactivated mixed glial cultures have an altered $p24^{gag}/p55^{gag}$ ratio in comparison with the activated cultures. Treatment with 5 μM saquinivir confirms that processing of $p55^{gag}$ under either condition is due to a saquinivir-sensitive protease. This figure is representative of four experiments performed with cells from separate donors.

efficient suppression of $p55^{Gag}$ processing by Western blot (Fig. 7). HIV-1 gag-specific quantitative RT-PCR also detected viral RNA in the supernatants from activated cultures regardless of Saquinivir treatment, but no viral RNA was detected in the supernatants from nonactivated infected cultures in the absence or presence of inhibitor. Therefore, premature $p55^{Gag}$ cleavage is associated with this quiescent infection, but is not the proximate mechanism restricting viral production and release. Our data indicate the existence of a replication block that has no immediate

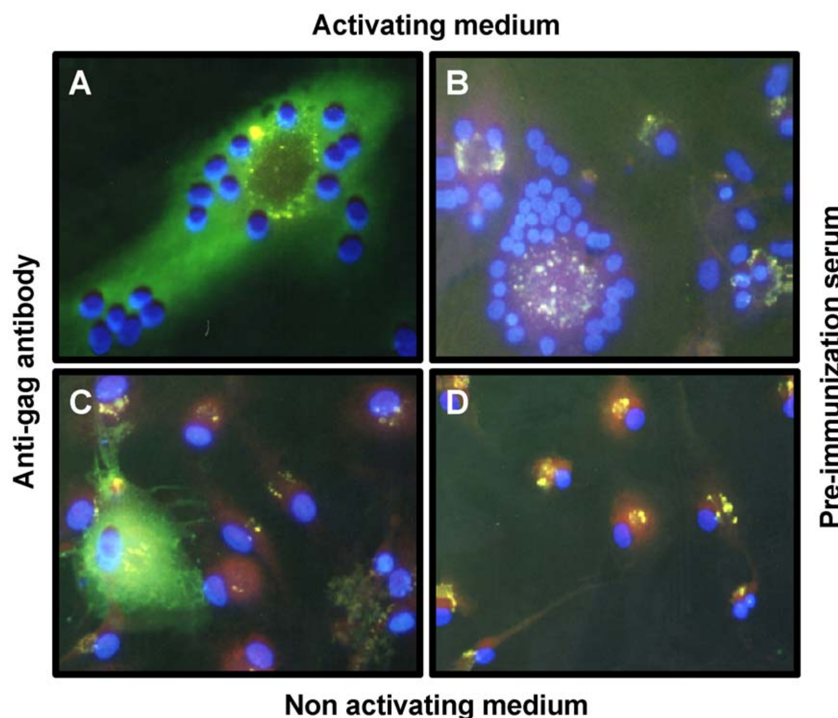

Fig. 6. Immunofluorescence microscopy detects HIV-1 Gag protein in infected cells cultured in either nonactivating or activating medium using a rabbit polyclonal antibody against Gag (UP598, a gift from Michael Malim, King's College London). Mixed glia, which morphologically appear to be microglia and in other experiments phagocytosed beads and stained for di-I-AcLDL (both microglial markers), were Gag positive in both activating (A) and nonactivating (C) conditions. This figure is representative of a total of six experiments.

parallels to any extant block seen with wild-type virus in primary cells or in other models of HIV latency.

Discussion

Because of their longevity and capacity to harbor lentiviral genomes for long periods of time, the microglia, the principal cell infected in the CNS (Bagasra et al., 1996; Koenig et al., 1986; Wiley et al., 1986), is a potential HIV reservoir (Cavert and Haase, 1998; Pierson et al., 2000; Ryzhova et al., 2002). Not coincidentally, microglia and brain macrophages are felt to be the key mediator of the neurologic disease caused by HIV, as they secrete neurotoxic products and viral proteins (reviewed in Kaul et al., 2001). However, the most consistent neuropathological correlation with HAD is the presence of activated microglia, as defined by the expression of immunocytochemical activation markers (Glass et al., 1995; Tyor et al., 1992; Wesselingh et al., 1993). Our experiments were designed to mimic the conditions that lead to activation by adding proteins known to activate microglia and brain macrophages. The nonactivating medium conditions represent the nonactivated state of the normal healthy CNS, while the activating medium conditions mimic the pathologically inflamed CNS found during disease and viral infection (Jordan et al., 1991; Strizki et al., 1996; Watkins et al., 1990).

Our experiments had three principal goals: (1) identification of the stage or stages at which the productive infection is blocked, (2) narrowing down the factors responsible for activation, and (3) most ambitiously, pinpointing the cellular pathways and specific molecules involved in the reactivation of HIV infection and the release of infectious virus.

Our initial bias was that our system would be similar to latent infection of T lymphocytes, and we were somewhat surprised to find that reverse transcription, integration, and viral transcription proceeded unimpeded, difficult as it is to quantify the Alu–LTR PCR results. Further investigation showed significant Gag expression in quiescently infected microglia, albeit to lower levels than those seen in a fully productive infection of cells cultured in activating conditions, suggesting that the block to viral production is posttranscriptional (Fig. 6). Therefore, in contrast to the long-term latency states in $CD4^+$ T lymphocytes, which are often due to inefficient initiation of transcription, decreased transcript elongation, or failure of export of unspliced viral mRNA (Butera, 2000), our model so far appears to demonstrate that HIV transcription and translation are proceeding in nonactivated cells.

Interestingly, we noted an unusual phenotype of alteration in the processing of $p55^{Gag}$. The premature processing was sensitive to inhibition by HIV protease inhibitors and is therefore unlikely to be due to a cellular protease. Krausslich (1992) reported a similar $p24^{Gag}$ accumulation phenotype in a transfected Gag/Protease system treated with low levels of a protease inhibitor, which is much different than our system. Additionally, we saw neither an increase in total Gag expression nor increased cytotoxicity in our nonactivated cultures in either the presence or absence of protease inhibitor (Kaplan and Swanstrom, 1991; Krausslich, 1992). Our intuitive hypothesis is that a key protein involved in viral assembly is upregulated or altered in the activated cultures, and while the altered processing is associated with this phenomenon, we do not believe it is the immediate mechanism of inefficient viral production.

We were not surprised that a wide range of cytokines and serum components made the microglia permissive for replication and reactivation of HIV (Fig. 2). Many different cytokines and cytokine receptors are expressed by activated macrophages, although the full set of molecules in the activation pathway has not been catalogued (Nau et al., 2002). Microarray data confirmed that the activating medium results in the robust activation of a specific set of genes, with significant increases in immune and cell signaling genes (Albright, unpublished data). Other than NEDD4, a factor reported to be involved in retrovirus budding or assembly (Kikonyogo et al., 2001; Strack et al., 2000), there were no obvious changes in proteins involved in HIV assembly (data not shown).

In conclusion, while reverse transcription and integration may be slightly impaired in nonactivating cultures, this alone cannot account for the greater than 10-fold difference in virus release between infected cells under the two conditions tested. Recent studies have helped delineate where and how HIV matures and buds in macrophages (Demirov et al., 2002; Ono and Freed, 2004; Pelchen-Matthews et al., 2003; Raposo et al., 2002), and as we continue to investigate the exact posttranslational replication block in our model, we are focusing on Gag biochemistry, protein localization, assembly, and budding. Because our model employs differential cellular activation or expression, future studies may lead to insights into late stage interactions between HIV and the host cell, as well as provide new therapeutic targets for HIV-1 infection.

Materials and methods

Isolation and culture of mixed macro- and microglia

We cultured microglia, astrocytes, and oligodendrocytes together in conditions that mirror those seen in a healthy CNS and did not favor viral replication (Albright et al., 2000b) through an adaptation of protocols previously published (Strizki et al., 1996; Yong and Antel, 1992). Three to 10 ml of fresh tissue, obtained as a by-product from individuals undergoing therapeutic surgery for epilepsy, were incubated with 0.25% trypsin and mechanically dissociated by forcing with a pipette through a cell strainer. The resulting cells and small debris were diluted in PBS

(without Ca^{2+} and Mg^{2+}) to remove the trypsin; notably, premium fetal calf serum (FCS; Atlanta Biologicals, GA) was not used for inactivation of trypsin. The mix of cells and debris was centrifuged for 10 min at 1750 × *g* and the resulting pellet was resuspended in culture medium with Dulbecco's modified Eagle medium (DMEM) supplemented with 4.5 g/l glucose, 50 mg/ml gentamicin, and 1 mM sodium pyruvate. FCS and giant cell tumor supernatant (GCTS; IGEN, Gaithersburg, MD), a fixture of this culture system, were omitted (nonactivating medium) (Table 1). The mix of cells and debris was plated directly onto either 6- or 96-well plates, or 8-well Lab-Tek Permanox chamber slides (Nalge Nunc International, Naperville, IL, USA) at a density of 1–2 ml of starting minced tissue per plate or chamber slide, and cultured in nonactivating medium. After 4–16 h, cultures were washed and cultured again in nonactivating medium. To increase yield, a 2nd plating of cells, not yet adhered to the plastic culture ware, was performed for an additional 24 h. All cells were then cultured for several weeks, with daily medium changes until all debris was gone, and more medium changes every 3–5 days thereafter.

Characterization of cultures

To determine the proportion of microglia in mixed glial cultures, a carboxyl polystyrene (latex) bead mix (Spherotech, Libertyville, IL) was added (1.6, 1.87, 2.17, 2.18, 3.18, 3.54, and 4.1 μm) in appropriate culture medium, with each bead type having a final concentration of 0.005% w/v. After incubation at 37 °C for 2–4 h, cells with more than 10 beads inside were considered to be live, functional microglia. Cell types, not of a macrophage lineage, that do not phagocytose beads were used as negative controls. Percentages of oligodendrocytes and astrocytes in mixed glial cultures were identified using immunofluorescence as previously described with antibodies against glial fibrillary acidic protein (GFAP, Novus) and GalCer (Albright et al., 1996).

HIV-1 infection of microglia cultured with or without FCS/GCTS

Microglia were cultured and infected at an approximate multiplicity of infection of one infectious virus units per cell, with several CCR5-tropic HIV isolates known to infect microglia: HIV-$1_{YU\text{-}2}$, HIV-1_{BaL}, and HIV-$1_{DS\text{-}br}$ (Albright et al., 1999; Popovic and Gartner, 1987). All viral stocks were titered, infectious units per ml (IU/ml), using an infectious MAGI assay as previously described (Albright et al., 2000a). The initial experiments were performed from Ba-L and DS-br viral stocks made in monocyte-derived macrophages (MDM). To obtain viral stocks in medium without FCS/GCTS, later experiments used the cloned HIV-$1_{YU\text{-}2}$ (RF-1), isolated from the brain of an individual with HIVD (Li et al., 1991) with its accessory genes repaired (Fouchier et al., 1997). Virus stocks were prepared by transfecting 293T cells that had been growing at log phase for at least 2 days. The cells were plated at a density of 300,000 or 3 × 10^5 cells/well and cultured in DMEM with 10% FCS after addition of 3 μg of the pYU-2(RF-1) plasmid for 4–16 h as previously described (Shieh et al., 1998). After transfection, the 293T cells were carefully washed and fed with nonactivating medium. Virus supernatants were collected 24 and 48 h after transfection and centrifuged for 10 min at 3000 rpm to remove cellular debris. Stocks were treated with 20 U/ml DNase for 1 h at 25 °C in 5 mM $MgCl_2$. To infect microglia, the virus was added and the nonactivating medium replaced, as indicated, with either standard microglia (activating) medium with 5% FCS (Atlanta Biologicals) and 5% GCTS (Fisher) (Strizki et al., 1996; Watkins et al., 1990), or nonactivating medium (as described above). In some experiments, efavirenz, a non-nucleoside reverse transcriptase inhibitor of HIV-1, was used at 200 nM, a concentration previously determined to be approximately 10 times greater than the in vitro IC_{90} concentration in microglia but that was not toxic to these cells (Albright et al., 2000a).

Virus release assays

Culture supernatants were assayed for the presence of (1) 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 (Albright et al., 2000b; Vodicka et al., 1997); and (2) Viral $p24^{Gag}$ antigen using the HIV-1 $p24^{Gag}$ ELISA kit according to manufacturer's instructions (Perkin-Elmer, Boston, MA).

Real-time PCR to detect HIV reverse transcription

To obtain DNA samples, cells were lysed in the following buffer: 100 mM KCl, 0.1% NP40, 20 mM Tris pH 8.4, 0.5 mg/ml Proteinase K, incubated at 56 °C for 2 h, and boiled for 15 min to inactivate the Proteinase K. Real-time PCR was then performed in 96-well plate format (USA Scientific Inc, #1404-9700) using an ABI Prism 7700 thermocycler and the following reagents: 0.25 mM dNTPs, 5 mM $MgCl_2$, 1 μM primers and probe, 1.25 U Ampli-Taq Gold. Cycling conditions were as follows: 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 60 °C for 1 min. ACH-2 DNA lysates were used to create a standard curve from which HIV copy number in experimental samples was calculated and statistically compared using the nonparametric Mann–Whitney *U* test for significance between two data sets. Primer and probe sequences are available as supplemental information. Gag DNA real-time PCR analysis utilized the published primers SK38 (nt 1542–1569) and SK39 (nt 1629–1656), and the probe SK19 (nt 1589–1620). All primers and probes were obtained from the University of Pennsylvania Center for

AIDS Research (CFAR). ACH-2 DNA was used to generate a standard curve for GAP-DH and data plotted as reverse transcripts per cell.

Alu–LTR PCR and Southern blotting to detect HIV integration

DNA samples were obtained as described above. Nested Alu–LTR PCR was performed with primers at a concentration of 0.5 μM (Alu) and 3 μM (LTR) for each round, and the following reagents: 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 2.5 U Ampli-Taq Gold. Cycling conditions for each round were 95 °C for 10 min; 40 cycles of 95 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min 40 s; and 72 °C for 7 min. After nested PCR, Southern blotting was performed to detect PCR products as per manufacturers instructions using the NEL624 Perkin-Elmer 3′-End labeling Biotin Kit with Streptavidin-AP. HIV-1 probe sequence was YU-2 LTR (nt 1–40). As a control, GAP-DH DNA was amplified simultaneously in parallel with the first round Alu–LTR PCR. Reaction conditions were identical, with 0.5 μM primer concentrations; all primer sequences are available as supplemental data.

Cytokines used for HIV reactivation

In the initial HIV reactivation experiments, quiescently infected microglia was treated with 500 ng/ml of the following growth factors and cytokines (R&D Systems, Minneapolis, MN): tumor necrosis factor-a (TNF-a), granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), fibroblast growth factor (FGF), and interferon-gamma (IFN). For later dose response curve experiments, the microglia was treated with 0–50 000 U/ml of growth factors or cytokines calculated using the WHO 1st International Standard Unit convention that is based on biological activity and R&D Systems' conversion factor for each growth factor or cytokine (available at www.rndsystems.com). Concentration ranges were based on the reported ED_{50} for each growth factor or cytokine.

Statistical analyses

Unless otherwise noted, the Student's *t* test was used for small samples ($n < 30$). In larger samples ($n > 30$), we tested the difference of means using *z* scores for normal curve area with the assumption of normality (Sokal and Rohlf, 1995). A type II error of less than 5% ($P < 0.05$) was allowed for all tests, unless otherwise indicated. To calculate 95% confidence interval estimates for the difference between two populations, we compared the averages of the two means (nonactivating vs. activating medium) and the appropriate *z* scores calculated (Sternstein, 1994).

Quantitative RT-PCR

Total RNA was isolated with Trizol Reagent (Invitrogen, Carlsbad, CA) 3–5 days postinfection. cDNA was made using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen #11904018) with Oligo (dT) as per manufacturer's instructions, and then used for real-time PCR analysis using molecular beacons (see above). Primers specific for gag and GAP-DH were used (see above), except for a different GAP-DH reverse primer specific for RNA sequences. The standard curve for relative GAP-DH values was based on dilutions of PBMCs from which RNA was isolated and cDNA synthesized.

Indirect immunofluorescence

Microglia cultured in 8-well Lab Tek chamber slides was infected and sampled as previously described (Albright et al., 2000b). Cells were washed twice in PBS, fixed with fresh 4% paraformaldehyde for 1 h at 37 °C to permeabilize the cell membrane, and incubated at room temperature with a blocking solution consisting of 0.1% BSA and 8% goat serum for 60 min (Albright et al., 2000b). Primary antibody staining was performed either with UP598, anti-Gag polyclonal rabbit antiserum (a gift from M. Malim), or with a pre-immunization antiserum from the same rabbit. Cells were then stained with a FITC-conjugated goat anti-rabbit IgG (whole molecule) (Sigma, #F-0511, St. Louis, MO), washed three times in PBS, and visualized with a Leitz Aristoplan microscope and I3 Ploemopak Fluorescent vertical illuminator. A 515-nm long pass suppression filter was used to distinguish FITC-positive microglia from background autofluorescence, which appears as a bright yellowish–green color, and is generally confined to perinuclear patches. The more conventional L3 illuminator, which has a 525/520 nm band pass suppression filter (Albright et al., 2000b), does not distinguish between FITC and autofluorescent signals. Photographs were taken with a Wild Leitz MPS46 Photoautomat camera exposing Kodak Ektachrome film (ASA400) for 12 s for all images such that the intensity of staining could be compared among different photographs.

Western blot analysis of Gag expression

Mixed glial cultures were infected with $HIV_{YU\text{-}2}$ as described above and cultured in the presence or absence of 5 μM Saquinivir mesylate. Seven days postinfection, cells were lysed in 0.5% Triton X-100 containing protease inhibitors (Complete Inhibitor Cocktail Tablets, Roche). Total protein concentration was determined using Bio-Rad DC Protein Assay, and 2 μg of total protein loaded per well. After SDS-PAGE and transfer to nitrocellulose, the membrane was blocked in 0.2% Tween 20 in PBS with 5% milk for 1 h at room temperature, followed by overnight incubation at 4 °C with mouse monoclonal antibody 24-3 (gift from M. Malim) that specially recognizes HIV $p24^{gag}$. A secondary

HRP-conjugated goat anti-mouse was used to detect p24^{gag}-containing Gag proteins and visualized using Pierce Super-Signal chemiluminescent reagents.

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