Morphological differentiation of human SH-SY5Y neuroblastoma cells inhibits human immunodeficiency virus type 1 infection

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We have studied human immunodeficiency virus type 1 (HIV-1) infection in human SH-SY5Y neuroblastoma cells at various stages of morphological differentiation. Two days' treatment of the cells with retinoic acid (RA) or dibutyryl cAMP (db-cAMP) resulted in the appearance of elongated neurites and enhanced production of 160K to 200K neurofilament proteins as shown by indirect immunofluorescence. DNA synthesis was reduced only in RA-treated cells as detected by 5-bromo-2'-deoxyuridine incorporation. The cells were infected with two T-lymphotropic virus strains (IIIB and NDK) and two fresh isolates (39001 and 46001) from bronchoalveolar lavage samples of AIDS patients. The latter two isolates were unable to form syncytia in infected CD4-positive T-lymphoblastoid C8166 cells which was in contrast to our T-lymphotropic virus strains. Interphase in situ hybridization showed that 14 to 16% of SH-SY5Y cells become positive for HIV-1 DNA. Regardless of the virus strain, morphological differentiation of the cells with RA or db-cAMP inhibited infection by 50% at a single cell in situ resolution. Nested PCR confirmed the presence of proviral DNA in the infected cells. These results show that human neuroblastoma cells, tumour cells of neuroectodermal origin, can be infected by different HIV-1 isolates and that the infection is inhibited by neurotypic cell differentiation.
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Fig. 1. Morphological differentiation of SH-SY5Y neuroblastoma cells after treatment with db-cAMP. Cells were treated for 2 days with 1 mM-db-cAMP and neurite outgrowth was monitored using a microscope equipped with phase-contrast optics. (a) Untreated cells. (b) db-cAMP-treated cells.

as H9, KARPAS and C8166 lymphoid leukaemia cell lines were maintained in RPMI-1640 medium (Gibco, Life Technologies) containing 10% fetal calf serum (FCS; Gibco) and antibiotics (streptomycin 100 μg/ml, penicillin 100 units/ml; Gibco). The human osteosarcoma cell line MG-63 (ATCC CRL 1427) was maintained in Eagle’s MEM supplemented with 10% FCS and antibiotics. Human peripheral blood lymphocytes (PBL) were obtained from HIV-seronegative donors and prepared using Ficoll-Hypaque gradients. Isolated lymphocytes were stimulated and cultured as described (Vesanen et al., 1991).

Human SH-SY5Y neuroblastoma cells were induced to differentiate up to 4 days either with 1 μM-retinoic acid (RA; Sigma), 1 mM-dibutyryl cAMP (db-cAMP; Sigma) or 50 ng/ml mouse nerve growth factor (NGF; Janssen Biochemica) in RPMI-1640 medium supplemented with 10% FCS and antibiotics. They were monitored daily by phase-contrast microscopy for the appearance of elongated neurites and the production of the large 160K to 200K neurofilament complex. According to appearance

of elongated neurites (Fig. 1), the treatments with RA and db-cAMP differentiated 80 to 90% of the cells within 2 days (Table 1). NGF treatment was less effective, because by 2 days elongated neurites were seen in only 20 to 30% of the cells (Table 1). In our previous study (Neuman et al., 1989) we have shown that treatment of SH-SY5Y cells with RA, 12-D-tetradecanoyl phorbol-13-acetate or NGF enhances production of mRNA for the 200K neurofilament protein. In the present study, we monitored the protein expression and performed immunofluorescent staining of the 160K to 200K neurofilament complex in SH-SY5Y cells with a polyclonal antibody specific for the complex (Vesanen et al., 1992). Two days of treatment of SH-SY5Y cells with RA, db-cAMP or NGF enhanced production of the neurofilaments. Human MG-63 osteosarcoma cells, which were used as a negative control, gave no staining.

We also followed the effect of cell differentiation on cellular DNA synthesis. Cells were seeded on 100 cm² dishes (Nunc) at 4 x 10⁵ cells per dish and induced to differentiate for 48, 72 and 96 h. They were pulsed with 50 μM-5-bromo-2'-deoxyuridine (BrdU; Sigma) for the last 4 h of differentiation. After labelling, the cells were washed twice with PBS, trypsinized and viability was calculated using trypan blue exclusion. The cell samples were centrifuged onto glass slides and fixed with ice-cold methanol for 5 min at 4°C. The cell layers were permeabilized by treating them first with 5 μg of pepsin (Sigma) per ml of 0.01 M-HCl for 3 min at 37°C and subsequently with 0.5% Nonidet P40 in PBS. The cells were stained with a monoclonal antibody against BrdU (Amersham) at a dilution of 1:8 in Tris-buffered saline supplemented with 0.3% BSA (Sigma). The secondary antibody, fluorescein isothiocyanate conjugated antimouse IgG (Dakopatts), was used at a dilution of 1:40 in PBS. The cells were counterstained with propidium iodide (1 μg/ml; Sigma). As shown in Table 1, cellular DNA synthesis was inhibited strongly by the RA treatment; at each time point studied 15% of the cells incorporated BrdU. In contrast, db-cAMP and NGF treatments had no effect on DNA synthesis as compared to untreated control cells of which 35 to 45% incorporated BrdU. The total cell count was decreased following RA treatment by 50%. By the three criteria of neural cell differentiation the most effective differentiation was obtained with RA which was effective in neurite formation, neurofilament production and inhibition of cellular DNA synthesis. Although effective in differentiation according to neurite formation and neurofilament production, db-cAMP did not inhibit cellular DNA synthesis. The least effective was NGF treatment which had no effect on cellular DNA synthesis and only a partial effect on cell morphological differentiation.
Table 1. Effect of RA, db-cAMP and NGF on morphological differentiation and DNA synthesis of SH-SY5Y neuroblastoma cells

<table>
<thead>
<tr>
<th>Cell cultures</th>
<th>Morphological differentiation of SH-SY5Y cells (%)</th>
<th>Effect of morphological differentiation on cellular DNA synthesis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated cells</td>
<td>5–10</td>
<td>43</td>
</tr>
<tr>
<td>RA-treated cells</td>
<td>80–90</td>
<td>14</td>
</tr>
<tr>
<td>db-cAMP-treated cells</td>
<td>80–90</td>
<td>37</td>
</tr>
<tr>
<td>NGF-treated cells</td>
<td>20–30</td>
<td>41</td>
</tr>
</tbody>
</table>

* Percentages of neurite-bearing cells after 2, 3 and 4 days of treatment analysed using a phase-contrast microscope.

† Cellular DNA synthesis after 48 h of treatment as measured by BrdU staining as described in the text. Percentages of stained nuclei are mean values of three independent experiments, in which 1000 nuclei were studied. After 72 and 96 h of treatment the proportions of stained nuclei were at the same level.

Fig. 2. Interphase *in situ* hybridization with digoxigenin-11-dUTP-labelled HIV-1 full-genomic cDNA probe on NGF-treated HIV-1 (NDK strain)-infected SH-SY5Y neuroblastoma cells. Cells were induced to differentiate for 2 days with 50 ng/ml mouse NGF prior to infection. After 1 day of infection the cells were fixed in 4% paraformaldehyde and *in situ* hybridization was performed. Cells were counterstained with acridine orange and analysed using phase-contrast microscopy. Signals are indicated by arrows.

The HTLV-IIIB strain of HIV-1 was propagated in H9 cells and the NDK strain in KARPAS cells. Human HIV-1 isolates 39001 and 46001, kindly provided by Dr Jukka Suni (Aurora Hospital, Helsinki, Finland), were isolated from BAL samples of two AIDS-stage patients. The fresh isolates were propagated in stimulated human PBL. During isolation and propagation these fresh isolates were cultured for 10 weeks with stimulated PBL. The ability of HIV-1 strains to form syncytia was studied with the C8166 cell line. Cells (2 × 10⁶) in 96-well microtitre plates (Nunc) were infected and monitored microscopically daily for syncytium formation up to 6 days. In contrast to the lymphocyte-adapted strains, our BAL isolates did not form syncytia in the CD4-positive C8166 T cells. This indicated that the fresh lung isolates of HIV-1 from AIDS patients are biologically different from our lymphocyte-adapted strains. Undifferentiated and differentiated SH-SY5Y cells (1 × 10⁶) were infected for 24 h with 1 ml of stock virus in 5 ml RPMI-1640 medium containing 10% FCS, antibiotics and the differentiating agent. Under these conditions of infection the 50% tissue culture infective dose was 5 · 10⁶/ml for the IIIIB and NDK strains. This dose corresponded to a 1:1000 dilution in p24 enzyme immunoassay (Organon Teknika) giving an absorbance value of 1.0, which was used also in the case of the 39001 and 46001 strains.

HIV-1 infections were monitored by the interphase *in situ* hybridization method (Vesanen et al., 1992) that provides quantification at the single cell level and thus enables direct determination of the proportion of infected cells in the cultures (Fig. 2). Cytospin preparations were made of undifferentiated and RA-, db-cAMP- or NGF-differentiated HIV-1-infected SH-SY5Y neuroblastoma cells after 1 day of infection. The uninfected SH-SY5Y cells were used as controls. As a control to study the influence of input viral DNA in the procedure, untreated cells were exposed to the virus for 2 h at 4°C before immediate fixing of the cells and preparing the cytospin slides. A full-genomic insert of HIV-1-specific cDNA carried by the pBH10-R3 plasmid (Biotech Research Laboratories) was used as a probe. The isolated insert was labelled with digoxigenin-11-dUTP by random priming according to the instructions of the kit supplier (DNA Labelling and Detection Kit; Boehringer Mannheim). After hybridization and signal development, the cells were counterstained with 0.006% (w/v) acridine orange (Merck) in PBS for 15 s and analysed using a phase-contrast microscope. The statistics were calculated using the standard R × C contingency table test.

Interphase *in situ* hybridization revealed that different HIV-1 isolates can infect undifferentiated human neuroblastoma cells (Table 2). HIV-1 infection itself had no effect on morphological differentiation. In untreated cell cultures 14 to 16% of the cells were HIV-1-positive. Usually only one signal per cell nucleus was seen. Thus, no clear differences could be demonstrated in the efficiency of the different virus strains to infect the SH-SY5Y neuroblastoma cells. Keys et al. (1991) reported similar results. Using PCR they showed that a human glioma cell line could be infected with different HIV-1 isolates, derived either from peripheral blood or brain.
Table 2. Proportion of HIV-1-infected SH-SY5Y interphase nuclei after morphological differentiation of the cells

<table>
<thead>
<tr>
<th>HIV-1 strain</th>
<th>Untreated cells (%)</th>
<th>RA-treated cells (%)</th>
<th>db-cAMP-treated cells (%)</th>
<th>NGF-treated cells (%)</th>
<th>Negative uninfected control cells (%)</th>
<th>Virus exposure on ice for 2 h †</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIIB</td>
<td>16-1</td>
<td>81</td>
<td>8-6</td>
<td>16-0</td>
<td>2-3</td>
<td>18</td>
</tr>
<tr>
<td>NDK</td>
<td>14-1</td>
<td>6-7</td>
<td>9-1</td>
<td>15-0</td>
<td>3-0</td>
<td>ND</td>
</tr>
<tr>
<td>39001</td>
<td>16-4</td>
<td>6-8</td>
<td>8-7</td>
<td>14-2</td>
<td>2-3</td>
<td>ND</td>
</tr>
<tr>
<td>46001</td>
<td>16-6</td>
<td>6-3</td>
<td>7-0</td>
<td>15-8</td>
<td>2-7</td>
<td>ND</td>
</tr>
</tbody>
</table>

* SH-SY5Y neuroblastoma cells were induced to differentiate by RA, db-cAMP or NGF for 2 days before infection with the different HIV-1 strains. Infection was studied by in situ hybridization using a digoxigenin-labelled HIV-1-specific probe which was performed as described in the text. Percentages of positive cells are mean values from three independent experiments, in which at least 1000 interphase cells were analysed.
† Untreated SH-SY5Y cells were incubated for 30 min on ice and were then exposed to the virus under the same conditions for 2 h before immediate fixation and preparation of the cytospin slides. In situ hybridization was performed as described.
‡ ND, Not detected.

We were interested to see whether the morphological differentiation of the SH-SY5Y cells would have an effect on virus infection. As compared to untreated cells, a 50% reduction in the proportion of positive cells was seen in both RA- and db-cAMP-differentiated cells for each viral strain (Table 2). The differences are highly significant for all strains (P < 0.0001). NGF treatment of the cells did not alter their susceptibility to infection; the fraction of positive cells was the same as in the untreated cell cultures (14 to 16%). No alterations could be demonstrated in the inhibition of infection when the differentiated cells were infected with separate virus strains. Of control cells 1-8 to 3% gave a positive signal.

Nest PCR was used as a control for the in situ experiments. Preparation of cell lysates from undifferentiated and differentiated HIV-1-infected SH-SY5Y neuroblastoma cells and nested PCR were performed as described (Vesanen et al., 1992), with minor modifications. In the primary PCR, 5 µl of the lysate (200 ng DNA) was used as a template. The primers used in first PCR were BJGAG1 (TAAAGGAATGTACACAGG-ATG) and BJPOL2 (TTGTATATTTAATCTGTTA-CAG). The secondary PCR employed two primers: MSGAG7 (GATGACAGCATGTCAGGGAG) and BJPOL3 (GTGACAGGGTGAGCTCTAC). The size of the prototypic amplified fragment was 674 bp. After the first round of amplification no products were detected with any of the samples. After the secondary nested PCR all samples were positive showing amplification of the specific bands (Fig. 3; lanes 1, 2, 3 and 4). We conclude that different HIV-1 strains can infect human SH-SY5Y neuroblastoma cells with the same efficiency. In addition, we have shown that induction of morphological differentiation of the cells inhibits HIV-1 infection. The inhibition was independent of cellular DNA synthesis as demonstrated by comparing RA- and db-cAMP-differentiated cells. Moreover, NGF, which was a mild differentiation inducer, had no effect on the susceptibility of SH-SY5Y neuroblastoma cells to HIV-1 infection. This indicates that the inhibition of HIV-1 infection was related to morphological alterations of the SH-SY5Y cells. Earlier, Sharpless et al. (1992b) studied infection of undifferentiated and differentiated neuronal HCN-1 cells with the lymphocytotropic HIV-1 LAV strain. Using PCR they showed that cells at a more differentiated stage were less well infected. Our study
confirms and extends their finding since PCR as a method does not define the proportion of positive cells as is the case with in situ hybridization. Sharpless and coworkers also showed that the monocyte/macrophage-tropic Jr-FL virus strain could not infect HCN-1 cells. On the other hand, our fresh HIV-1 isolates from BAL samples which did not induce syncytia in C8166 cells could efficiently infect neuroblastoma cells (14 to 16%).

Wigdahl & Kunch (1990) have shown that primary neuronal cell populations isolated from human fetal dorsal root ganglia can be infected with HIV-1. However, no convincing data have been published so far on HIV-1 infection in neurons in vivo although numerous attempts have been made using immunohistochemical methods and in situ hybridization. It should be noted that the methods for detecting virus production do not reveal whether the neurons are actually infected. We have previously shown (Vesanen et al., 1991) that the low level productive infection in SH-SY5Y neuroblastoma cells can be detected only by co-cultivation. In situ hybridization of viral mRNA (our unpublished data) or immunochemical detection of viral protein were not sensitive enough for identifying virus although 20% of the cells were infected at the proviral DNA level.

The entry of HIV-1 into neuronal cells which appears to be CD4-independent may be differentiation-restricted. Our results do not exclude infection of fully differentiated neuronal cells but suggest that neuronal cells are preferentially susceptible to HIV-1 infection at an early stage of morphological cell differentiation. In our previous in situ hybridization studies we have shown that in cultures of the SK-N-MC cell line 50% of the cells were infected with HIV-1 (H1B strain) (Vesanen et al., 1992) and in a CD4-independent fashion. Harouse et al. (1991) have reported that infection of SK-N-MC neuroblastoma cells with HIV-1 can be inhibited by antibodies to galactosyl ceramide. Moreover, Yahi et al. (1992) have shown that the same antibodies inhibit infection of human colon epithelial HT29 cells. Thus, an alternative mechanism which permits the virus entry may function in CD4-negative cells. This could be related to the findings of the present study where morphological differentiation inhibits virus infection.

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