The expression of HIV-1 *tat* and *nef* genes induces cell-specific changes in growth properties and morphology of different types of rat cells

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Abstract. Among the viral regulatory genes the *tat* and *nef* genes of HIV-1 encode the proteins playing a central role in viral replication and exerting pleiotropic effects on the survival and growth of the cells. These effects differ in various cell types, possibly due to the use of genes from different HIV-1 isolates. In this work, we studied the effects of the *tat* and *nef* genes on three types of cultured rat cells: primary embryo fibroblasts, pseudonormal Rat-2, and pheochromocytoma PC12. Both genes affected growth properties and morphology of cells, the effects being cell-specific. The proliferative activity of both Rat-2 and PC12 cells was considerably increased after transfection with the *tat* gene. In primary rat embryo fibroblasts the *tat* gene induced multilayered foci. More importantly, it was shown that the efficiency of transformation was higher in cells coexpressing *tat* and *nef*. The *nef* gene caused considerable suppression of Rat-2 cell proliferation, but no changes in their morphology. The *nef* gene transfection of PC12 cells also led to suppression of their proliferative activity. In addition, cellular agglomerates which were morphologically similar to multinuclear syncytial cells were detected in these cells for the first time.

INTRODUCTION

Human immunodeficiency virus (HIV), the etiological agent of AIDS, is a complex retrovirus which contains several structural and accessory (regulatory) genes. Among viral regulatory genes the *tat* and *nef* genes encode the proteins playing a central role in viral replication and exerting pleiotropic effects on the survival and growth of different cell types (Cullen 1998).

The HIV Tat protein is a pleiotropic factor that influences cellular physiology by affecting the expression of cellular genes (Westendorp *et al.* 1994; Rautonen *et al.* 1994; Rasty *et al.* 1996; Sastry *et al.* 1996). Apart from its function as a transactivator, the Tat protein is known to induce some pathologies associated with AIDS (Vogel *et al.* 1988; Kim *et al.* 1992; Corallini

et al. 1993; Gavriil *et al.* 1999). Extensive research has been carried out in order to understand the action of the *tat* gene on cell proliferation and apoptosis. However, the data obtained are contradictory (Ensoli *et al.* 1993; Campioni *et al.* 1995; Zauli *et al.* 1995; Patki *et al.* 1996; Sastry *et al.* 1996). Campioni *et al.* (1995) showed that the Tat protein stimulated cell proliferation in transgenic mice and protected them from apoptosis in conditions of serum starvation. Zauli *et al.* (1995) observed apoptosis inhibition by Tat in Jurkat T-cell lines and primary PBMC. In contrast, Sastry *et al.* (1996) showed that T-lymphocytes transfected with constitutively-expressed *tat* gene resulted in rapid apoptosis when grown under serum-free conditions. According to data reported by Patki and Lederman (1996), the HIV-1 Tat protein induced apoptosis and inhibited lymphocyte proliferative responses. Several reports have provided evidence that the *tat* gene has oncogenic potential (Vogel *et al.* 1988; Corralini *et al.* 1993; Kim *et al.* 1992). Moreover, Tat suppressed butyric acid-induced differentiation in the haemopoietic progenitor cell line K562 (Mondal & Agrawal 1996).

The *nef* gene encodes a protein with structural features of a signal transducing molecule of the *ras* family, whose functions, however, remain unknown. Data are gradually accumulating that point to the role of the *nef* gene, not only in the regulation of expression of HIV-1 genes and in the replication of the virus (Kestler *et al.* 1991; Jamieson *et al.* 1994), but also in the repression of individual cellular gene expression (Garcia *et al.* 1991; De *et al.* 1998). Besides, the effect of the *nef* gene on the endocytosis of some cellular membrane-localised proteins (Schwartz *et al.* 1996) and its regulatory effect in the formation of syncytia of T-lymphocytes and macrophages (Meylan *et al.* 1998) were reported. The changes in lymphocyte proliferation were observed in transgenic mice containing the *nef* gene (Mudrik *et al.* 1992; Skowronski *et al.* 1993). The Nef protein significantly reduced the proliferative response to platelet-derived growth factor (Graziani *et al.* 1996), but induced proliferation of human peripheral blood mononuclear cells (Torres *et al.* 1996). Stable expression of the *nef* gene in human astrocytes changes growth properties and activation state of these cells (Kohleisen *et al.* 1999).

Thus, the data available provide evidence of the multifunctional action of HIV-1 *tat* and *nef* genes on cells from different tissues. However, the role of these genes in the development of pathologies in HIV-infected patients has not been elucidated to the full. Transfection of different nonpermissive cultured cells with these viral genes can provide an approach to be used to study the function of individual regulatory genes of HIV-1.

MATERIALS AND METHODS

Plasmids

To construct a *tat* gene-containing pMT-*tat* plasmid, the pRIP7 plasmid (kindly provided by Dr. S. Kim from the USA) (Kim *et al.* 1989) bearing HIV-1 provirus (isolate HXB-2) was used. A 2.7 kb pRIP7 *EcoRI/BamHI*-fragment containing two translated exons of the *tat* gene were subcloned in the pBS vector (plasmid pBStat). Then a 1.75 kb *BglIII/XbaI*-fragment from pRIP7 containing a part of HIV-1 LTR (regions R and U5) and its flanking human DNA genome region (plasmid pBStatLTR) were inserted into pBStat. After deletion of a part of the flanking region (the 1.4 kb *PstI*-fragment) a 0.84 kb *EcoRI/SalGI*-fragment containing the murine metallothioneine gene (MT) promoter was inserted into the resulting plasmid. The recombinant plasmid pNef with the *nef* gene under the control of cytomegalovirus (CMV) promoter and the control plasmid pNef-fs with a frame-shift mutation in the *nef* gene were kindly provided by Dr. S. Hammes (Hammes *et al.* 1989). Plasmids containing the *c-Ha-ras1* [pEJ6.6 (Shih &

Weinberg 1982)], *c-myc* [pSVc-myc-1 (Land *et al.* 1983)] and *neo^r* [pSV2neo (Southern & Berg 1982)] genes were also used.

Cell cultures

A pseudonormal line Rat-2 of rat fibroblast cells, primary fibroblasts of 16- and 17-d-old rat embryos and rat pheochromocytoma PC12 cells were used in experiments. Rat-2 cells were grown in Eagle's medium with 10% fetal bovine serum (FBS). Primary rat embryo fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS. PC12 cells capable of neuronal differentiation under the action of NGF (Green & Tischler 1976) were grown in RPMI-1640 medium with the addition of 5% horse serum and 10% FBS.

Isolation of stable transformed polyclonal cell cultures

The standard electroporation procedure was used in all transfection experiments. Cell cultures at the log phase of growth (10^6 cells per chamber) were suspended in 60 μ l of $1 \times$ HEBS buffer (25 mM HEPES, 140 mM NaCl, 0.75 mM Na_2HPO_4 , pH 7.1) and were treated with 11 μ g plasmid DNA. The following parameters were used for different cell cultures: impulse duration, 1.5 ms, voltage 500 V for primary fibroblasts, 600 V for Rat-2 cells and 450 V for PC12 cells. Each of the plasmids pMT-*tat*, pNef and pNef-fs was cotransfected with the plasmid pSV2neo in a 10:1 ratio. The control transfection was performed with the pSV2neo plasmid (1 μ g/ 10^6 cells). After the treatment cells were seeded at $2-3 \times 10^5$ cells per dish ($d = 60$ mm) in standard media. The selection of transformed cells was performed by the addition of G418 (0.5 mg/ml) to the culture medium after 24 h past electroporation. In order to obtain polyclonal stable transformed cultures, the pooled colonies were harvested after two weeks of growth in selective medium. To test the presence of the transgene in cells and its expression, PCR and RT-PCR were performed. For this purpose, in all the cases, at least 10 single colonies were used which were isolated and cultivated under G418 selective pressure. Polymerase chain reaction (PCR) and Reverse transcriptase-PCR (RT-PCR) was performed with the following oligonucleotide pairs: 5'-GAGCCAGTAGATCCTAGACTAGAGC-3' and 5'-TCTGATGAGCTCTTCGTCGCTGTC-3' for the *tat* gene and 5'-GGTGGCAAGTGGTCAAAAAGTAGTG-3' and AATCAGGGAAGTAGCCTTGTGTGTG-3' for the *nef* gene. PCR on cDNA and genomic DNA was performed using a DNA thermal cycler (Perkin-Elmer Cetus Norwalk, Connecticut, USA), 50 ng of each primer, and an annealing temperature of 55 °C. In all cases, > 90% of isolated G418-resistant colonies consisted of transgene. The transgene expression was detected in more than 60% G418-resistant single colonies.

Cell proliferation rate and differentiation assay

Cell proliferation rate was estimated from the number of cells every 2 days during a week by the optical density indices with the use of MTT (3-(4,5-dimethylthiasole-2il)-2,5-diphenyltetrasolium bromide) (Shearman *et al.* 1994). For the induction of the *tat* gene expression from MT promoter, ZnSO_4 was added to the culture medium to a final concentration of 0.2 mM.

In order to examine neuronal differentiation of PC12 cells 100 ng/ml NGF was added to the culture medium every 3 days for 10 days.

RESULTS

Effects of the HIV-1 *tat* gene

Polyclonal cultures of the *tat*-transfected Rat-2 and PC12 cells were characterised in terms of growth properties (proliferation, focus formation) and morphology.

Figure 1. Growth rate of *tat*-transformed polyclonal cell cultures. The number of PC12 (squares) and Rat-2 (circles) cells was counted in control pSV2*neo*-transfected variants (open symbols) and in pMT-*tat*/pSV2*neo*-transfected variants in the presence of ZnSO₄-inductor (x-centre symbols) and in its absence (solid symbols). The graph represents the results of three independent experiments; standard deviations are indicated by bars.

Tat-expressing cells revealed a considerably higher proliferative activity compared with control cells (Fig. 1). Most differences were detected in the presence of an inductor (ZnSO₄) of metallothioneine promoter of the *tat* gene. Addition of 0.2 mM ZnSO₄ to the medium of control cells did not effect their proliferation.

Tat-transfected Rat-2 cells formed multilayer colonies, and the morphology of these cells was noticeably changed as compared with the control cells (Fig. 2). However, the morphology of PC12 cells transfected by the *tat* gene remained unchanged. Cultivation of *tat*-containing PC12 cells in conditions leading to neuronal cell differentiation (addition of NGF) revealed no statistically reliable effect of the HIV-1 *tat* gene on either the process of their neuronal differentiation or cell proliferation rate (data not shown).

To determine whether the *tat* gene could transform primary rat embryo fibroblasts, we used a standard focus-formation assay (Table 1). We used expression plasmids, single and in combination, containing the following DNA sequences: the *tat* gene, the cellular oncogene Harvey rat sarcoma viral oncogene homologue (c-Ha-*ras*1) and the *c-myc* oncogene. We assessed transformation by monitoring the appearance of foci 10–14 d after transfection. The *tat* gene alone induced the formation of foci. Also, cotransfection of *tat* and c-Ha-*ras*1 genes increased the number of foci. The increase was statistically significant ($P < 0.05$) after cotransfection of c-Ha-*ras*1 and *c-myc*. The number of multilayer colonies was also observed to be remarkably increased after cotransfection of *tat* and *c-myc* genes.

Effects of HIV-1 *nef* gene

After transfection of Rat-2 cells with the *nef* gene or with the plasmid carrying the *nef* gene with a frame-shift mutation (control), the proliferation of the normal *nef* gene containing cells was found to be considerably lower than that of the control cells (Fig. 3).

Figure 2. Morphology of (a) Rat-2 cells transfected with pMT-tat/pSV2neo and (b) control Rat-2 cells transfected with pSV2neo. Magnification $\times 320$.

Table 1. Foci formation in rat embryo fibroblasts after transfection with different plasmids

Transfected DNA	Number of foci per 1.5×10^5 cells*	Efficiency of transformation $\times 10^{-5}$
mock-transfection	0	0
pMTtat	6.5 ± 1.0	0.43
pMTtat + pEJ6.6	13.2 ± 2.1	0.88
pMTtat + pSVc-myc-1	9.0 ± 1.9	0.60
pMTtat + pNef	9.3 ± 1.7	0.62
pnef	0	0
pSVc-myc-1	0	0
pEJ6.6	0	0
pEJ6.6 + pSVc-myc-1	15.1 ± 1.5	1.00

*The average of four independent experiments.

Transfection of rat embryo fibroblasts with the *nef* gene gave no multilayer foci formation (Table 1). However, the number of multilayer foci was somewhat higher after cotransfection of *tat* and *nef* than that observed after transfection of the *tat* gene alone.

The morphology of rat embryo fibroblasts and Rat-2 cells transfected with the *nef* gene did not differ from control pSV2 *neo*-transfected cells. At the same time, the morphology of the *nef* containing PC12 cells appeared to be considerably changed as compared with the control. Formation of large structures similar to the syncytium (multinuclear cells) were observed in the light microscope (Fig. 4). The aggregates were viable and their size increased during 14 to 20 d of cultivation without replating. Parallel to this effect, the visually observed number of cells grew very slowly, which is probably due either to the decrease in the proliferative activity of the cells, or to the formation of a syncytia, which subsequently were lost during cultivation. After replating, practically all syncytial aggregates perished and the number of cells capable of morphological transformation was reduced from passage to passage. The estimation of the proliferative activity of *nef*-transfected cells after a number of replating procedures showed practically no difference in the growth parameters as compared with control (data not shown). PCR analysis of a cellular line cultivated during a month revealed the absence of the HIV-1 *nef* gene.

Figure 3. Growth rate of *nef*-transformed Rat-2 polyclonal cell cultures. The number of cells was counted in pNef/pSV2*neo*-transfected variant (solid circles) and in two control variants: transfected with pSV2*neo* (open circles) and with pNef-fs/pSV2*neo* (+-centre circles). The graph represents the results of three independent experiments; standard deviations are indicated by bars.

Figure 4. Syncytium-like structures in the pNef/pSV2*neo*-transfected PC12 cells. Magnification × 320.

DISCUSSION

The pathogenic manifestation of HIV-1-induced AIDS observed in different tissues include impaired hematopoiesis, immunopathology, cytopathology in the central nervous system, myopathy and different types of malignancy. Investigations of the effects of the *tat* and *nef* genes suggest that they play an important role in HIV pathogenesis.

To study the effect(s) of HIV regulatory genes we used various rat cell lines and not human cells usually infected by this virus. This choice was determined by the intention to discriminate between universal and specific mechanisms of action of the *tat* and *nef* gene products on cells of various species and types. It should be noted that some rat-derived cells, including Rat2, are able to produce infectious HIV-1 and support substantial levels of HIV-1 genes expression (Keppler *et al.* 2001).

Our results, obtained for three different types of rat cell cultures, point in all cases to cell proliferation enhancement under the action of HIV-1 *tat* gene. This fact correlates with the data published by other authors (Ensoli *et al.* 1993; Lotz *et al.* 1994; Campioni *et al.* 1995; Zauli *et al.* 1993, 1995; Seve *et al.* 1999). Even though the *tat* gene suppresses apoptosis (Zauli *et al.* 1995), it can not appreciably affect the results obtained as the content of apoptotic cells in growing cell cultures does not exceed 5%.

In contrast to a previous report about an important role of Tat in abrogating erythroid differentiation in K562 cells (Mondal & Agrawal 1996), we have no evidence for any effect of the *tat* gene on PC12 neuronal differentiation induced by NGF. At the same time, the expression of a pMT-*tat* plasmid in mouse embryonic stem cells disturbs the formation of embryoid bodies (our unpublished data). These findings suggest that, similar to proliferation, the effect of the *tat* gene on differentiation varies in different types of cells.

Besides, in our experiments the oncogenic potential of the *tat* gene was demonstrated on rat embryo fibroblasts. Malignant transformation induced by the *tat* gene in cell cultures and in transgenic mice had been observed earlier (Vogel *et al.* 1988; Corallini *et al.* 1993; Kim *et al.* 1992).

In the light of published and our own data, one may believe that the oncogenic potential of the *tat* gene is realised by the proliferative activity enhancement in cells and elimination of apoptosis. The data presented allow us both to conclude that the effect of the *tat* gene is different in different types of cells and indicate the multifunctional character of this gene's product.

In addition, the cooperative effect of *tat* and *nef* genes on the neoplastic transformation was detected for the first time (Table 1). Recently a cooperative effect was observed between the *nef* and the *hck* genes (Briggs *et al.* 2000). This may prove that the *nef* gene is able to induce the oncogenic activity of some genes. Moreover, the results recently obtained for stable *nef*-transfected human astrocytes have demonstrated the oncogenic potential of *nef* gene alone (Kohleisen *et al.* 1999). These data indicate that oncogenic effect of HIV-1 *nef* gene is probably cell type and/or species-specific, as was shown for HIV-1 *vpr* and *vpx* genes (Chang *et al.* 2000).

The effect of the *nef* gene on Rat-2 and PC12 cell proliferation is on the whole contrary to the effect of the *tat* gene. Our results correlate with the data showing that the *nef* gene product decreases the proliferative response to the action of the platelet-derived growth factor (Graziani *et al.* 1996) and reduces the lymphocytes proliferative activity (Greenway *et al.* 1995). This effect is opposite to the ability of the exogenous Nef protein to stimulate the proliferation of the peripheral blood mononuclear cells (Quaranta *et al.* 1999). Obviously the effect of the *nef* gene, as that of the *tat* gene, is different in different cell types and depends on the target cell's metabolism.

In experiments with PC12 cells, direct indication of the involvement of the *nef* gene in the mechanism of multinuclear syncytium formation, which generally appears after HIV-1 infection of human T-lymphocytes and macrophages was obtained for the first time. Data describing the regulatory effect of the *nef* gene on syncytium formation are scarce and contradictory (Otake *et al.* 1994; Meylan *et al.* 1998). Different effects on syncytium formation in CD4⁺ T-lymphocytes by the *nef* genes of different HIV-1 isolates was observed (Meylan *et al.* 1998). However, the syncytium forming action of the *nef* gene from the same HIV-1 isolate may obviously depend on the type of cells subjected to its action, as this effect was observed in PC12 cells only.

Thus, the results reported for three different rat cell lines demonstrate the pleiotropic but basically different action of the regulatory *nef* and *tat* genes of HIV-1 on rat cells of different tissue origin. Moreover, the results obtained show that each particular *tat* or *nef* gene taken from one viral isolate exerts various effects on different rat cell types. Therefore, the regulatory effects of HIV-1 accessory genes are cell-specific.

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