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Productive infection of primary murine astrocytes, lymphocytes, and macrophages by human immunodeficiency virus type 1 in culture

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A mouse model of human immunodeficiency virus type 1 (HIV-1) infection would be extremely valuable for evaluation of therapies and vaccines; however, multiple blocks to productive infection of NIH 3T3 and other mouse cell lines have been reported. The authors investigated the replication of HIV-1 in primary mouse astrocytes, lymphocytes, and macrophages in culture by infection with intact HIV-1 pseudotyped with the vesicular stomatitis virus G envelope glycoprotein (VSV-G) or with the envelope glycoprotein of amphotropic murine leukemia virus. Astrocytes, lymphocytes, and macrophages were susceptible to productive infection as variously assayed by detection of p24 and Tat proteins, viral protease-mediated processing of Gag, appropriately spliced viral RNA, and infectious progeny virus. As expected, NIH 3T3 cells were not susceptible to productive infection by VSV/NL4. Susceptibility mapped neither to the *Fv* locus nor to a possible polymorphism in cyclin T1. This study indicates that there are no intrinsic intracellular barriers to HIV-1 replication in primary mouse cells when virus entry is efficient. *Journal of NeuroVirology* (2004) **10,** 400–408.

Keywords: HIV-1; tropism; mouse model; pseudotype

Introduction

Human disease and death associated with the HIV-1 pandemic demand better model systems for the evaluation of effective, inexpensive therapies, and especially for therapeutic or protective vaccines. Many investigators attempted to capitalize on the advantages of the mouse to develop an animal model of human immunodeficiency virus type 1 (HIV-1) infection or disease. As early as 1988, it was clear that cells of the central nervous system from rodents were susceptible to cytopathic effects of HIV-1: rodent neurons could be killed directly by HIV-1 through exposure to viral gp120 or killed by exposure to neurotoxins produced by human macrophages activated by HIV-1 (Brenneman *et al*, 1988; Dreyer *et al*, 1990). Murine macrophages also could be activated by HIV-1 to secrete neurotoxins (Merrill *et al*, 1992) and rodent astrocytes were shown to respond to gp120 by defects in several defects in ion transport (Benos *et al*, 1994). But HIV-1 replication was not easily modeled in the mouse. By 1986 it was clear that the murine fibroblast

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cell line, NIH 3T3, failed to transcribe genes driven by the HIV-1 long terminal repeat (LTR) (Levy *et al*, 1986) and that the expression of human CD4 failed to confer susceptibility to HIV-1 to these and other transformed mouse cells (Maddon *et al*, 1986). Independent, intracellular blocks to HIV-1 infection of murine fibroblast cell lines were shown at the level of both Tat and Rev function (Winslow and Trono, 1993). Indeed, blocks to HIV-1 replication have been reported at the level of virus entry, transcription, RNA transport, protein processing, and virion assembly (Bieniasz and Cullen, 2000; Mariani *et al*, 2000). The deficiency in Tat function leading to an overall reduction in HIV-1 transcription in murine fibroblast cell lines was attributed to the inability of murine cyclin T1 to form a functional complex with Tat and TAR RNA (Kwak *et al*, 1999). In recent studies, another defect was described in 3T3-derived cell lines in excessive splicing of viral RNA (Zheng *et al*, 2003), leading to expression of proteins encoded by multiply spliced RNA (Hatziioannou *et al*, 2004).

However, reports of virus production from mice transgenic for the HIV-1 genome indicate some greater permissiveness to virus replication in primary cells than in transformed cells. In large studies of mice carrying various HIV-1 genomes under the control of a complex CD4 promoter, viral RNA and both structural and regulatory viral proteins were detected in spleen and particularly in thymus cells (Hanna *et al*, 1998a, 1998b). In this model, Tat-mediated transactivation is not required and the proposed Rev defect did not impair virus production. In a different mouse model in which HIV-1 transgenes integrated in tandem into the cellular genome, viral capsid antigen was present in plasma at constitutive levels of 100 to 400 pg p24 per milliliter, values similar to those observed in aquired immunodeficiency syndrome (AIDS) patients prior to therapy (Doherty *et al*, 1999; Ho *et al*, 1989). Similar results were obtained in another HIV-1 transgenic mouse strain (Browning Paul *et al*, 2000). These HIV-1 constructs rely on the HIV-1 LTR to direct transcription. These studies clearly suggest that although many transformed murine cell lines restrict HIV-1 replication, primary cells are permissive to infection, once the block to virus entry is circumvented.

We have a long-standing interest in HIV-1–associated neuropathogenesis and reported that CD4-negative primary human astrocytes, despite restrictions in entry of native HIV-1, can be infected efficiently by HIV-1 pseudotyped with the vesicular stomatitis virus G (VSV-G) envelope glycoprotein, a virus that carries both VSV and HIV-1 envelope glycoproteins (Canki *et al*, 2001). This observation raises the possibility that this approach will permit detectable HIV-1 replication in murine cells in culture. In the present study, using intact NL4-3 pseudotyped with VSV-G or amphotropic murine leukemia virus (MLV) envelope glycoproteins, we found that primary murine astrocytes, lymphocytes, and macrophages were susceptible to productive HIV-1 infection. Infected primary murine cells produced Tat and mature Gag protein, appropriately singly spliced viral RNA, and infectious progeny virus. As consistently reported elsewhere, (Levy *et al*, 1986; Maddon *et al*, 1986), NIH 3T3 cells failed to produce structural protein during infection by pseudotyped NL4-3. We conclude that there are no major intrinsic intracellular restrictions in HIV-1 replication in primary mouse cells in culture.

Results

Mouse astrocytes, splenic lymphocytes, and peritoneal macrophages produce HIV-1 p24 upon infection in culture

In direct analogy to our previous study using human fetal astrocytes, we tested murine neonatal astrocytes for their susceptibility to VSV/NL4 in culture by assay of extracellular HIV-1 core antigen p24 over time after infection (Figure 1A). Cells reached a peak of

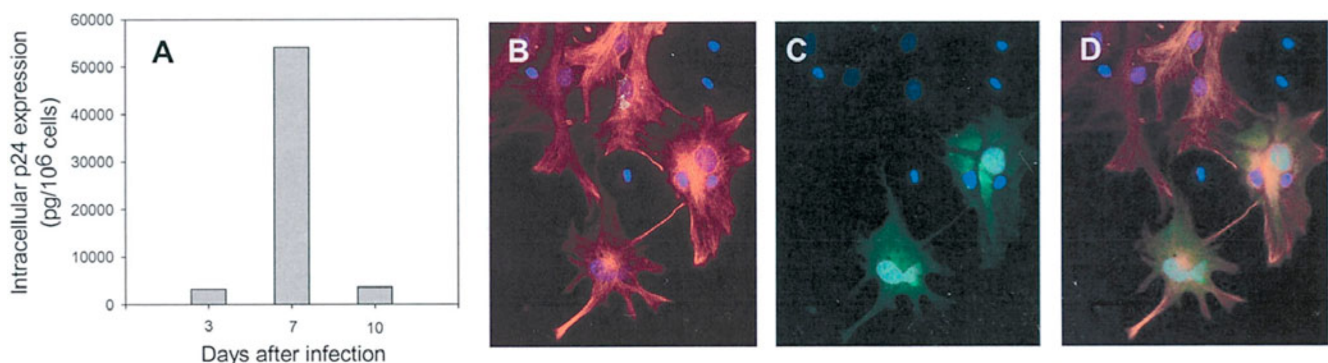

Figure 1 VSV/NL4 infection of primary murine astrocytes monitored by p24 or GFP marker protein expression. (**A**) Neonatal C57BL/6 astrocytes were infected in culture at 1.0 pg p24 per cell and supernatant was sampled over time for p24 content by ELISA. (**B–D**) Neonatal C57BL/6 astrocytes were infected in culture with VSV/NL4-GFP and 48 h later infected cells were stained for GLT-1 as a marker of astrocytes and for detection of nuclei and examined under a fluorescence microscope as described. **B**, GLT-1 (*red*); **C**, GFP (*green*); **D**, overlay of **B** and **C**. The actual magnification was 400×.

p24 production 7 days after infection. To estimate the frequency of infection, murine astrocytes were infected with an HIV-1 in which the green fluorescent protein (GFP) coding region was inserted into Nef so that productively infected cells express GFP early in infection, a time when Nef expression is normally detected in HIV-1–infected cells (Klotman *et al*, 1991). Cells were examined microscopically 2 days after infection (Figure 1B). About 10% of cells were productively infected. We then extended the murine cell types tested to include macrophages and lymphocytes, the principal targets of HIV-1 in human beings. Peritoneal macrophages or splenic lymphocytes were isolated from adult BALB/c mice and infected in culture with VSV/NL4. Cells were cultured in the presence or absence of 2 μM 2′,3′-dideoxycytidine (ddC), an inhibitor of reverse transcription (Mitsuya and Broder, 1986). NIH 3T3 cells and human monocyte-derived macrophages were infected in parallel. Infection was monitored by the production of intracellular core antigen p24 over time after infection (Figure 2A). Mouse macrophages were susceptible to VSV/NL4 and their production of p24 increased with time after infection; the inhibition of p24 production by ddC treatment measured at all time points demonstrates that p24 production observed in untreated cells derives from virus replication (Figure 2B). In contrast and consistent with previous studies (Levy *et al*, 1986; Maddon *et al*, 1986), NIH 3T3 cells failed to produce p24 and human macrophages produced 7.5×10^5 pg p24 per 10^6 cells at the peak of infection, about 10-fold more than in mouse macrophages. Similar results were obtained using NL4-3 pseudotyped with MLV envelope or strain 129 macrophages (not shown). HIV-1 infection in lymphocytes was monitored in intracellular and extracellular compartments, p24 levels in cell supernatants are displayed in the inset (Figure 2B). Replication was detectable at day 5, was substantial by day 7, declined by day 11, and was completely inhibited by ddC. Similar results have been obtained using splenic lymphocytes from C57BL/6 mice with peak p24 levels in the range of 200,000 to 500,000 pg per 10^6 cells (not shown). These findings indicate that murine astrocytes, lymphocytes, and macrophages are susceptible to productive infection by pseudotyped HIV-1. However, most of the p24 produced was retained in the intracellular compartment and little extracellular p24 was detected, as reported elsewhere for murine cell lines (Bieniasz and Cullen, 2000; Mariani *et al*, 2000).

Relationship between susceptibility to HIV-1 and Fv *locus and cyclin T1 polymorphism*

There is a strain specific restriction among mice for replication of murine retroviruses conferred by the host *Fv* locus (Jolicoeur, 1979). BALB/c and C57BL/6J are $Fv^{b/b}$ and Swiss, AKR, DBA/2, and 129 are $Fv^{n/n}$. In an analogous system, human cells pose a restriction similar to $Fv^{b/b}$ (Towers *et al*, 2000). Viruses classified as N-tropic, a determinant present in Gag, can replicate only in $Fv^{n/n}$ hosts; conversely, B-tropic viruses replicate only in $Fv^{b/b}$. If HIV-1 behaves as a B-tropic virus, then this distinction may explain the susceptibility of BALB/c cells and the resistance of NIH 3T3 cells that are derived from a Swiss mouse. We obtained spleen cells from AKR, BALB/c, and DBA/2 spleen cells and infected them with VSV/NL4 at 0.1 and 0.5 pg p24 per cell. Two

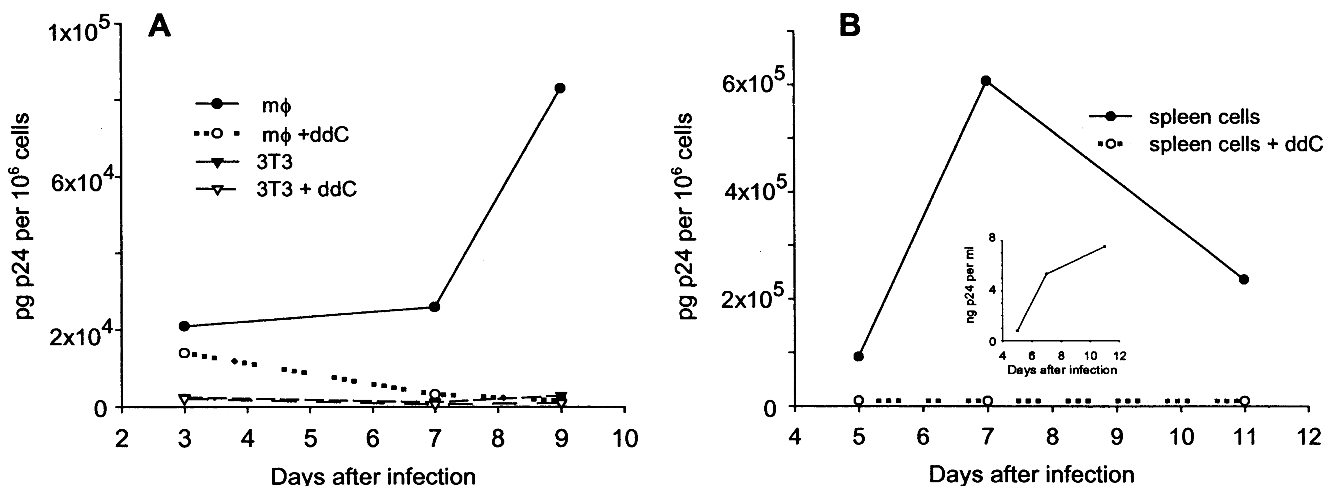

Figure 2 VSV/NL4 infection of BALB/c macrophages or lymphocytes monitored by p24 production. (**A**) Peritoneal macrophages or NIH 3T3 cells were exposed to VSV/NL4 at 1.0 pg p24 per cell, washed, and returned to culture in the presence or absence of 2 μM ddC. Cells were collected at the indicated times for the measurement of p24 production by ELISA. (**B**) Murine splenic lymphocytes were stimulated with mitogens for 2 days in culture prior to infection with VSV/NL4 at 1.0 pg p24 per cell. After infection and washing, cells were returned to culture and supernatant and cells were harvested at the indicted times for the measurement of p24 production by ELISA. The inset shows the levels of p24 in cell supernatants.

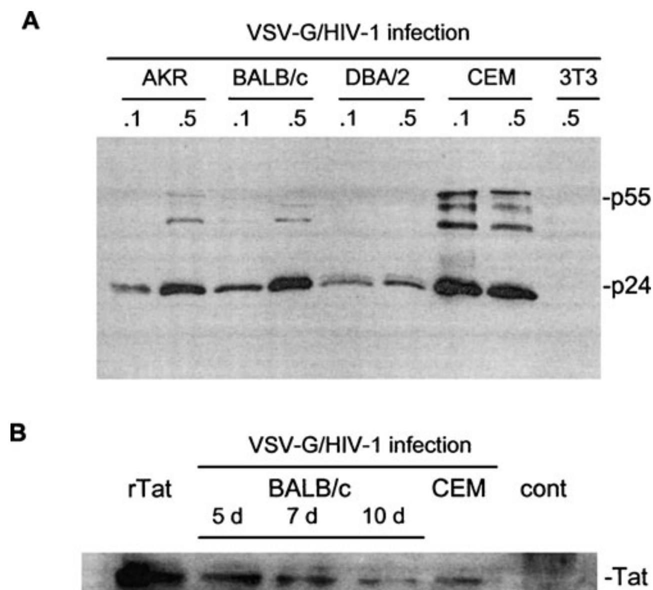

Figure 3 VSV/NL4 infection of lymphocytes of different mouse strains monitored by Western blot staining for Gag or Tat. (**A**) Lymphocytes or NIH 3T3 cells were cultured and infected as described in the legend to Figure 3, except different mouse strains and different virus doses were used as indicated. Cells were harvested 10 days after infection for Western blot staining with monoclonal anti-p24. (**B**) BALB/c lymphocytes were infected with VSV/NL4 at 0.5 pg p24 per cell and harvested at the indicated times for Western blot, staining with polyclonal anti-Tat antibody. As positive controls, CEM cells were infected in parallel and harvested 5 days after infection.

doses were employed because the *Fv* restriction can be overcome at high virus doses (Jolicoeur, 1979). Human CEM cells and NIH 3T3 were infected in parallel (Figure 3A). Cells were tested for Gag expression by Western blot. Lysates were standardized by cell number. Spleen cells from each mouse strain were susceptible to productive HIV-1 infection, although not as extensive as human CEM cells infected in parallel. By contrast, NIH 3T3 cells contained no detectable Gag, indicating that they are not susceptible to HIV-1 replication and confirming that the viral core antigen detected in mouse spleen cells is newly synthesized. The efficient processing of the Gag p55 to mature core antigen p24 that requires viral protease demonstrates that both Gag and Gag-Pol transcripts were synthesized, transported from the nucleus, and translated and that p55 associated closely with either Gag-Pol or HIV-1 protease, probably in assembly complexes, for orderly protease-mediated processing to take place. A similar infection of BALB/c spleen was conducted and evaluated by Western blot staining for viral Tat (Figure 3B). Recombinant Tat was run in parallel. Tat was produced within 3 days of infection, but its expression in infected mouse cells declined with time. Thus both $Fv^{n/n}$ (AKR and DBA/2) and $Fv^{b/b}$ (BALB/c) cells are susceptible to VSV/NL4, indicating no *Fv* restriction to HIV-1 replication in mouse cells.

It has been reported that due to a bulge conferred by tyrosine 261, murine cyclin T1 fails to substitute for human cyclin T1 in formation of a complex with Tat and TAR required for efficient HIV-1 transcription (Kwak *et al*, 1999). We investigated the possibility that inbred mouse strains carry different alleles of cyclin T1 that might function more efficiently with Tat and TAR. DNA was isolated from NIH 3T3 cells and from BALB/c, C57BL/6, and 129 spleen cells and a region of the cyclin T1 gene encoding tyrosine 261 was amplified by polymerase chain reaction (PCR) and sequenced (data not shown). All sequences were identical to the reference sequence (accession number NM 009833). On this basis, we cannot attribute the susceptibility of the mouse strains tested to a cyclin T1 sequence that might facilitate complex formation with Tat. It is worth noting that rat cyclin T1 also carries tyrosine 261 and that Tat has been directly observed to function well in rat cells (Keppler *et al*, 2001). Perhaps other nuclear factors substitute for human cyclin T1 in certain rodent cells, but not in NIH 3T3.

Appropriate viral RNA splicing during HIV-1 infection of mouse spleen cells

Another defect reported during HIV-1 replication in NIH 3T3 cells was excessive splicing of viral RNA, leading to a paucity of unspliced and singly spliced RNA for translation (Zheng *et al*, 2003). We investigated the level of singly spliced RNA in mouse spleen cells after infection with VSV/NL4 by reverse transcriptase (RT)-PCR, amplifying a region present only in singly spliced Vif mRNA (Figure 4). Vif mRNA was detected in two independent infections and increased with time after infection. These findings indicate that some viral RNA is appropriately spliced during infection of mouse cells and can be used for translation, consistent with our detection of viral Gag translated from an unspliced mRNA.

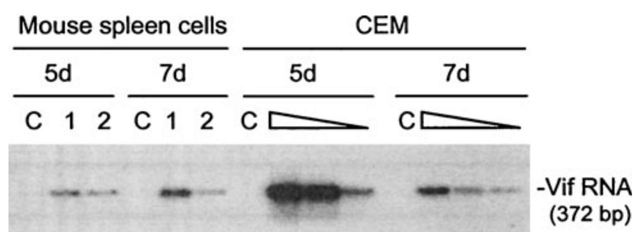

Figure 4 VSV/NL4 infection of BALB/c lymphocytes monitored by RT-PCR amplification of singly spliced Vif transcript. Cells were cultured and infected as described in the legend to Figure 3, except the virus dose was 0.5 pg p24 per cell. At day 5 and day 7 after infection, cells were harvested and RNA was extracted for amplification of a region in the Vif transcript flanking the single splice site. C−: uninfected mouse lymphocytes; M_1: infected cells from mouse 1; M_2: infected cells from mouse 2. CEM cells infected in parallel served as positive controls, the cDNA was diluted 10-, 50-, and 250-fold relative to cDNA from mouse cells prior to amplification.

Production of infectious progeny virus by HIV-1–infected primary mouse cells

To investigate the completion of the viral life cycle in HIV-1–infected mouse cells, we tested the infectivity of both cell-free and cell-associated progeny virus. HIV-1 present in supernatant of MLV/NLHXADA-GP–infected mouse macrophages or human H4 cells was titered for infectivity on MAGI-CCR5 cells (Chackerian *et al*, 1997). The infectivity of progeny virus was comparable, H4-derived virus contained 1 infectious unit per pg p24, virus produced by infected mouse spleen cells contained 0.5 infectious unit per pg p24. The staining for β-galactosidase production by the indicator cell line exposed to murine-derived virus is shown in Figure 5A. Figure 5B shows the response to supernatant of mock-infected murine cells, and Figure 5C shows the infectivity of human cell derived HIV-1. We assayed also cell-to-cell transmission of HIV-1 from mouse cells using 1G5 cells, an indicator cell line that expressed luciferase upon HIV-1 infection (Aguilar-Cordova *et al*, 1994) (Figure 5D). NL4-3–infected CEM cells served as positive controls. Infectious progeny virus transmissible by cocultivation was produced by mouse spleen cells and by CEM. Our studies indicate that when virus entry is efficient, HIV-1 can complete its life cycle in primary murine cells.

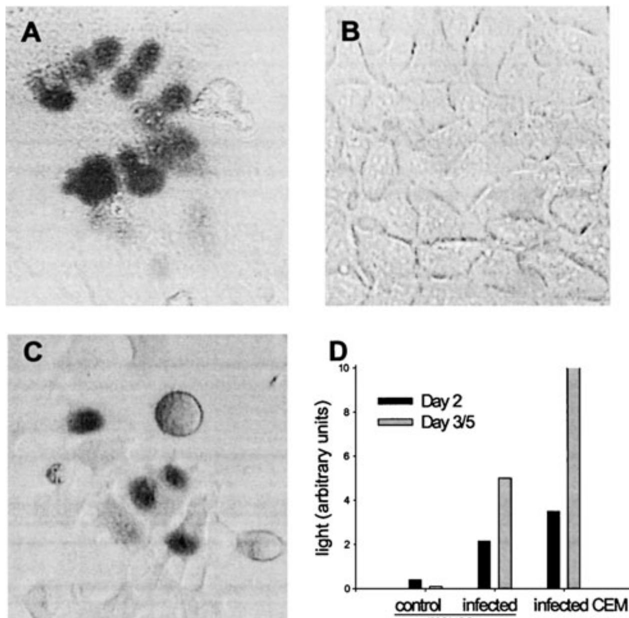

Figure 5 Production of infectious HIV-1 by infected mouse macrophages or lymphocytes. (**A–C**) BALB/c macrophages or human H4 cells were infected with MLV/NLHXADA-GP, washed, and returned to culture and supernatants were harvested after 10 days for MAGI assay. Staining of CCR5-MAGI cells is shown (**A**) exposed to macrophages supernatant, (**B**) mock infected, (**C**) exposed to H4 cells supernatant. (**D**) 1G5 cells were cocultured with VSV/NL4-infected mouse spleen or CEM cells. At the times indicated, cultures were harvested and standardized by protein content for assay of luciferase activity, shown in arbitrary light units.

Discussion

Our results demonstrate that there is no intrinsic species-specific barrier to HIV-1 replication in mouse cells, once the block to virus entry is circumvented. In primary astrocytes, lymphocytes, and macrophages, productive HIV-1 infection is readily established and replication can be completed through the production of infectious progeny virus, although there is a relative defect in export of virus particles. Overall, our findings recommend a reevaluation of attempts to create a mouse model of HIV-1 infection that reproduces many or all phases of the virus life cycle.

Many investigators have reported that murine neural cells are susceptible to HIV-1 driven cytopathic effects, including changes in gene expression as well as cell death (Benos *et al*, 1994; Dreyer *et al*, 1990; Merrill *et al*, 1992). In the present work, we demonstrated a complementary response of murine cells to HIV-1 that is productive infection. Astrocytes, lymphocytes, and macrophages, were consistently susceptible to infection by pseudotyped HIV-1 (Figures 1 to 5). Virus replication could be scored by traditional means, protein detection by enzyme-linked immunosorbent assay (ELISA), immunocytochemistry, Western blot, and assay of infectious progeny virus, as well as by assay of appropriate viral RNA splicing. In contrast, NIH 3T3 presented blocks to HIV-1 replication after efficient virus entry. The observed production of p24 protein by VSV/NL4-infected primary cells of several mouse strains and not by NIH 3T3 cells indicates a fundamental difference in susceptibility between primary cells of multiple lineages and the cell line. The defects in HIV-1 replication in NIH 3T3, including Tat function (Winslow and Trono, 1993), Rev function (Zheng *et al*, 2003), or expression of regulatory but not structural proteins (Hatziioannou *et al*, 2004; Mariani *et al*, 2000), do not adequately reflect the behavior of HIV-1 in murine cells.

Even when the block to virus entry is circumvented, HIV-1 replication may be limited to a greater extent in transformed cells than in primary cells. Considering CD4-negative human astrocytes, HIV-1 Rev functions poorly in some transformed cell lines due to excessive nuclear export and infected cells express little viral structural protein (Ludwig *et al*, 1999). However, primary human astrocytes show appropriate expression of all three HIV-1 transcript classes and productive infection, once the virus entry barrier is surmounted (Canki *et al*, 2001). Among the impediments to full HIV-1 expression in NIH 3T3, a Rev defect was recently described that can be corrected by expression of human p32 protein, an inhibitor of splicing (Zheng *et al*, 2003). Although we did not directly assay Rev activity, the expression of Gag in primary mouse cells and the production of infectious progeny virus demand that unspliced genomic viral RNA reach the cytoplasm for translation.

Specific features of NIH 3T3 also may be responsible for the extensive defect observed in Tat activity that can be complemented by expression of human cyclin T1 (Kwak *et al*, 1999). However, not all rodent fibroblast cell lines present such barriers to HIV-1 replication. Rat 2, a rat fibroblast cell line, showed efficient Tat-mediated transactivation and was permissive to HIV-1 expression conferred by transfection in our studies and those of others (Keppler *et al*, 2001; Mizrachi *et al*, 1992). The mouse appears to resemble the rat in permitting Tat function despite the conformation of cyclin T1 (Keppler *et al*, 2001), perhaps through the action of a different nuclear protein to facilitate the Tat/TAR interaction.

Our results indicate that virion export is inefficient in mouse lymphocytes (Figure 2), as was previously reported in mouse cell lines (Bieniasz and Cullen, 2000; Koito *et al*, 2003; Mariani *et al*, 2000). This restriction may also apply to rat lymphocytes in which the extent of viral (or marker) protein expression in cell lysates was much greater than in cell supernatants (Keppler *et al*, 2001). The authors interpret the difference as one of early versus late viral gene expression, but the alternative interpretation may also apply. This behavior is reminiscent of HIV-1 assembly in primary human macrophages, in which the bulk of particles bud into intracellular membranes rather than through the plasma membrane (Orenstein *et al*, 1988) and is also characteristic of the prototypical lentivirus, visna (Narayan *et al*, 1982). It is interesting that in an simian immunodeficiency virus (SIV) model of infection, macrophages can constitute the primary reservoir of virus and maintain high viral burdens in the animal (Igarashi *et al*, 2001). This observation suggests that high levels of virion export are not required for persistent virus infection in a host.

Because our results show that bypassing virus entry permits productive infection of murine cells, why has construction of mouse cells transgenic for human CD4 and either CXCR4 or CCR5 failed to confer susceptibility to HIV-1? In these lines, the human proteins are expressed by lymphocytes and macrophages that already express the analogous rodent receptors. Mice transgenic for human CD4 and CXCR4 displayed altered T-cell migration, attributed to excess receptors for the chemotactic factor (stromal derived factor-1) SDF-1, and their cells were poorly susceptible to virus in culture (Sawada *et al*, 1998), as were lymphocytes from mice transgenic for human CD4 and CCR5 (Browning *et al*, 1997). However the expression of human CD4 and CCR5 in these animals was at least 10-fold lower than expression of murine CD4 and CXCR4, and both receptor density and association are critical to efficient HIV-1 cell-fusion (Tuttle *et al*, 1998; Wu *et al*, 1997). In fact, coreceptors have been observed to compete for association with CD4 on cell surfaces and HIV-1 fusion efficiency can be reduced by a competing coreceptor expressed on susceptible cells by transfection (Lee *et al*, 2000). These studies indicate that creating a complex of human receptors on murine cells able to mediate efficient HIV-1 entry depends on receptor ratios and endogenous surface protein display as well as receptor display itself. More recently, transgenic rats that express human CD4 and CCR5 were constructed and their macrophages were permissive to HIV-1 replication in culture (Keppler *et al*, 2001), but the initial studies revealed only a modest infection in the animal (Keppler *et al*, 2002).

We demonstrate that primary murine astrocytes, lymphocytes, and macrophages are permissive to HIV-1 replication in vitro when virus entry is efficient, although HIV-1 production in mouse cells does not match production in human cells. Preliminary studies are being conducted to optimize HIV-1 entry into murine cells in vivo. Development of a useful mouse model of infection will require further research to improve both HIV-1 entry and HIV-1 production in murine cells. Similar findings regarding the susceptibility of murine cells to pseudotyped HIV-1 have recently been published by Hinkula et al (2004).

Materials and methods

Primary murine cells

All animal studies were conducted with the approval of the St. Luke's-Roosevelt Hospital Center and the University of Rochester Medical Center institutional animal care and utility committees. Adult female AKR, BALB/c, C57BL/6, DBA/2, and 129×1 mice were purchased from Jackson Labs. Mice were sacrificed by carbon dioxide asphyxiation, spleens were surgically removed, and peritoneal macrophages were harvested by peritoneal wash. A single-cell suspension of spleen cells was prepared by gentle homogenization in a TenBroeck homogenizer and lysis of erythrocytes using ACK, lysing buffer (BioWhittaker) according to the manufacturer's instructions. Mouse cells were cultured in RPMI-1640, supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol. For the first 2 to 3 days of culture, spleen cells were stimulated with 1 μg per milliliter concanavalin A (Con A) and 10 units per milliliter murine interleukin-2 (Sigma, St. Louis, MO), Con A was then omitted. For preparation of neonatal astrocytes, 2-day-old C57BL/6 mice were sacrificed, brains were surgically removed, and astrocytes were isolated as described (Fedoroff and Richardson, 2001). After 3 weeks in culture and confirmation for expression of glial fibrillary acid protein (GFAP) by >98% of cells, infection by HIV-1 was tested.

Cell lines

NIH 3T3, 293T, MAGI-CCR5 (Chackerian *et al*, 1997), and H4 cells were cultured as monolayers in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal FBS, glutamine, and

antibiotics. 1G5 cells, which carry a firefly luciferase gene under the control of the HIV-1 LTR, were obtained from the AIDS Research and Reference Reagent Repository and were cultured in RPMI-1640, 5% FBS, glutamine, and antibiotics; the human T-cell leukemia cell line CEM was cultured in the same medium.

Viruses and infection

Infectious HIV-1/NL4-3 expressing GFP was constructed by PCR using the plasmids pNL4-3 and pGFP-C1. Details will be published elsewhere (Li, manuscript in preparation). Intact HIV-1/NL4-3 pseudotyped with the VSV-G glycoprotein or NLHXADA-GP (Westervelt *et al*, 1991) pseudotyped with amphotropic MLV envelope glycoprotein (Page *et al*, 1990) were prepared by transfection of 293T cells as described previously (Canki *et al*, 2001). Virus stocks were titered by assay of p24 content by ELISA using the HIV Ag kit (Coulter, Hialeah, FL). Cells were infected at 0.1 to 1 pg p24 per cell for 1 to 2 h at 37°C, followed by washing in phosphate-buffered saline (PBS). Infection was monitored by measurement of p24 content in cell supernatants and cell lysates by ELISA. Cell lysates were prepared using the instructions and lysis buffer supplied by the manufacturer and standardizing to the number of viable cells.

Fluorescence microscopy

The mouse fetal astrocytes were grown and infected with VSV/NL4GFP on round coverslips in 12-well plates. Two days after infection, cells were fixed with 2% paraformaldehyde, counter-stained with 1 ng per milliliter of 4,6-diamidino-2-phenylindole (DAPI), and coverslips were mounted on glass slides for microscopy. Slides were stained with rabbit anti-GLT-1 (glutamate transporter-1) and Texas Red conjugated anti-rabbit immunoglobulin (Ig) to visualize astrocytes. Fluorescence images were captured by using a digital video imaging microscope system consisting of a Zeiss Model Axioplan 2 microscope (Carl Zeiss) with a HAMAMATSU ORCA-ER digital camera (HAMAMATSU Corp) and Openlab software (Improvision).

Western blot

Cell extracts were prepared in lysis buffer (137 mM NaCl, 50 mM Tris, pH 7.2, 3 M $MgCl_2$, 0.5 mM dithiothreitol (DTT), 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, and 100 μM Leupeptin) at 1–2 $\times$ 10^7 cells per milliliter. Extracts were electrophoresed in 10% polyacrylamide gels (Bio-Rad), proteins were transferred and immunoblotted with monoclonal anti-p24 obtained from the AIDS Research and Reference Reagent Repository or with polyclonal anti-Tat kindly provided by Dr. A. Nath. Blots were then stained with horseradish peroxidase–conjugated secondary antibodies before visualization by enhanced chemiluminescence system (Amersham).

Cyclin T1 sequencing

Total genomic DNA was isolated from NIH 3T3 cells and from spleen cells from BALB/c, C57BL/6, and 129 $\times$ 1 mice using DNazol (Invitrogen). Primers were designed based upon the murine cyclin T1 sequence (accession number NM 009833) to amplify a coding region including tyrosine 261. PCR amplification was conducted as reported (Kim *et al*, 2003) using the forward primer (5′-CCCCACCTCCAGTAAACAAA-3′) and the reverse primer (5′-GTTGACATGCTCATTAATCC-3′). The PCR products were purified by electrophoresis in agarose and sequenced at the Columbia University Comprehensive Cancer Core Facility using the forward primer as a sequencing primer.

RT-PCR

RNA was isolated from spleen cells or CEM cells using Trizol (Invitrogen) and converted to cDNA using the Superscript First Strand Synthesis System (Invitrogen). Primers were designed to amplify a region around the single splice site in Vif mRNA. PCR was conducted using forward primer (5′-GTGTGGAAAATGTCTAGCAGTGGCGC-3′) and reverse primer (5′-ACCAGTCCTTAGCTTTCCTTGAAATATAC-3′) for 40 cycles, the PCR product of 372 bases was subjected to electrophoresis and Southern blot and detected by hybridization using the ^{32}[P]-labeled probe (5′-GGCAAGTAGACAGGATGAGGA-3′).

Assay of infectivity of HIV-1 produced by mouse cells

Ten days after infection of murine peritoneal macrophages by MLV-pseudotyped NLHXADA-GP, supernatant was collected, standardized by p24 content, and used for infection of indicator MAGI-CCR5 cells (Chackerian *et al*, 1997). Two days after infection, the number of cells producing β-galactosidase was determined exactly as described (Kimpton and Emerman, 1992). Human H4 cells infected in parallel served as positive controls. Alternatively, a quantitative assay for cell-to-cell transmission of HIV-1 was designed using 1G5 cells, which express firefly luciferase under the control of the HIV-1 LTR (Aguilar-Cordova *et al*, 1994). Mouse spleen cells were harvested 5 days after VSV/NL4 infection and were cocultivated with 1G5 cells at a ratio of 5:1. After 2 and 5 days of cocultivation, cultures were harvested, standardized by protein content, and luciferase activity was determine with the dual luciferase assay kit from Promega according to the manufacturer's instructions. As positive controls, CEM were infected with NL4-3 at 0.4 pg p24 per cell and harvested 6 days after infection. They were cocultivated with 1G5 cells at a ratio of 2.5:1 and were harvested after 2 and 3 days of cocultivation for luciferase assay.

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