Molecular analysis of the differential restriction of human immunodeficiency virus type 1 replication in neuronal cell lines

Karen Hsia,1 Deborah H. Spector2,3 and Stephen A. Spector1,3,4

Departments of Pediatrics (Clinical Sciences Building, Room 427)1 and Biology2, Center for Molecular Genetics3 and Center for AIDS Research4, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0672, USA

Human immunodeficiency virus type 1 (HIV-1) replication is restricted partially in SK-N-MC and completely in SK-N-SH neuronal cells. To investigate the molecular mechanism of this differential restriction of HIV-1 replication, cells infected with HIV-1 were analysed for their steady-state levels of: total and unintegrated HIV-1 DNA by DNA PCR, different species of HIV-1 RNA by RT–PCR, and HIV-1 p24 protein production by an ELISA procedure. We found that the kinetics of the infection were slower and there was a lower level of accumulation of HIV-1 macromolecules (total and unintegrated circular DNA, unspliced and spliced RNAs and viral proteins) in the SK-N-MC cells than in the permissive CEM cells. In SK-N-SH cells, HIV-1 DNA was only transiently detected during the first 24 h post-infection, and the unspliced RNA was detected up to 1 week post-infection. However, the HIV-1 spliced RNAs and the 2-LTR circular DNA were not detected at all during the course of infection. Both SK-N-MC and SK-N-SH cells showed higher levels of HIV-1 DNA, RNA and p24 protein when infected with an HIV-1 (amphotropic retrovirus) pseudotype, HIV-1B. However, the level of HIV-1 replication was still lower in SK-N-SH than in SK-N-MC cells. Moreover, although the kinetics of viral protein production were comparable in SK-N-MC cells infected with HIV-1B and CEM cells infected with HIV-1, the overall level of virus replication was still much lower in HIV-1B-infected SK-N-MC cells. These data suggest that the restriction of HIV-1 replication in neuronal cell lines takes place at both virus-entry and post-entry levels, and cellular factors may be involved in the differential restriction of HIV-1 replication in these cells.

Introduction

Infection with human immunodeficiency virus type 1 (HIV-1) frequently is complicated in its late stages by the AIDS dementia complex, a neurological syndrome characterized by abnormalities in cognition, motor performance and behaviour (reviewed by Price et al., 1988; Spencer & Price, 1992; Simpson & Tagliati, 1994). The pathogenetic mechanisms of the nervous system disorders in HIV-1-infected individuals are not yet understood. Previous studies have suggested that the monocyte/macrophage lineage of cells, including brain microglial cells, are the major targets of HIV-1 infection in the central nervous system (CNS) in vivo (Gabuzda et al., 1986; Koenig et al., 1986; Wiley et al., 1986; Pumarola-Sune et al., 1987). However, with the use of the highly sensitive in situ PCR technique, HIV-1-infected neurons can also be detected in affected brain tissues (Nuovo et al., 1994). Whether the functional impairment of neurons in HIV-1-infected individuals is due to the infection of neuronal cells, or due to some indirect effects of HIV-1 infection remains unknown.

Different tumour cell lines of neuronal origin have been examined for their susceptibility to HIV-1 in vitro. While some cell lines (e.g. SK-N-MC, HCN-1A) can support a low level of HIV-1 replication (Li et al., 1990; Harouse et al., 1991; Shapshak et al., 1991; Vesanen et al., 1991; Sharpless et al., 1992; Truckenmiller et al., 1993; Jault et al., 1994; Harouse & Gonzalez-Scarano, 1996), other lines (e.g. SK-N-SH) appeared to be resistant to the infection (Li et al., 1990; Vesanen et al., 1991; Jault et al., 1994). The molecular mechanisms underlying this differential susceptibility of the various neuronal cell lines to HIV-1 infection, however, have not yet been elucidated.

To examine the molecular basis of restriction of HIV-1 replication in cells of neuronal origin, we infected two neuronal cell lines (SK-N-MC and SK-N-SH) with the LAV strain of HIV-1. Infected cells were monitored for 8 weeks to determine the kinetics of virus production, and the steady-state level of intracellular HIV-1 DNA and RNAs. Furthermore, to study the
regulation of HIV-1 replication in neuronal cell lines beyond the step of virus entry, similar studies were carried out with an HIV-1 (amphotropic retrovirus) pseudotype. The HIV-1 pseudotypes have previously been shown to grow in high titres in some (e.g. SK-N-MC) but not in other (e.g. SY5Y, which is a clonal derivative of the SK-N-SH cells) neuronal cell lines (Jault et al., 1994). Our results show that restriction of HIV-1 replication in cell lines of neuronal origin occurs at both the virus-entry and post-entry levels. In addition, the differential susceptibility of HIV-1 infection in these two neuronal cell lines, even in the case of HIV-1 pseudotypes, suggests that host cell factor(s) play an important role in the restriction of HIV-1 replication.

Methods

- **Cells and viruses.** The human neuroblastoma-derived cell lines, SK-N-MC (HTB10) and SK-N-SH (HTB11), were obtained from the ATCC. These cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1-glutamine (0.26 mg/ml), nonessential amino acids and 1 mM sodium pyruvate. The human T-cell lines, CEM and CEM-1B, were obtained and propagated as previously described (Spector et al., 1990). The CEM-1B cells were derived from CEM cells that had been persistently infected with amphotropic retrovirus (Spector et al., 1990).

- **Infection of cells.** SK-N-MC, SK-N-SH and CEM cells were seeded in 1-25 flasks approximately 20 h prior to infection, at a density of 2 × 10^6 cells per flask. The culture media were then removed, and cells were exposed to HIV-1 and HIV-1B (approximately 10^6 pg viral p24 antigen per flask) for 2 h at 37 °C. Prior to infection, the virus stocks were pretreated with 30 U/ml of RNase-free DNase for 45 min at room temperature. After infection, the virus inocula were removed, and cells were washed three times with PBS before culturing at 37 °C.

- **DNA PCR.** At various times after infection, cell aliquots (1× or 10×) were pelleted in microcentrifuge tubes and stored at −20 °C until DNA PCR analysis. Before PCR, thawed cell pellets were brought up to 80 µl with H2O and cells were lysed by repeated freezing–thawing (three times) before treatment with 0.1 mg/ml of proteinase K (Boehringer Mannheim) for 1 h at 60 °C. The proteinase K was then heat inactivated at 95 °C for 15 min, and the digested cell lysates were either subjected to PCR immediately, or stored at −20 °C. For PCR, lysates containing the equivalent of 10^6 or 10^4 cells were added to the PCR reaction tubes containing 1 × PCR buffer (Perkin-Elmer), 1.5 mM MgCl2, 0.2 mM of each deoxynucleotide, 15 pmol of each primer and 1.25 units of Taq DNA polymerase (Perkin-Elmer), in a total volume of 100 µl per tube. In this study, two primer pairs were used to detect two populations of HIV-1 DNAs. The primers SK38 and SK39 (Ou et al., 1988) were used to detect the total full-length HIV-1 DNAs in cell lysates equivalent to 10^2 (HIV-1-infected CEM and HIV-1B-infected SK-N-MC and SK-N-SH) and 10^4 (HIV-1-infected SK-N-MC and SK-N-SH) cells, while the primers LR20S (5′ TCT AGT AAC TAG GGA ACC CAC TG) and L20AS (5′ TGA TCC CTG GCC CTG GTG TG) were used to amplify a DNA fragment spanning the circle junction in HIV-1 2-LTR circular DNA in cell lysates equivalent to 10^5 cells. The PCR was run for 35 cycles in a programmable thermal cycler (Perkin-Elmer), with denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 3 min for each cycle.

After PCR amplification, 10 µl aliquots of the PCR products were denatured at 94 °C for 5 min and immediately hybridized for 10 min at 55 °C with a 32P-end-labelled SK19 or L37S probe in oligomer hybridization buffer containing 15 mM NaCl and 10 mM Na2EDTA (pH 8.0) (Hsia & Spector, 1991). The SK19 probe (Ou et al., 1988) was used to hybridize the 115 bp DNA fragment amplified with the SK38/SK39 primer pair; the L37S (5′ TGC CTG GAG TGC GCC TCT AAC TAG TGT GTG CCC TGC TGT T) probe was used to hybridize the 251 bp DNA amplified with the LR20S/L20AS primer pair. Approximately 5 × 10^5 c.p.m./0.1 pmol of each 32P-end-labelled probe was used in hybridization reaction containing 10 µl of PCR product. The resulting heteroduplexes were then analysed by PAGE (8% gel) and autoradiography, as described previously (Hsia & Spector, 1991).

- **RT–PCR.** Total cellular RNAs were isolated with RNAzol-B (CINNA/BIOTEX Laboratories) according to the manufacturer’s instructions. The RT–PCR analysis of RNA samples was performed according to the method of Sakse et al. (1993, 1994) with minor modifications. Briefly, 2 µg RNA in 20 µl of water was heated for 5 min at 80 °C and then reverse-transcribed into cDNA for 1 h at 42 °C in a total of 40 µl of 1 × RT buffer (GIBCO-BRL) containing 0.4 mM of deoxynucleotides (dNTPs), 24 units of RNasin (Promega), 10 pM of random hexamers (Boehringer Mannheim), 2.5 mM dithiothreitol (GIBCO-BRL) and 400 units of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL). An aliquot (2 µl) of each cDNA was added to the PCR mixture (in a total of 50 µl) containing 1 × PCR buffer II (Perkin-Elmer), 1.5 mM MgCl2, 50 pM each unlabelled dNTP (Perkin-Elmer), 100 ng of each primer, 0.25 µl of [α-32P]dCTP (3000 Ci/mmol, ICN), and 1.25 units of Taq polymerase (Perkin-Elmer). After overlay with mineral oil, the reaction mixtures were subjected to 33 cycles of PCR with denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min, and extension at 72 °C for 1 min per cycle. The amplified 32P-labelled PCR products were then analysed by PAGE and autoradiography.

The primers used for studying unspecific (US) and multiply spliced (MS) tat/re cDNA, described by Sakse et al. (1993), generate two amplicons of 160 bp and 131 bp, respectively. An additional primer pair SK (SK38 and SK39) (Ou et al., 1988), located in the p24 coding region of the gag gene, was also used to study the full-length unspecific HIV-1 RNAs. The upstream sense primer (US1) used for vif, vpr, nef mRNAs is the same as that for the US RNA. The downstream antisense primers for these mRNAs are: 5′ CAC CTG CCA TCT GTT TTC CAT 3′ for vif (VF2A); 5′ GCT ACT GCC AAC TAC TGT AGA 3′ for vpr (VP2R); and 5′ TCT CTC TCC ACC TTC TTC TT 3′ for nef (NF3A). The sense (RPM) and antisense (VPU2) primers for the vpu mRNA are: 5′ TCT CGA CGG AGG ACT CCG ACC ACC ACC ACC TCC 3′ and 5′ GGT CGC AAC AAC TAT TCC TA 3′, respectively. The amplicon sizes for SK, vif, vpr, nef and vpu mRNAs are 115, 271, 225, 258 and 217 bp, respectively.

Results

Kinetics of HIV-1 replication in CEM cells and cells of neuronal origin

To determine the kinetics of HIV-1 infection in CEM cells and two neuronal cell lines (SK-N-MC and SK-N-SH), we first analysed the amount of HIV-1 p24 antigens present in the culture supernatant at different times post-infection (up to 8 weeks). For these experiments, fresh medium was added to each culture 24 h before each harvest, and the amount of HIV-
HIV-1 replication in neuronal cells

Table 1. Detection of HIV-1 p24 antigen in culture supernatants of CEM and neuronal cells infected with HIV-1 or HIV-1B

Cells were infected with HIV-1 or HIV-1B at 10^7 pg p24 antigen per 2 x 10^6 cells in each T-25 flask, for 2 h at 37 °C, as described in Methods. Fresh medium was given to each culture at 24 h before each time-point (with the exception of the 1 day point, which had the fresh medium for about 22 h), at 4 ml per T-25, or 7 ml per T-75 flask for later time-points (2–8 weeks). Starting at 1 week post-infection, infected cells were harvested weekly and seeded to T-75 flasks at 5 x 10^6 per 7 ml per T-75. At each time-point, culture supernatants and cells were harvested. Cell numbers were determined with a haemacytometer. The amount of p24 antigen in each 24 h supernatant was determined by an ELISA procedure (Abbott) and was normalized with the number of cells in that flask.

<table>
<thead>
<tr>
<th>Time post-infection</th>
<th>HIV-1 infected p24 Ag (pg/10^6 cells/day)</th>
<th>HIV-1B infected p24 Ag (pg/10^6 cells/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CEM</td>
<td>SK-N-MC</td>
</tr>
<tr>
<td>1 day</td>
<td>52200</td>
<td>14300</td>
</tr>
<tr>
<td>2 days</td>
<td>1280000</td>
<td>91</td>
</tr>
<tr>
<td>1 week</td>
<td>5830000</td>
<td>376</td>
</tr>
<tr>
<td>2 weeks</td>
<td>281000</td>
<td>2400</td>
</tr>
<tr>
<td>3 weeks</td>
<td>224000</td>
<td>14500</td>
</tr>
<tr>
<td>4 weeks</td>
<td>1550000</td>
<td>15000</td>
</tr>
<tr>
<td>5 weeks</td>
<td>465000</td>
<td>13200</td>
</tr>
<tr>
<td>6 weeks</td>
<td>1150000</td>
<td>8900</td>
</tr>
<tr>
<td>7 weeks</td>
<td>800000</td>
<td>1730</td>
</tr>
<tr>
<td>8 weeks</td>
<td>700000</td>
<td>1590</td>
</tr>
</tbody>
</table>

Fig. 1. HIV-1 DNA in infected CEM, SK-N-MC and SK-N-SH cells. CEM, SK-N-MC and SK-N-SH cells infected with HIV-1 or HIV-1B were harvested at 7 h, 1 day (d), 2 d, 1 week (w), 2 w, 4 w, 5 w, 7 w and 8 w post-infection. After digestion with 0-1 mg/ml proteinase K, cell lysates equivalent to 10^7 HIV-1-infected CEM, HIV-1B-infected SK-N-MC and SK-N-SH cells, and 10^6 HIV-1-infected SK-N-MC and SK-N-SH cells were subjected to PCR with primers SK38 and SK39. The PCR products were then hybridized in solution with 32P-end-labelled SK19 probe and analysed by PAGE and autoradiography, as described in Methods.

Fig. 2. Unintegrated HIV-1 2-LTR circular DNA in infected CEM, SK-N-MC and SK-N-SH cells. CEM, SK-N-MC and SK-N-SH cells infected with HIV-1 or HIV-1B were harvested at 7 h, 1 d, 2 d, 1 w, 2 w, 4 w, 5 w, 7 w and 8 w post-infection. After digestion with 0-1 mg/ml proteinase K, cell lysates equivalent to 10^6 infected cells were subjected to PCR with primers LR20S and L20AS. The PCR products were hybridized with 32P-end-labelled L37S probe followed by analysis with PAGE and autoradiography.
1 p24 antigen accumulated in the supernatant during the 24 h period was normalized to the number of cells present in the culture. As shown in Table 1, there was little or no HIV-1 production in the infected SK-N-SH culture, while the SK-N-MC cells were able to support a low level of HIV-1 replication. However, the kinetics of virus production appeared to be significantly slower in the SK-N-MC cells than in the CEM cells, with the peak of virus production occurring at 3–5 weeks post-infection in the SK-N-MC cells versus 1 week post-infection in the CEM cells. Further analysis of viral titre with the tissue culture infectious dose (TCID) assay revealed that the virus particles released into the culture supernatant of the infected SK-N-MC cultures were biologically active, with TCID titres of $10^5$, $10^4$ and $10^3$/ml at 1 week, 4 weeks and 8 weeks post-infection, respectively.

**Comparison of steady-state HIV-1 DNA in infected CEM and neuronal cell lines**

The amount of HIV-1 DNA in the infected cells was determined by PCR amplification of the cellular DNA with SK38 and SK39 primers (Ou et al., 1988). The kinetics of HIV-1 DNA accumulation in SK-N-MC and in CEM cells (Fig. 1) were similar to the kinetics of virus release into the culture supernatant of these cells (Table 1). In the nonproducer SK-N-SH cells, HIV-1 DNA could only be detected at 7 h and 24 h post-infection, even when the amount of input DNA was 100-fold higher than that used for the HIV-1-infected CEM cells (equivalent to $10^2$ cells) (Fig. 1).

Because the circular forms of retroviral genome were detected only in the nuclear fraction (Varmus & Swanstrom, 1985; Bukrinsky et al., 1992), they can be used as markers for completed viral DNA genome after nuclear transport (Bukrinsky et al., 1992). To determine whether the presence of 2-LTR circular DNA is associated with a productive virus infection, DNA from 2-LTR circular DNA was detected in the nonproducer SK-N-SH cells at any time during the 8 weeks of study (Fig. 2). The accumulation of the 2-LTR circular DNA in infected CEM and SK-N-MC cells (Fig. 2) again showed kinetics similar to viral protein production (Table 1), with similar molar ratios between these two cell types at various times post-infection. These results suggest that accumulation of HIV-1 2-LTR circular DNA does correlate with a productive HIV-1 infection in these cells.

**Quantitative comparison of HIV-1 unspliced and spliced RNAs in infected CEM and SK-N-MC cells**

HIV-1 RNAs in the infected cells were studied extensively for their relative abundance and for the kinetics of their accumulation. Total RNAs extracted from each infected culture at different times of infection were reverse-transcribed into cDNAs, which were then amplified by PCR using primers specific for each HIV-1 RNA species. In cases where there was a large amount of viral RNA (such as the unspliced full-length HIV-1 RNA), serial dilution of cDNA was needed for optimal PCR amplification. In a pilot experiment with pooled cDNAs from infected CEM and SK-N-MC cells at 1 d, 2 d, 1 w, 2 w, 6 w and 8 w post-infection, it was found that the optimal PCR conditions for detection of the unspliced HIV-1 RNA required that the cDNAs be serially diluted to the equivalent of $10^{-3}$ µg RNA for the CEM cells, or $10^{-3}$ µg RNA for the SK-N-MC cells (Fig. 3).

For amplification of the unspliced HIV-1 RNA, we used two primer pairs. The SK primers (SK38 and SK39) were used to amplify a DNA fragment in the p24-coding region of the
Table 2. Relative abundance of HIV-1 RNAs in infected CEM and SK-N-MC cells

<table>
<thead>
<tr>
<th>RNA</th>
<th>CEM</th>
<th>SK-N-MC</th>
<th>CEM/SK-N-MC Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK (gag)</td>
<td>11300.0</td>
<td>195.0</td>
<td>57.9</td>
</tr>
<tr>
<td>US (gag)</td>
<td>23500.0</td>
<td>460.0</td>
<td>51.1</td>
</tr>
<tr>
<td>MS (tat/rev)</td>
<td>2850.0</td>
<td>48.0</td>
<td>59.4</td>
</tr>
<tr>
<td>vif</td>
<td>150.0</td>
<td>1.0</td>
<td>93.8</td>
</tr>
<tr>
<td>vpr</td>
<td>41.8</td>
<td>0.2</td>
<td>209.0</td>
</tr>
<tr>
<td>vpu</td>
<td>1210.0</td>
<td>0.8</td>
<td>1512.5</td>
</tr>
</tbody>
</table>

The gag gene, and the US primers (US1 and US2A) amplified a DNA fragment in the U5 region of LTR, which traversed the major splice junction for all the spliced HIV-1 RNAs (Saksela et al., 1993). Comparable results were obtained with both sets of primers (Fig. 3). The multiply spliced tat/rev RNA (MS) was studied with a set of primers from the two coding exons of tat and rev, which yields a 131 bp amplified product (Saksela et al., 1993). Other spliced HIV-1 mRNA species (vif, vpr, vpu and nef) were studied with sets of primers separated by the intron between the major splice donor site for all of the spliced HIV-1 RNAs and their respective splice acceptor sites in the coding exons. The relative abundance of each HIV-1 RNA species in infected CEM and SK-N-MC cells was measured by PCR amplification of serially diluted cDNAs which were pooled following reverse transcription of RNAs extracted from the cells at 1 day, 2 days, 1 week, 2 weeks, 6 weeks and 8 weeks post-infection. Fig. 3 shows that while PCR products representing the MS and vpu RNAs were detectable after amplification of cDNA generated from 10^{-4} to 10^{-5} µg of RNA from infected CEM cells, a higher concentration of cDNA was needed to detect vif, vpr and nef RNAs. The relative abundance of each HIV-1 RNA species in the infected CEM and SK-N-MC cells was calculated by counting the radioactivity of gel fragments in Fig. 3 at a cDNA dilution representative of the optimal PCR amplification. As Table 2 shows, the relative abundance of all HIV-1 RNAs except vpu was 50 to 200-fold higher in the CEM cells than in the SK-N-MC cells. In the case of vpu, the difference was significantly greater, with an approximately 1500-fold higher level of mRNA present in the CEM cells than in the SK-N-MC cells. These results suggest that the restriction of HIV-1 replication in SK-N-MC cells is associated with a relatively lower level of vpu mRNA expression.

To further estimate the amount of the US and MS RNAs present in the infected CEM and SK-N-MC cells, serial dilutions of known amounts of RNA, prepared by in vitro transcription of cloned US and MS DNA (kindly provided by Roger Pomerantz) (Seshamman et al., 1992), were subjected to RT–PCR in parallel with the RNA extracted from the infected cells, and the products were analysed by gel electrophoresis. For each RNA species, we determined the cDNA dilution that was optimal for PCR amplification, and measured the amount of label present in the PCR product. By comparing the values for the RNA from the infected cells with known amounts of RNA from the in vitro transcription reactions, we calculated that there were approximately 1·55 × 10^{3} and 6·76 × 10^{3} copies of US and MS RNA, respectively, in each infected CEM cell, and 3·42 × 10^{3} and 1·63 × 10^{3} copies of US and MS RNA, respectively, in each infected SK-N-MC cell. These data are consistent with the results in Table 2 showing a molar ratio of approximately 50 for both US and MS RNAs in infected CEM and SK-N-MC cells.

Kinetics of HIV-1 RNA accumulation in infected CEM cells and cells of neuronal origin

In the above experiments, the cDNAs from the given time-points were pooled for PCR analysis. To measure the kinetics of accumulation of HIV-1 unspliced RNA, multi-spliced tat/rev RNAs, and other RNA species of accessory genes (vif, vpr, vpu) and regulatory gene ( nef ) in infected CEM and neuronal cell lines, we proceeded to analyse the RNAs obtained from separate time-points by RT–PCR with specific primers. In some cases where the specific HIV-1 RNA concentrations were high (e.g. the unspliced RNA), the cDNAs were diluted for optimal PCR amplification. In general, there was a delay in accumulation of all the HIV-1 RNA species in infected SK-N-MC cultures. The peak in these cultures occurred at 6 weeks post-infection as compared to a peak of HIV-1 RNA in the CEM cultures at 2 days to 2 weeks post-infection (Fig. 4).

One interesting finding in the study of kinetics of HIV-1 RNA accumulation is the transient detection of HIV-1 unspliced RNA in infected SK-N-SH cells. This species of HIV-1 RNA was detected in infected SK-N-SH cells for as long as 1 week post-infection (Fig. 4 A). The detection of HIV-1 unspliced RNA by RT–PCR was not due to contamination by HIV-1 DNA in the RNA preparations, because a negative control RT–PCR experiment without adding reverse transcriptase did not show any amplification of HIV-1 sequences in the same RNA preparations (data not shown). It is possible that this RNA represents the input RNA since at no time-point were we able to detect the HIV-1 RNAs of the regulatory genes ( tat , rev and nef ) (Fig. 4 B, F), and of the accessory genes ( vpu , vpr and vif ) (Fig. 4 C, D, E). These results indicate that the absence of detectable HIV-1 protein in the culture supernatants of infected SK-N-SH cells (Table 1) is due to the very low levels
K. Hsia, D. H. Spector and S. A. Spector

Fig. 4. HIV-1 RNAs in infected CEM, SK-N-MC and SK-N-SH cells. Total cellular RNAs were extracted from HIV-1- or HIV-1B-infected CEM, SK-N-MC and SK-N-SH at 1 d, 2 d, 1 w, 2 w, 6 w, and 8 w post-infection as indicated. Two µg of each RNA was reverse-transcribed in a total volume of 40 µl, and 2 µl of the undiluted (representing 100 ng RNA) or 101 to 104-fold diluted cDNA product (representing 10–0 ng RNA) was subsequently PCR amplified for the HIV-1 (A) unspliced (US), (B) multiple-spliced (MS) tat/rev, (C) vif, (D) vpr, (E) vpu and (F) nef RNAs as described in Methods. Dilution of cDNA with sterile Milli-Q water was found to be necessary to achieve an optimal PCR amplification in several cases as indicated. The RT–PCR products were subsequently analysed by PAGE and autoradiography.
Infection with pseudotyped HIV-1 still shows a differential restriction of HIV-1 replication in the SK-N-MC and SK-N-SH cells

To determine if the differential restriction of HIV-1 replication in the two neuronal cell lines is the result of differential inhibition of virus entry by these cells, SK-N-MC and SK-N-SH cells were infected with HIV-1 that was pseudotyped with an amphotropic murine leukaemia virus (designated HIV-1B). Presumably, this could enhance significantly virus entry into cells otherwise nonsusceptible to infection by HIV-1. Cells infected by HIV-1B were monitored for the presence of intracellular HIV-1 DNA, 2-LTR circular DNA and different species of HIV-1 RNA, as well as the HIV-1 p24 antigen level in the culture supernatants at different times post-infection, as described previously with those infected with HIV-1.

Table 1 shows that both SK-N-MC and SK-N-SH cells support better replication of HIV-1B than HIV-1, although the level of HIV-1 p24 protein production is still significantly higher in HIV-1B-infected SK-N-MC than the infected SK-N-SH cultures. In HIV-1B-infected SK-N-SH cultures, a low level of viral p24 protein was produced persistently throughout the 8 weeks of study, in contrast to a lack of consistent detection of this protein in the same cells infected with HIV-1. Interestingly, the SK-N-MC cells infected with HIV-1B not only showed a significantly higher level of HIV-1 p24 protein production than those infected with HIV-1, but also showed an early peak of virus replication (peaking at 1 week post-infection), similar to the HIV-1-infected CEM cells (Table 1). However, despite a significant enhancement in virus replication found in SK-N-MC when infected with the pseudotyped HIV-1, the level of viral protein produced was still much lower than that produced in HIV-1-infected CEM cultures (Table 1). These results suggest that the differential restriction of HIV-1 replication in cells of neuronal origin is not due solely to an inhibition of virus entry into these cells.

Studies on HIV-1 DNA in the SK-N-MC and SK-N-SH cells infected with HIV-1B showed that in parallel to an increased production of p24 antigen, consistently higher levels of HIV-1 DNA were also detected in both cell types from as early as 7 h post-infection (Fig. 1). In contrast to a transient detection of HIV-1 DNA in SK-N-SH cells infected with HIV-1, HIV-1 DNA was persistently detected in these cells infected with HIV-1B, showing that HIV-1B was able to stably infect the SK-N-SH cells. The levels of HIV-1 DNA detected in HIV-1B-infected SK-N-SH cells, however, were lower than those in HIV-1B-infected SK-N-MC cells, which in turn were lower than those in HIV-1-infected CEM cells (Fig. 1).

Consistent with an early peak of HIV-1 p24 antigen production from HIV-1B-infected SK-N-MC cells, an early peak of accumulation of HIV-1 2-LTR circular DNA was also observed in these cells, at as early as 24 h post-infection (Fig. 2). The infection with HIV-1B appeared to enhance the accumulation of HIV-1 2-LTR circular DNA not only in SK-N-MC, but also in SK-N-SH cells (Fig. 2). The similar kinetics of p24 antigen production and accumulation of viral 2-LTR circular DNA in cells infected with HIV-1 and HIV-1B suggests that the accumulation of HIV-1 2-LTR circular DNA does correlate with a productive infection in these cells.

To study the kinetics of HIV-1 RNA expression in HIV-1B-infected neuronal cell lines, cDNAs reverse-transcribed from 100 ng RNA at 1 day, 2 days, 1 week, 2 weeks, 6 weeks and 8 weeks post-infection were subjected to PCR for different HIV-1 RNAs with different primer pairs, as described above. In cases of abundant RNA species, cDNAs were serially diluted to reach an optimal PCR amplification. Fig. 4 shows that infection of SK-N-MC with HIV-1B results not only in an earlier peak of accumulation of HIV-1 US, MS, vpu, vpr and vif RNAs (with a peak of 2 weeks instead of 6 weeks post-infection), but also in an enhancement in the levels of these RNAs (by at least 10-fold), as compared to the same cells infected with HIV-1. In SK-N-SH cells infected with HIV-1B, HIV-1 US, MS and vpr RNAs were detected persistently throughout the 8 weeks of study, in contrast to a transient detection of only US RNA in cells infected with HIV-1 (Fig. 4A, B, E). These results indicate that at least some of the restriction of HIV-1 replication in neuronal cells can be overcome by infection with a pseudotyped HIV-1 which bypasses the CD4 dependence of virus entry. However, the absence of detectable HIV-1 vpr and vif RNAs in HIV-1B-infected SK-N-SH cells (Fig. 4C, D), and the fact that levels of HIV-1 RNAs in HIV-1B-infected SK-N-SH cells are lower than those in similarly infected SK-N-MC cells (which are in turn lower than those in HIV-1-infected CEM cells), suggest that the differential restriction of HIV-1 replication in neuronal cells is due to regulation by cellular factors at the post-virus entry level.

Discussion

A productive HIV-1 infection requires that the host cell provides virus receptors for effective virus entry, and an intracellular environment to support a high level of virus replication. The results presented in this study are consistent with previous findings by Li et al (1990), Vesanen et al (1991) and Jault et al (1994) who showed a differential restriction of HIV-1 replication in two neuronal cell lines: a nonproductive infection in SK-N-SH cells and a low level of persistent infection in SK-N-MC cells. In this study, the molecular basis for this differential restriction of HIV-1 replication was examined by studying the steady-state levels of intracellular HIV-1 DNA, spliced and unspliced viral RNAs, and viral protein production from infected cells at multiple time-points post-infection for up to 8 weeks. As compared with the fully
permissive CEM cells, HIV-1 showed late kinetics of virus replication in SK-N-MC cells (peaking at 3–5 weeks post-infection instead of 1 week). This was observed with studies on intracellular accumulation of viral DNA (especially the 2-LTR circular DNA) and all species of viral RNAs studied, including the unspliced RNA and spliced tat/rev, vpu, vpr, vif and nef RNAs and viral proteins. In addition to the late kinetics, the level of virus replication was also found to be much lower in SK-N-MC than the permissive CEM cells.

To determine if the partial restriction of viral protein production from HIV-1-infected SK-N-MC cells is the result of general restriction of expression of HIV-1 DNA, or due to a preferential block in transcription of a particular HIV-1 RNA, the relative abundance of each HIV-1 RNA species in the infected cells was examined and compared with the average p24 protein production. While the HIV-1-infected CEM produced an average of 500 to 600-fold higher p24 antigen than the HIV-1-infected SK-N-MC, there was only about 50-fold difference observed with the full-length unspliced HIV-1 RNA and the multiply spliced tat/rev RNAs, and approximately 100 to 200-fold difference with vif and vpr RNAs. The vpu RNA, however, was approximately 1500-fold more abundant in the CEM cells. It is not clear why the vpu gene transcription is preferentially blocked in the HIV-1-infected SK-N-MC cells. One possibility is that the utilization of the splice acceptor site for the vpu/env bicistronic RNA was inefficient (Bruggeman et al., 1994). The vpu gene of HIV-1 has been shown to encode a well-characterized accessory protein 16 kDa in size (Cohen et al., 1988; Strebel et al., 1988; Terwilliger 1993; Subbramanian & Cohen, 1994). Although not essential for virus replication in vitro, the expression of vpu has been shown to enhance viral particle release from infected cells (Strebel et al., 1988, 1989; Terwilliger et al., 1989; Vincent & Jabbar, 1995). Thus, it is possible that the restricted expression of vpu is responsible for the low level of HIV-1 production in HIV-1-infected SK-N-MC cultures, especially during the early phase of infection.

In contrast to the partial permissiveness of the SK-N-MC cells to HIV-1 infection, the SK-N-SH cells do not appear to support a productive infection by this virus. This is in agreement with previous findings by Li et al. (1990), but disagrees with those of Shapshak et al. (1991). Although we failed to produce progeny virus, HIV-1 DNA and the unspliced HIV-1 RNA were transiently detected in the infected SK-N-SH cells. Our finding that the full-length HIV-1 RNA was detectable in infected SK-N-SH cells even at 7 days post-infection suggests that the virus did enter the cells. Whether this RNA and DNA is due to the input HIV-1 or a low level of progeny RNA transcribed from the HIV-1 DNA genome remains to be elucidated. Nevertheless, the infection in the SK-N-SH cells appeared to be abortive, and the 2-LTR circular DNA and the spliced HIV-1 RNAs were not detected at any time in the course of infection. It is possible that the transient detection of HIV-1 DNA in HIV-1-infected SK-N-SH cells could be due to the nonspecific adherence of virus particles in the inoculum to the cell membrane at early times. In fact, HIV-1 DNA was still detectable from DNase-treated virus inoculum containing only 10^5 pg viral p24 antigen (0.001% of that used for infection) (unpublished data).

The absence of detectable HIV-1 circular DNA and virus replication in SK-N-SH cells suggest that the presence of HIV-1 circular DNA does correlate with a productive infection. The HIV-1 2-LTR circular DNA is a marker of nuclear transport of the preintegrated complexes (Bukrinsky et al., 1992), due to its exclusive localization in the nuclear fraction (Varmus & Swanstrom, 1985; Bukrinsky et al., 1992). Although the function of HIV-1 2-LTR circular DNA in infected cells has not been well established, their accumulation has been associated with a reinfection process in both acutely and chronically infected cells (Pauza et al., 1990; Besansky et al., 1991). In vivo, accumulation of HIV-1 unintegrated DNA has been found in brain tissue of AIDS dementia patients (Pang et al., 1990), and the amount of which has been shown to be decreased in the peripheral blood mononuclear cells (PBMC) of these patients after antiretroviral therapy (Dickover et al., 1992). Our results indicating an association between the presence of HIV-1 2-LTR circular DNA viral protein production, both in kinetics and in quantity, also suggest that the accumulation of the 2-LTR circular DNA may be a marker of productive infection by HIV-1 in vitro.

The restriction of HIV-1 replication in neuronal cell lines could be due to a lack of CD4 receptors on the cell surface. In fact, the infection of neuronal cell lines has been thought by several investigators to take place via a CD4-independent pathway, because of their lack of detection of CD4 mRNA by Northern blot hybridization and lack of blocking of infection with monoclonal antibodies to CD4 (Harouse et al., 1989, 1991; Li et al., 1990; Truckenmiller et al., 1993). Harouse et al. (1989, 1991), using antibodies against galactosyl ceramide (GaC), have suggested that the entry of HIV-1 into CD4-negative neuronal cells may involve GaC or highly related glycolipid receptors.

One way to enhance the efficiency of virus entry into nonpermissive or partially permissive cells is to use the pseudotyped virus for infection. HIV-1 pseudotyped with murine amphotropic or xenotropic retroviruses, or with human T-cell leukaemic virus type I (HTLV-I), has been shown to have expanded cellular tropism (Chesebro et al., 1990; Lusso et al., 1990; Spector et al., 1990; Landau et al., 1991). Both SK-N-MC and SY5Y (a SK-N-SH-related cell line) cells have previously been found to support better replication of HIV-1 when pseudotyped with a murine amphotropic retrovirus (Jault et al., 1994). In this study, we found an increase in the amount of intracellular HIV-1 DNA and RNA as well as an increase in the amount of viral protein production from both SK-N-MC and SK-N-SH cells infected with a pseudotyped HIV-1 (HIV-1B). In SK-N-MC cells infected with HIV-1B, the kinetics of appearance of HIV-1 circular DNA, HIV-1 RNAs (unsplitted,
tat/rev, vpu and vif), and HIV-1 p24 protein were very similar to those in HIV-1-infected CEM cells, suggesting that the kinetics of HIV-1 replication is related to the efficiency of virus entry into host cells. Others have also noticed that the apparent permissiveness of host cells for HIV-1 infection is related to the efficiency of entry (Srivastava et al., 1991; Fernandez-Larsson et al., 1992). In SK-N-SH cells, a stable infection was found only with HIV-1B, but not with HIV-1. In the former case, this stable infection was associated with the persistence of detectable levels, albeit low, of HIV-1 DNA, HIV-1 unspliced RNA, spliced tat/rev and vpu RNAs, and viral p24 antigen.

Although the infection with pseudotyped HIV-1 shows an enhancement in virus replication in both SK-N-MC and SK-N-SH cells, the level of virus replication observed in the HIV-1B-infected SK-N-SH cells is still much lower than that in the HIV-1B-infected SK-N-MC cells. It is possible that the efficiency of entry of the pseudotyped virus into the two cell lines differs, perhaps due to the difference in the level of amphotropic virus receptors on these cells. However, it seems more likely that the cellular mechanisms operating after virus entry are primarily responsible for the differential restriction of HIV-1 replication observed in these two cell lines of neuronal origin. The cellular factors can act either positively as stimulants, or negatively as inhibitors of virus replication (Chesebro et al., 1990; Weiner et al., 1991; Stefano et al., 1993). Thus, the restriction of HIV-1 replication in neuronal cell lines might be due either to a lack of a cellular cofactor for a productive virus replication, or to the over-production of some inhibitors.

In conclusion, our studies on the SK-N-MC and SK-N-SH neuronal cell lines infected with HIV-1 and pseudotyped HIV-1 (HIV-1B) suggest that the restriction of HIV-1 replication can take place at both virus entry and post-entry levels, and host cellular factors may be involved in the differential restriction of HIV-1 replication in cells of neuronal origin.

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