Activation of integrated human immunodeficiency virus type 1 in human neuroblastoma cells by the cytokines tumour necrosis factor alpha and interleukin-6

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Human immunodeficiency virus type 1 (HIV-1) infection was studied in two different human neuroblastoma cell lines, SK-N-MC and SH-SY5Y. Results from immunofluorescence analysis indicate that SK-N-MC cells express a 68K neurofilament, and SH-SY5Y cells express additionally a 160K to 200K neurofilament complex and thus represent a more differentiated state. HIV-1 infection in these cell lines was demonstrated by nested polymerase chain reaction and further characterized by in situ hybridization, which showed that about 50% of SK-N-MC cells and 20% of SH-SY5Y cells were infected by HIV-1 and contained integrated proviral HIV-1 DNA. Among the cytokines and growth factors studied, tumour necrosis factor alpha (TNF-α) enhanced virus production in both cell lines, but to a differing extent, according to our mRNA and p24 antigen capture assay. In SK-N-MC cells the enhancement of HIV-1 mRNA was detected after 24 h of stimulation, and declined to the control level by 48 h. In SH-SY5Y cells a clear-cut stimulation was seen at both time points. By contrast, interleukin-6 (IL-6) enhanced the virus replication only in SK-N-MC cells, as shown at the mRNA level. Immunohistochemical staining showed no differences in the proportion of HIV-1-positive cells after 48 h of stimulation by TNF-α or IL-6 when compared to the control cells. In addition, based on a thymidine incorporation assay, TNF-α inhibited, but IL-6 strongly increased, the DNA synthesis in SK-N-MC cells, whereas in the SH-SY5Y cell line no such differences were seen. We discuss the possibility that developing, less-differentiated neurons may be more readily infected by HIV-1 than fully differentiated neurons, and that cytokines such as TNF-α and IL-6, which are elevated in HIV-1-infected individuals, may enhance HIV production.

Introduction

Human immunodeficiency virus (HIV) infection in children and adults frequently manifests symptoms of central nervous system (CNS) diseases, which involve many types of neurological dysfunction and may develop to a severe dementia at a late stage of infection (Cornblath et al., 1987; McArthur, 1987; Epstein et al., 1988; Johnson et al., 1988; Price et al., 1988). However, the mechanism of HIV neuropathogenesis is not fully understood. To date, it is known that the predominant source of the virus in the brain in vivo is the monocyte/macrophage lineage of cells including brain microglial cells, but capillary endothelial cells, multinucleated giant cells and astrocytes are also infected (Epstein et al., 1984–1985; Ho et al., 1985; Sharer et al., 1986; Stoler et al., 1986; Wiley et al., 1986; Vazeux et al., 1987; Watkins et al., 1990). Several in vitro studies show that tumour cells of neuroectodermal origin are susceptible to infection (Chiodi et al., 1987; Dewhurst et al., 1987; Koyanagi et al., 1987; Harouse et al., 1989; Li et al., 1990; Vesanen et al., 1991). However, HIV-mediated CNS damage could also be due to the effects of cytokines/growth factors. Elevated levels of tumour necrosis factor alpha (TNF-α) (Lähdevirta et al., 1988; Grimaldi et al., 1991) and interleukin-6 (IL-6) (Gallo et al., 1989; Birx et al., 1990; Breen et al., 1990; Laurenzi et al., 1990) in the cerebrospinal fluid/serum have been described in HIV-1-infected patients. Several reports indicate that TNF-α may be neurotoxic, mediating demyelination of neurons (Robbins et al., 1987; Selmaj & Raine, 1988).

We have previously demonstrated that a persistent inapparent HIV-1 infection, apparently independent of the CD4 receptor, occurs in human SK-N-MC, SH-SY5Y and IMR-32 neuroblastoma cells (Vesanen et al., 1991). The infection was productive, however, with differing low levels of virus expression as determined by p24 antigen capture assays and Northern blotting. The cultured HIV-1-infected SK-N-MC cells expressed detectable levels of p24, whereas infected SH-SY5Y and

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IMR-32 cells had a low level of expression, which was seen only upon cocultivation with the HIV-sensitive lymphoid cell line C8166.

In the present study we have further characterized the HIV-1 infection in SK-N-MC and SH-SY5Y cell lines using in situ hybridization and polymerase chain reaction (PCR). The effects of different cytokines/growth factors on virus expression were also studied. Our results imply that less differentiated neuroblastoma cells are more readily infected than more differentiated cells. We speculate that in the foetus, direct HIV-1 infection of developing neuronal cells may occur, and production of the virus could be enhanced by cytokines, thus contributing to the neuronal dysfunction seen in HIV-infected children.

Methods

Cell lines. The human neuroblastoma cell line SK-N-MC (Biedler et al., 1973; ATCC HTB10) and the human osteosarcoma cell line MG-63 (ATCC CRL 1427) were obtained from the ATCC. The cells were maintained in Eagle's medium (MEM) supplemented with 10% foetal calf serum (FCS) (Gibco) and antibiotics (streptomycin at 100 μg/ml, penicillin at 100 units/ml) (Gibco). The human neuroblastoma cell line SH-SY5Y (Pählim et al., 1981) was kindly provided by Dr S. Pählim (Karolinska Institute, Stockholm, Sweden) and was maintained in RPMI 1640 medium (Gibco) containing 10% FCS and antibiotics.

Immunofluorescent staining of neurofilaments in neuroblastoma cell lines. SK-N-MC, SH-SY5Y and MG-63 cell lines were fixed with 4% (w/v) paraformaldehyde for 15 min at room temperature and then stained using the indirect method. Polyclonal rabbit antiserum recognizing all neurofilament species (68K, 160K and 200K), kindly provided by Dr Ismo Virtanen (Department of Anatomy, University of Helsinki, Finland), gave an absorbance value of 1.0. After 24 h of incubation, the cells on cytospin preparations were denatured in 70% formamide/2 x SSC pH 7.0 for 2 min at 70 °C and dehydrated at 4 °C. The preparations were fixed in 4% (w/v) paraformaldehyde. Metal phase spreads were prepared from infected SK-N-MC neuroblastoma cells using conventional cytogenetic procedures. The uninfected SH-SY5Y and SK-N-MC cell lines were used as controls. Each experiment was performed at least twice.

Before hybridization, the preparations were treated with 5 μg of pepsin per ml (Sigma) for 5 to 10 min at 37 °C (Burns et al., 1987) and with 200 μg of RNase A per ml (Boehringer Mannheim) for 60 min at 37 °C in 2 x SSC (Pinkel et al., 1986).

The hybridization mixture consisted of 50% formamide, 5% (w/v) Dextran sulphate, 2 x SSC, 5 x Denhardt's solution, 0.5 mg/ml of herring sperm DNA and 3 μg/ml of the biotin- or DIG-labeled probe. The cells on cytospin preparations were denatured in 70% formamide/2 x SSC pH 7.0 for 2 min at 70 °C and dehydrated at 4 °C. Denaturation of the DIG-labeled probe was performed at 95 °C for 10 min. Hybridization buffer (10 μl) containing the DIG-labeled probe was pipetted on the cytospin slides. Denaturation of the biotin-labeled probe and the target DNA on conventional cytogenetic preparations of SK-N-MC cells was done simultaneously at 75 °C for 3 to 4 min after the hybridization buffer was put onto the slides. Hybridizations were done overnight at 42 °C. After hybridization the slides were washed

Nested PCR

(i) Preparation of cell lysates for PCR. Cell lysates were prepared after 1 month of HIV-1 infection (10th passage) by minor modifications of the method of Higuchi (1989). Trypsinized cells were collected by centrifugation for 30 min at 200 g, washed twice with PBS and counted. Washed cells were resuspended in cell lysis buffer (50 mm-KCl, 10 mm-Tris-HCl pH 8.3, 2.5 mm-MgCl2, 0.45% Tween 20, 0.45% NP40, 0.1 mg/ml gelatin) to a density of 6 x 106 cells/ml. Proteinase K (Boehringer Mannheim) was added to a final concentration of 60 μg/ml and cells were incubated at 55 °C for 1 h followed by 10 min at 95 °C to inactivate the protease.

(ii) Nested PCR. PCR was performed in two steps with bracket and nested primers (Ayehunie et al., 1991; Salminen, 1992). The primer coordinates according to the 1990 Los Alamos database HIVHXB2 coding sequences are (+ denotes coding, − non-coding strand): gag, +1027 to +1049 (BJGAG1), −1286 to −1306 (BJGAG3) and pol, −243 to −267 (BJGAG2). The primers were used in a nested combination to amplify HIV-1 subgenomic gag fragments. Amplifications were performed in a volume of 50 μl using 25 μl of cell lystate in the primary PCR and 5 μl from the first reaction as the template in the secondary PCR. Reactions were overlaid with 20 μl mineral oil (Sigma). Native Taq polymerase (2.5 units) (Perkin-Elmer Cetus), 0.8 μM of each primer and 200 μM of each dNTP (Perkin-Elmer Cetus) were used in a buffer consisting of 50 mm-KCl, 1.5 mm-MgCl2, 10 mm-Tris-HCl pH 8.3. Temperature cycling was performed using a Techn e PHC-1 thermal cycler. Profiles were 1 min at 98 °C initial denaturation followed by 30 cycles of 30 s at 98 °C, 1 min at 55 °C and 3 min at 72 °C. After cycling a final incubation for 5 min at 72 °C was included. In the secondary PCR an annealing temperature of 53 °C was used. PCR products were run on 3.5% NuSieve agarose gels (FMC Biolabs) stained with ethidium bromide and visualized by u.v. transillumination. Several negative and positive controls (nine reagents, two HIV-1 negative human DNA and three HIV-1 IIIB strain-infected CEM-SS DNA) were included in all PCR steps.

In situ hybridization. In situ hybridizations were performed both with the biotin-labeled, full-genomic HIV-1-specific cDNA probe pHBl0-R3 (Biotech Research Laboratories) and with a digoxigenin (DIG) labeled 9 kb insert of the pHBl0-R3 plasmid. Biotin-11-dUTP (Sigma) was incorporated into the plasmid by nick translation (nick translation kit, BRL). DIG-11-dUTP was added to the viral insert by random priming (DNA labelling and detection kit, Boehringer Mannheim).

Cytospin preparations were made of cultured SK-N-MC and SH-SY5Y neuroblastoma cells after 1 month of infection (10th passage). The preparations were fixed in 70% formamide/2 x SSC at 4 °C and dehydrated at 4 °C. The preparations were hybridized with a 35S-labeled, full-length genomic HIV-1 probe. The probe consists of a 9.9 kb BamHI-E fragment of HIV-1 IIIB genomic DNA (Vesanen et al., 1991). The probe was labeled with [35S]dCTP using the random priming procedure as described above. The preparations were hybridized overnight at 42 °C and exposed to X-Omat AR film (Eastman Kodak Co.) for 1 week.
with three changes of 50% formamide, 2 × SSC pH 7-0 at 40 °C for 5 min each change, in two changes of 2 × SSC at 40 °C for 15 min each, once in 1 × SSC and once in 0-1 × SSC for 15 min at room temperature.

The biotin-labelled hybrids were revealed by avidin-FITC (5 μg/ml; Vector) according to Pinkel et al. (1986). The DIG-labelled hybrids were detected with AP-conjugated anti-DIG antibody using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium salt (NBT) as substrates as described by Seibl et al. (1990).

**Stimulation of HIV-1 by cytokines/growth factors.** Following HIV-1 infection the cells were cultured for 1 month before the start of the stimulation experiments. The cytokines and growth factors used were human IL-1 (100 units/ml; Endogen), human recombinant IL-2 (100 units/ml; Janssen Biochemica), human recombinant IL-6 (100 units/ml; Janssen Biochemica), human recombinant TNF-α (100 units/ml; Genzyme), bovine brain-derived basic fibroblast growth factor (bFGF) (5 ng/μl; R&D Systems), porcine platelet-derived transforming growth factor beta (TGF-β) (5 ng/ml; R&D Systems), human recombinant epidermal growth factor (EGF) (10 ng/ml; Chiron Corporation) and mouse nerve growth factor (NGF) (2.5 S) (50 ng/ml; Janssen Biochemica). The stimulations were performed in MEM and RPMI 1640 medium containing 10% FCS

**Northern blot analysis.** The poly(A)+ mRNA fraction was isolated and 10 μg of each sample was electrophoresed, blotted and hybridized with a 9 kb insert of the pBH10-R3 plasmid labelled by random priming (BRL) as described by Vesanen et al. (1991). A probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to monitor the amounts of mRNA. Densitometric scanning analysis was performed to detect the intensity of signals.

**[3H]Thymidine incorporation assay.** The assay was performed in 24-well plates (Nunc), in which 1 × 10^5 cells were subcultured per well. After overnight growth the cells were washed once and starved for 6 h in MEM containing 0.2% BSA (Sigma). After starvation the cells were stimulated with the cytokines for 24 h in MEM containing 0.2% BSA, and for the last 6 h in the presence of [3H]thymidine (1 μCi/ml) and the incorporated [3H]thymidine was measured. Each assay was carried out in triplicate.

**Results**

**Immunofluorescent staining of neurofilaments in neuroblastoma cell lines**

Indirect immunofluorescence staining was performed to investigate the neurofilament expression in SK-N-MC and SH-SY5Y neuroblastoma cell lines. Two polyclonal antibodies were used, one which recognizes the 68K, 160K and 200K neurofilaments and the other which recognizes the 160K to 200K neurofilament complex. The results showed that the SH-SY5Y cell line expressed the 160K to 200K neurofilament complex, whereas the SK-N-MC cell line expressed only the 68K neurofilament (data not shown). The MG-68 osteosarcoma cell line, used as a negative control, showed no fluorescence when stained with these antibodies.

**Amplification of HIV-1 gag sequence by nested PCR**

PCR amplification of gag sequences in infected neuroblastoma cells resulted in the appearance of bands of the correct size as measured by agarose gel electrophoresis and ethidium bromide staining (Fig. 1). No amplification products were seen in uninfected cells or normal HIV-1-negative lymphocyte lysates. In HIV-1-infected SK-N-MC cells amplification products were seen after one round of amplification, whereas two rounds of amplification using nested primers were needed to amplify gag sequences in HIV-1-infected SH-SY5Y cells.

**Detection of HIV-1 by in situ hybridization**

**In situ** hybridization was performed to determine the proportion of HIV-1-positive cells in cultured SK-N-MC and SH-SY5Y cells after 1 month of infection. Both fluorescence and enzymatic in situ hybridization methods were used. At least 200 HIV-1-infected and uninfected interphase cells were studied for each cell line (Table 1). Of SK-N-MC interphase cells 48 and 51% were infected according to fluorescence and enzymatic detections, respectively (Fig. 2a). Of SH-SY5Y interphase cells 20% were HIV-1-positive according to enzymatic detection. The number of hybridization signals in infected interphase cells varied from one to five; at least 85% of the interphase cells contained only one signal. In uninfected control slides 2 and 2-5% of the

![Table 1. Frequency of HIV-1-positive interphase cells in infected SK-N-MC and SH-SY5Y cell lines by in situ hybridization](attachment:table1.png)

* Detection was performed with AP-conjugated anti-DIG antibody with BCIP and NBT as substrates or FITC-conjugated avidin (A-FITC); Bio, biotin.
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Fig. 1. Ethidium bromide-stained agarose gel after electrophoresis of 5 µl aliquots of PCR products from (a) first and (b) second (nested) amplifications of lysed neuroblastoma cells. Lanes 1, negative reagent control; lanes 2, negative human DNA control; lanes 3, positive control (CEM-SS cells infected by HIV-1 IIIB strain); lanes 4, SH-SY5Y cells infected by HIV-1 IIIB strain; lanes 5, SK-N-MC cells equally infected; lanes 6, uninfected SH-SY5Y cells; lanes 7, uninfected SK-N-MC cells. Amplified HIV-1 gag fragments are (a) 812 bp and (b) 278 bp.

Enhancement of HIV-1 production by cytokines at the protein level

HIV-1-infected SK-N-MC and SH-SY5Y cells were treated with IL-1 (100 units/ml), IL-2 (100 units/ml), IL-6 (100 units/ml), TNF-α (100 units/ml), NGF (50 ng/ml), TGF-β (5 ng/ml), bFGF (5 ng/ml) or EGF (10 ng/ml). After 24 h of TNF-α stimulation we were able to measure elevated levels of p24 antigen from the culture fluid, which were further increased when the antigen was tested after 48 h of stimulation (Fig. 3). In the case of the other cytokines tested, no alterations were seen in HIV-1 production at the protein antigen level when compared to the unstimulated control cells (data not shown).

The proportion of HIV-1-positive cells at the protein level was determined after 48 h of TNF-α stimulations using an immunohistochemical staining procedure (APAAP) to detect the p24 antigen. Compared to the unstimulated control cells, no changes were seen in the proportion of positive cells in either cell line studied.

Enhancement of HIV-1 production by TNF-α and IL-6 at the mRNA level

To characterize further the HIV-1 up-regulation by TNF-α, mRNA was isolated and hybridized in Northern blotting using a full-genomic HIV-1 cDNA probe. In SH-SY5Y cells a clear-cut augmentation of the mRNA
HIV-1 infection in neuroblastoma cells

Fig. 3. Detection of HIV-1 production in infected neuroblastoma cells at the protein level after TNF-α (▲; 100 units/ml) and IL-6 (●; 100 units/ml) stimulation. (a) SH-SY5Y cells, (b) SK-N-MC cells. Controls are shown (□). Protein p24 was measured from culture supernatant fluid after 24 and 48 h stimulation using EIA. The lines represent mean values of several independent experiments. The cut-off level (-----) in the assay was approximately 25 pg/ml.

was detected after 24 and 48 h of stimulation (Fig. 4a). In SK-N-MC cells the mRNA level increased 3.5-fold after 24 h of stimulation, declining to the level of control mRNA at 48 h (Fig. 4b). Thus TNF-α was capable of enhancing the viral mRNA production in both cell lines, although to a different extent.

The effect of IL-6 on HIV-1 expression was also studied at the mRNA level. In HIV-1-infected SK-N-MC cells we could demonstrate a 2.5-fold enhancement of HIV-1 mRNA after 48 h of stimulation (Fig. 4b). No changes were seen when HIV-1-infected SH-SY5Y cells were examined (Fig. 4a).

Effects of TNF-α and IL-6 on cell DNA synthesis

The effect of TNF-α and IL-6 on cell DNA synthesis was studied using the thymidine incorporation assay. In SK-N-MC cells, TNF-α inhibited by approximately 33% whereas IL-6 enhanced cellular DNA synthesis strongly by threefold, when compared to the control. In SH-SY5Y cells, TNF-α and IL-6 showed no differences in DNA synthesis. Moreover, in both cell lines TNF-α and IL-6 had no detectable morphological effects on cell differentiation.
Discussion

The mechanism of CNS dysfunction in HIV-positive children and adults is not understood. It is not known whether neuronal cells are directly infected by HIV and so contribute to the HIV neuropathogenesis, or whether the dysfunctions are caused indirectly via other cells infected in the CNS, for example monocyte/macrophages and microglial cells, cells which are the predominant source of the virus in the brain (Wigdahl & Kunsch, 1990; Dickson et al., 1991). We have previously characterized the persistent productive and seemingly CD4-independent HIV-1 infection in three different human neuroblasticoma cell lines (Vesanen et al., 1991). We have now characterized two of these cell lines in more detail and studied their infection with HIV-1. Indirect immunofluorescence staining of neurofilaments showed that both cell lines express the 68K neurofilament, but only the SH-SY5Y cell line expresses the 160K to 200K neurofilament complex. Thus the SH-SY5Y cells may be considered more differentiated than SK-N-MC cells.

Results obtained by PCR analysis agree well with results obtained by in situ hybridization. Although our PCR assay has not been optimized for quantification of HIV-1 sequences, others have shown that PCR can be quantitative (Dahlen et al., 1991). Thus, detection of amplification of gag sequences in the first round of PCR in the case of the infected SK-N-MC cells and detection of gag sequences only after nested PCR in the HIV-1-infected SH-SY5Y cells indicates that the former cell line has acquired a more abundant persistent infection. The PCR assay, however, does not distinguish whether this difference is accounted for by multiple copies of the provirus per cell or whether it is a result of fewer infected cells in the SH-SY5Y cell line. However, our interphase in situ hybridization studies revealed that the proportion of infected nuclei in the SH-SY5Y cell line was at least 30% lower than in the SK-N-MC cell line. Moreover, most of the positive cells contained only one signal.

In situ hybridization studies on SK-N-MC metaphase chromosomes indicate that the virus in this cell line existed in the integrated form. This may also explain the persistence of the HIV infection in the SK-N-MC cell line as shown in our previous studies (Vesanen et al., 1991).

Our study on the up-regulatory capacity of various cytokines showed that TNF-α and IL-6 enhance HIV-1 production in human neuroblasticoma cells. Such a stimulatory effect by these cytokines has also been demonstrated in other cell lines. TNF-α/β and IL-6 enhanced virus production in the promonocytic U1 cell line, whereas in the T lymphocytic ACH-2 cell line only TNF-α and -β were capable of producing such a stimulatory effect (Clouse et al., 1989; Folks et al., 1989; Poli et al., 1990a, b). According to our results, TNF-α up-regulates the virus production in infected SK-N-MC cells after 24 or 48 h of stimulation as measured using the p24 antigen capture assay. mRNA studies showed a 3.5-fold increase at 24 h, which then declined to the control level at 48 h. The decline in mRNA seen after 48 h might be due to a cytotoxic action of TNF-α on neurons, as described by others (Robbins et al., 1987; Selmaj & Raine, 1988). Based on the thymidine incorporation assay, inhibition by TNF-α of SK-N-MC cell DNA synthesis occurred.

IL-6 also enhanced the virus production in the SK-N-MC cells. A 2.5-fold enhancement was seen at the mRNA level after 48 h of stimulation. Furthermore, IL-6 strongly increased the cell proliferation, possibly correlating with the increased level of HIV-1 mRNA detected.

In contrast, in infected SH-SY5Y cells the only cytokine with up-regulatory capacity was TNF-α. After 24 h of stimulation we could detect a clear-cut enhancement of HIV-1 mRNA, which was maintained at 48 h. At the protein level (p24 antigen) a constant increase in virus production was measured after 24 and 48 h of stimulation. No effects of TNF-α and IL-6 on cellular DNA synthesis were seen when compared to the control.

We performed immunohistochemical staining (APAAP) to see whether the enhanced production of the virus is also apparent at the cellular level. We have shown previously that without stimulation about 0-1% of the SK-N-MC cells were HIV-1 p24 antigen-positive. No positive cells (<0.001%) were seen when SH-SY5Y cells were examined (Vesanen et al., 1991). In the present study we could not see any alterations in the proportion of the HIV-1-positive cells after 48 h of stimulation by TNF-α or IL-6, although enhanced protein production can be measured from culture fluid using the p24 EIA.

In conclusion, our in vitro results raise the possibility that fully differentiated neurons in adults, if compared to developing neurons, may be less susceptible to HIV-1 infection in vivo. This possibility is further supported by our recent results in which HIV-1 infection in differentiation-induced SH-SY5Y cells is lower than that in non-differentiated cells (unpublished data). Thus, the CNS dysfunction seen in adult patients might be caused by an indirect mechanism. Such mechanisms are suggested to be due to toxic effects of different cytokines/growth factors, e.g. TNF-α (Wigdahl & Kunsch, 1990; Merrill & Chen, 1991). Moreover, we have shown that TNF-α and IL-6 enhanced the virus production in vitro. Activated lymphocytes, astrocytes and microglial cells are known to have the ability to enhance secretion of TNF-α and IL-6 (Frei et al., 1987, 1989; Nakajima et al., 1989;
Rieckmann et al., 1991). Elevated levels of these cytokines are detected in the cerebrospinal fluid of HIV-infected patients (Lähdevirta et al., 1988; Bix et al., 1990; Breen et al., 1990; Grimaldi et al., 1991). Thus, developing neurons in the foetus and infants might be infected more easily than in adults and virus production might be enhanced by cytokines such as TNF-α and IL-6.

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