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Nucleoside reverse transcriptase inhibitors and human immunodeficiency virus proteins cause axonal injury in human dorsal root ganglia cultures

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Distal symmetric polyneuropathy (DSP) has emerged as the most common complication of human immunodeficiency virus (HIV) infection, which is associated with neuronal injury in the dorsal root ganglion (DRG). With the advent of highly active antiretroviral therapy, especially nucleoside analogs, patients are living longer. Some of the antiretroviral drugs used to treat HIV infection have been associated with neuropathies. The pathogenesis of these neuropathies remains poorly understood. Utilizing a human fetal DRG model of predominantly nociceptive fibers, the authors investigated the effects of HIV gp120 and $Tat_{1–72}$, alone or in combination with nucleoside analogs on both morphological and ultra-structural changes in DRG neurons. Nucleoside analogs and HIV proteins both caused a significant decrease in the mean axonal length. However, ddI was the most potent, followed by ddC, d4T, and AZT. Despite the combined exposure to toxic dosages of HIV proteins and nucleoside analogs, there appeared to be a ceiling effect on the amount of axonal retraction, indicating that the proximal and distal axon are differentially regulated. In conclusion, both HIV proteins and nucleoside reverse transcriptase inhibitors (NRTIs) cause axonal damage by inducing mitochondrial injury and rearrangement of microtubules. *Journal of NeuroVirology* (2007) **13,** 160–167.

Keywords: AIDS; antiretroviral; dorsal root ganglia; HIV; neuropathy; nucleoside analogs

Introduction

Distal symmetrical polyneuropathy (DSP) is not only the most common neurological complication associated with human immunodeficiency virus (HIV) infection (Berger and Nath, 2000; McArthur *et al*, 2005; Pardo *et al*, 2001), it can be a very painful and debilitating ailment as well. The most common clinical features of DSP are sensory loss and neuropathic pain in the distal extremities (Joseph *et al,* 2004; Keswani *et al*, 2002). Although the mechanisms underlining DSP remain unknown, it has been suggested that mitochondrial dysfunction may play a key role (Moyle, 2005).

Since the advent of highly active antiretroviral therapy (HAART), more patients are living longer and the quality of life for those infected with HIV has improved. However, during the post-HAART era, there has been an increase in the number of cases of toxic neuropathies (McArthur, 2004). This sensory neuropathy is phenotypically identical to DSP but has been termed antiretroviral toxic neuropathy (ATN). Although some therapeutic agents, particularly the nucleoside reverse transcriptase inhibitors (NRTIs), have been associated with peripheral neuropathies, this association is not well understood. Patients receiving NRTI therapy develop a distal symmetric small fiber dying back axonal neuropathy, as seen in DSP (Dalakas *et al*, 2001; Joseph *et al,* 2004; Pardo *et al*, 2001). It remains unknown if NRTIs alone are sufficient to cause a neuropathy. Rodents treated with very high dosages of NRTIs do not develop

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any neuropathic changes (Hoke and McArthur, Johns Hopkins University, personal communication). It has thus been suggested that NRTIs may exacerbate an already existing HIV-associated peripheral neuropathy or alternatively humans may be more susceptible to the effects of NRTIs.

Although the mechanisms underlying the pain experienced by patients with DSP and/or ATN remain unknown, several animal studies have revealed both biochemical and anatomical effects of NRTIs that could contribute to the development of peripheral neuropathy as a result of mitochondrial toxicity (Dalakas *et al,* 2001; Joseph *et al,* 2004; Keswani *et al,* 2003a; Pardo *et al*, 2001). Notably, HIV proteins have been shown to be neurotoxic (Mattson *et al,* 2005; Nosheny *et al*, 2004) and induce mitochondrial dysfunction (Dalakas *et al,* 2001; Walker *et al,* 2002). It has been suggested that NRTIs cause an enhanced release of neurotransmitters from primary afferent nociceptors and secondary mitochondrial toxicity contributes to the enhanced nociception (Joseph *et al,* 2004). To address these issues, we have established and characterized a human dorsal root ganglion (DRG) culture model. Using this model, we have evaluated the effects of NRTIs that have been implicated in ATN and HIV proteins on nociceptive DRG neurons.

Results

HIV proteins cause dose-dependent axonal retraction

To determine if HIV gp120 and Tat proteins induced a dose-dependent decrease in the mean axonal length, we conducted initial experiments with human fetal nociceptic DRG sensory neuronal cultures, because previous studies have shown that patients with DSP develop a dose-dependent "dying back" pattern. Both HIV gp120 and Tat proteins caused a dose-dependent decrease in the mean axonal length (Figure 1), with significant decreases seen at 250 and 400 nM concentrations of Tat and at 500 pM for gp120 ($P < .001$). Furthermore, there were no morphological changes seen in the supporting cells and there was no evidence of cell death in the neurons including those that exhibited shortening of neurites as determined by Hoechst staining.

NRTIs have a differential effect on axonal retraction

In simultaneous experiments, we determined if the NRTI drugs could induce neurotoxicity in human DRG cultures. Human fetal nociceptic DRG sensory neurons were exposed to varying dosages of NRTI and the cultures were monitored by light microscopy, immunostaining for axons and by electron microscopy. As shown in Figure 2, an abrupt shortening of the axons was noted in the NRTI-treated cultures. These effects were most pronounced in the ddI-treated cultures. At equivalent dosages, axonal retraction was highest with ddI, followed by ddC and then by d4T. No effect of AZT on axonal length was seen at fivefold higher concentrations compared to the highest dose used of the other NRTIs.

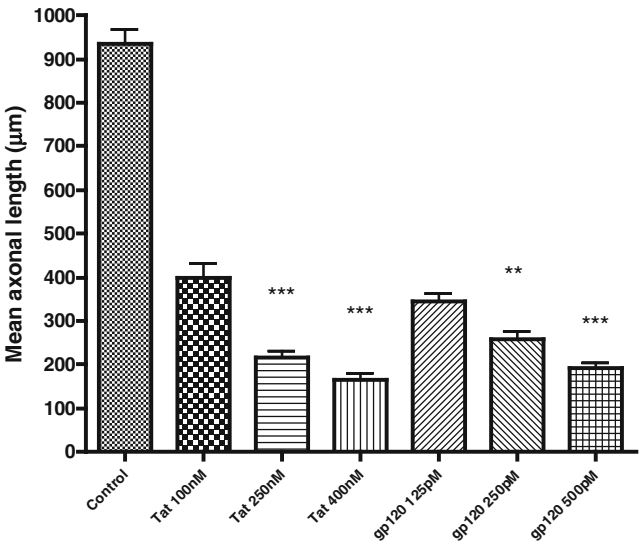

Figure 1 Neuritic trimming by HIV proteins. Dissociated human DRG cultures were treated with HIV proteins for 72 h. Axonal length was measured following immunostaining with antisera to MAP-2. Dose-dependent decrease in axonal length is noted in human DRG cultures with HIV-Tat and gp120. Data represents mean ± SEM. from six experiments done in triplicate ($^{**}P < .01$; $^{***}P < .001$).

NRTIs and HIV proteins show a ceiling effect on axonal retraction

Considering the possibility that DRG neurons of HIV-infected patients on HAART may be exposed to both HIV proteins and NRTIs, we conducted experiments with combinations of HIV gp120 and/or Tat with NRTIs in the human fetal nociceptic DRG sensory neuronal cultures. Both HIV gp120 and Tat alone induced a significant, but similar decreases in the mean ± SEM axonal length (224 ± 6 μm and 219 ± 14 μm, respectively) in comparison to vehicle control (521 ± 22 μm) ($P < .05$) (Figure 3). The combination of gp120 and Tat did not induce any further decreases in the mean ± SEM axonal length (211 ± 7 μm), suggesting a ceiling effect.

Each of the NRTI alone caused a decrease in the mean axonal length (Figure 3), with ddI having the most significant decrease, similar to that seen in Figure 2 when either HIV gp120 or Tat were combined with ddC or d4T (Figure 3A). However, the combination of gp120, Tat, and d4T or ddC did not cause any further decreases in axonal length with the mean ± SEM axonal lengths of 212 ± 10 μm and 240 ± 9 μm, respectively. Notably, ddI alone resulted in a mean ± SEM axonal length of 222 ± 12 μm, and combination with gp120 (mean ± SEM axonal length 211 ± 10 μm), Tat (mean ± SEM axonal length

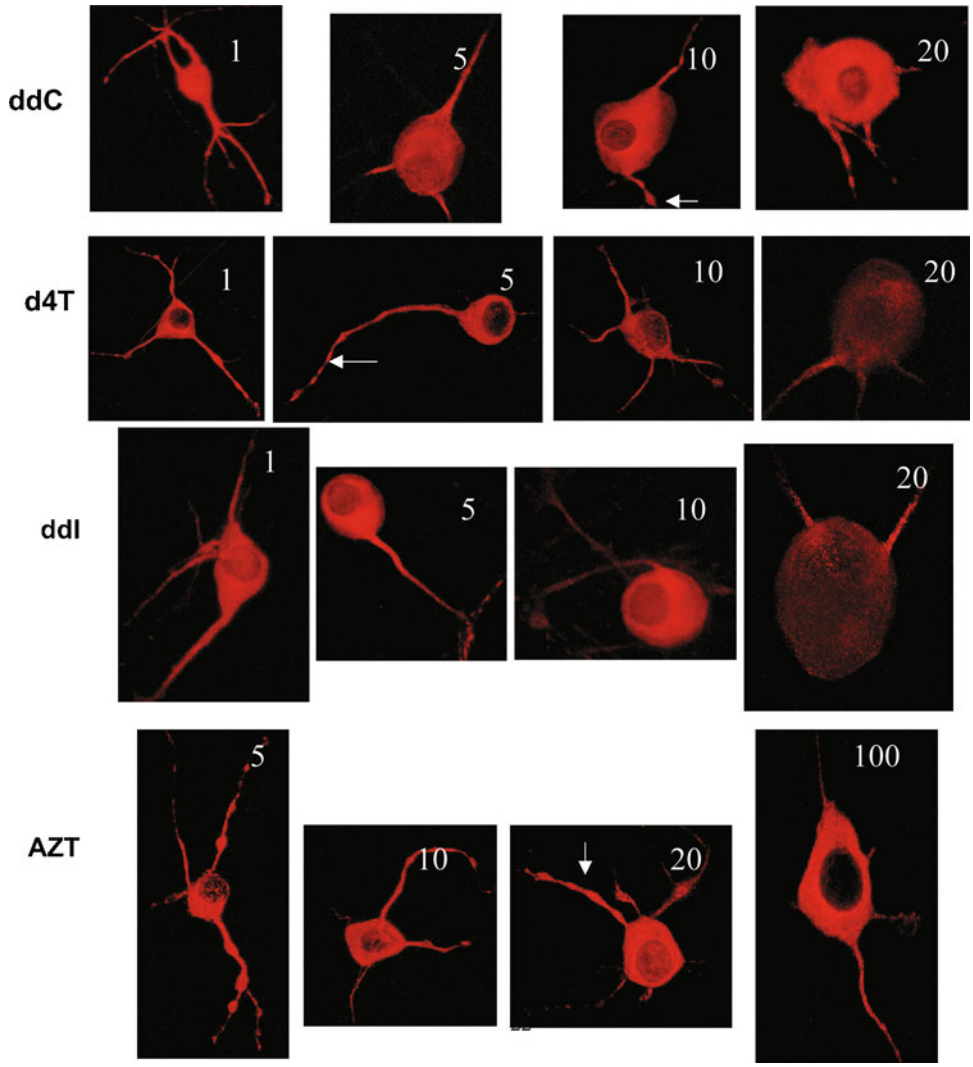

Figure 2 Effects of NRTI on DRG neurons. Dissociated DRG cultures were treated with various NRTI as shown for 72 h and then immunostained with antisera to MAP-2. The numbers in each panel represent the dosage of each of the compounds in μg/ml. Progressive morphological changes were noted with each of the NRTIs in a dose-responsive manner. Beading of the axons is noted following treatment with each of the NRTI. At the highest dose tested, ddC, d4T, and ddI show ballooning of the cytoplasm and loss of neurites, although the proximal ends of the neurites are preserved.

214 ± 11 μm), or all together (mean $\pm$ SEM axonal length 202 ± 10 μm) did not cause any further decrease in axonal length. Together these results suggest that irrespective of the viral protein or NRTI used, the human DRG axons do not retract beyond 200 μm. None of the above combinations of viral proteins and NRTIs caused neuronal cell death (Figure 3B).

Ultrastructural changes in axons with HIV proteins and NRTIs

Ultrastructural examination of the neurites by electron microscopy was performed to determine if there were any morphological correlates to the neurite retraction seen by light microscopy in response to NRTIs and HIV proteins. The neurites revealed accumulation of glycogen-like granules and loss of cristae in the mitochondria with each of the NRTIs, which are suggestive of oxidative stress. Microtubule rearrangement was seen in the NRTI-treated cultures, in addition to varying sizes of vacuoles (Figure 4). The HIV proteins gp120 and Tat also showed similar changes in mitochondria and in microtubules (Figure 4). No major morphological differences were noted between the effects of any of the NRTIs or HIV proteins.

Discussion

We have established a human *in vitro* model for HIV-associated sensory neuropathies that affect primarily small nociceptic sensory (C-fiber) neurons. We found that both HIV proteins and NRTIs cause axonal retraction in the DRG neurons without causing

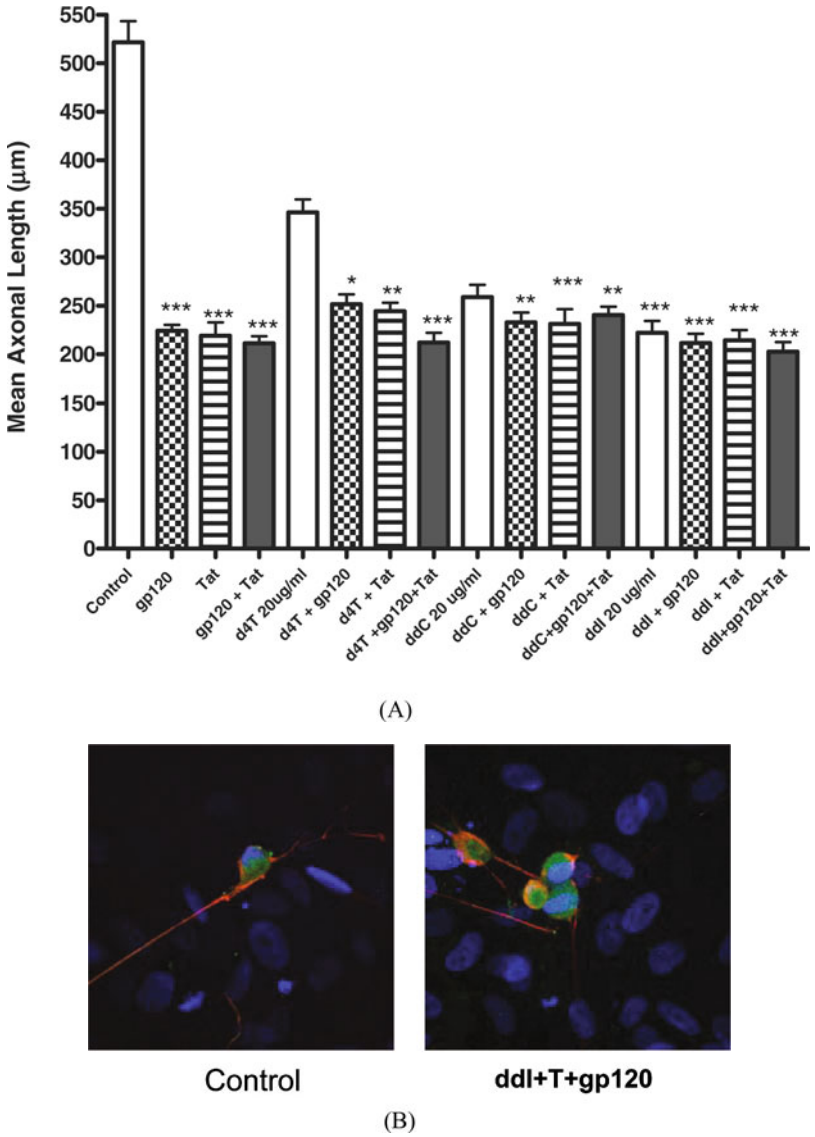

Figure 3 (**A**) Neuritic trimming caused by HIV proteins and nucleoside analogs. Dissociated DRG cultures were treated with HIV proteins Tat and gp120 and NRTI. Tat and gp120 (together and alone) caused a significant decrease in mean length of neuronal processes in human fetal DRG cultures. ddI at 20 μg/ml caused a significant decrease in the length of processes, followed by ddC and d4T. All the P values were less than .05, with the exception of d4T (P value, .06). The combination of d4T with HIV proteins caused further decreases in axonal length. However, there was a ceiling effect at about 200 μM. Data represents mean ± SEM from six experiments done in triplicate ($^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$). (**B**) Lack of nuclear fragmentation by HIV Tat and antiretroviral drugs on human fetal dorsal root ganglia cultures. DRG cultures were treated with ddI (20 μg/ml) + Tat (250 nM) + gp120 (500 pM) for 72 h. The cultures were fixed in 4% paraformaldehyde and immunostained with mouse polyclonal anti-beta-tubulin (red) and IB4 (green) and counter-stained with goat anti-mouse alexa fluro 568. Although some shortening of neurites is noted in the treated cultures, the nuclear morphology appears normal.

cell death. Similarly, in patients with DSP and ATN, there is a loss of epidermal nerve fibers of unmyelinated nerves, suggestive of axonal retraction. The DRG neurons and nerve fibers are likely exposed to both HIV proteins and NRTI via blood circulation. Additionally, HIV-infected macrophages may infiltrate the DRG and nerve fibers, which may be also release HIV proteins.

Previous work has shown that total neuritic length per neuron was significantly affected after exposure to ddC (Keswani *et al*, 2004). Interestingly, we now show that although both HIV proteins and NRTIs caused a dose-dependent decrease in axonal length, a ceiling effect was seen with each of the compounds, such that no further shortening beyond 200 μm was noted. This was also the case even when toxic dosages of HIV proteins and NRTIs were combined together. This suggests that the proximal part of the axon may be differentially regulated compared to the distal axon. Several investigators using a variety of models have shown that some compounds may have differential toxic properties on the axon and the cell body of DRG neurons (Hol *et al,* 1994; Melli *et al*, 2006). Our observations suggest that the proximal

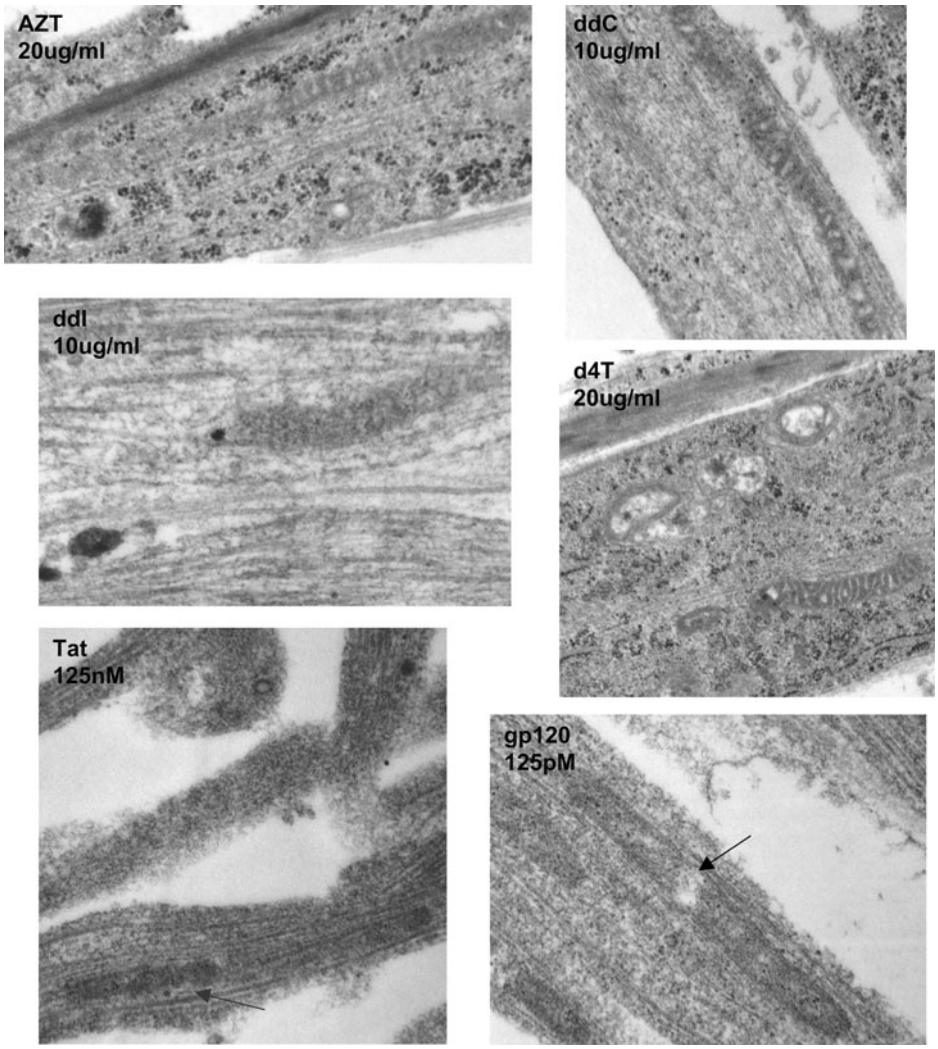

Figure 4 Effect of chronic exposure of NRTI on human DRG cultures. The ddC panel shows some loss of cristae in mitochondria and vacuoles in the neurite associated with dense particles, whereas the ddI panel reveals dense particles between microtubules and in the mitochondria. The cristae in the mitochondria have lost their normal architecture. However, the d4T panel reveals microtubules and neurofilaments gathered together with loss of normal structure. Many clusters of ribosomes are scattered in areas with sparse microtubules and neurofilaments. Dense particles are also present in the mitochondria. Exposure to Tat shows that the mictochondria have poorly defined cristae *(arrow)*, but microtubules and neurofilaments look normal. gp120 exposure shows numerous small dense particles scattered between the microtubules, neurofilaments, and in the mitochondria. The mitochondria have lost the normal architecture of the cristae and a vacuole is noted in the mitochondria *(arrow)*.

axon is likely regulated closely by the neuronal cell body.

Although the mechanisms by which the HIV proteins and NRTIs cause axonal injury are not fully understood, several investigators have shown axonal beading in a variety of pathological and neurological conditions (Coleman, 2005; Dalakas *et al,* 2001; Joseph *et al,* 2004). The clumping of microtubules seen in with HIV proteins and NRTIs may indicate that there is disruption of axonal flow, which may in turn cause axonal beading due to accumulation of vesicular cargo and transport proteins. Therefore, the data presented herein provides evidence that axonal injury induced by either HIV proteins and/or NRTIs correlates with neuronal dysfunction and precedes neuronal cell death. Similar morphological changes in axons have also been noted in brain of patients with HIV encephalitis (Masliah *et al*, 1992), suggesting that similar mechanisms of axonal injury may exist in the brain as well.

Additionally, studies using brain-derived cultures suggest that HIV proteins may directly interact with neurons, which leads to the neurotoxic effects via interactions with glial cells and the release of various cellular factors (Mattson *et al,* 2005). Another study suggests that the continuous presence of HIV proteins is not necessary, as they initiate a cascade of events that perpetuates, termed a "hit and run" phenomenon (Nath *et al*, 1999). Furthermore, HIV proteins may be transported distally along axonal pathways (Bruce-Keller *et al*, 2003). A recent study has also shown that HIV gp120 neurotoxicity is indirectly mediated

through Schwann cells (Keswani *et al,* 2003b). In our mixed human DRG cultures, Schwann cells constitute about one fourth of the total cellular population. Hence, the HIV proteins may cause axonal injury by both direct interactions with the axons or indirectly mediated via effects on the Schwann cells.

Consistent with our observations, several lines of evidence suggest that NRTIs cause mitochondrial impairment. DRG neurons harvested from healthy rabbits fed ddC exhibited abnormal mitochondria that were comparable to that shown in sural nerve biospies from patients with ddC neuropathy (Dalakas *et al,* 2001). Abnormal mitochondria had thin membranes, lacked visible cristae, and were enlarged. This was associated with vacuolization of the sural nerve (Dalakas *et al,* 2001). NRTI-treated neurons develop mitochondrial depolarization (Cossarizza and Mussini 2002), inhibition of mitochondrial DNA polymerase-γ (Keswani *et al,* 2002), and decreased activity of respiratory complexes (Skuta *et al*, 1999).

We found that there was a clear differential effect of the NRTI on the DRG neurons. Notably, ddI was the most potent, followed by ddC and d4T. AZT was the least toxic; it caused some beading of the neurites but no significant retraction was noted. The plasma levels of the NRTIs in HIV-infected patients are as follows: AZT (106 μg/ml) (Fischl *et al,* 1987), d4T (0.08 μg/ml) (Havlir *et al,* 2000), ddC (0.03 μg/ml) (Katzenstein *et al*, 1996), and ddI (800 μg/ml) (Dolin *et al,* 1995). Thus at concentrations three times the plasma level, AZT was found to show minimal toxicity, whereas concentrations of ddI that were much lower than plasma levels caused neuronal retraction. In comparison, concentrations of d4T and ddC that exceeded plasma levels were needed to cause toxicity. These observations suggest patients exposed to ddC would be at greatest risk for developing a neuropathy and is consistent with clinical studies with ddC (Dalakas *et al,* 2001; Famularo *et al,* 1997).

In conclusion, in an *in vitro* system of human nociceptic DRG sensory neurons, we have shown that HIV proteins and NRTIs induce axonal injury in a dose-dependent manner that occurs independent of changes in the cell body. Furthermore, we show that cytoskeletal rearrangement and ultrastructural abnormalities may have a key role in axonal degeneration of nociceptic sensory neurons. Therefore, our *in vitro* model would be a powerful tool for investigating the neurotoxic effects associated with HIV gp120, HIV Tat, and NRTIs and the mechanisms of sensory neuropathies associated with HIV/AIDS (acquired immunodeficiency syndrome). In addition, this model may be useful in developing new therapeutic strategies for ATN and DSP.

Materials and methods

Cell culture

Human fetal DRG were isolated from fetuses with a mean gestational age of 60 days $\pm$ 5 days, with consent, from women under going elective termination of pregnancy and approval by the Johns Hopkins Institutional Review Board. The human fetal samples were kept separate during the isolation procedure. The DRG were collected and treated with a 0.25% trypsin/DNase solution in normal Dulbecco's modified Eagle medium with F-12 supplement (DMEM/F12) for 10 min and centrifuged for 4 min at 1×10^3 rpm. The supernatants were removed and the pellets were resuspended in 1 ml of DMEM/F12 and dissociated using a sterile modified Pasteur's glass pipette with a cotton plug. Cells were then filtered using a Millipore filtering apparatus with a 70-μm mesh followed by counting, separately without pooling of samples from similar or different gestational ages, at a 5×10^4 cells/ml concentration. Cells were cultured in 25 ml of DMEM/F12 with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin, 0.20 μl/ml B-27, 1 μg/ml linoleic acid, 25 μg/ml insulin, and 15 ng/ml of nerve growth factor-β at 37°C with 5% CO_2. The cells were maintained in culture for 1 to 2 weeks with medium change once a week. These cultures contained cell that were nearly 100% isolectin-B4 (IB4), 85% calcitonin gene–related peptide, and 85% vanilloid receptor-1 positive, as determined by immunohistochemical staining, suggesting that these cultures contained predominantly nociceptic neurons.

HIV proteins

Recombinant $gp120_{HTLV\text{-}IIIB/H9}$ was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. gp120 was produced in peripheral blood mononuclear cells (PBMCs) and was >95% pure (Popovic *et al,* 1984; Ratner *et al,* 1985). Recombinant Tat protein was produced in our laboratory as previously described and was >98% pure (Ma and Nath, 1997).

Hoechst 33342 staining

Apoptotic cell death was monitored by using Hoechst 33342 staining. Culture medium was removed from each well and 400 μl of 4% paraformaldehyde (at room temperature) was added. After a 30-min incubation at room temperature, the paraformaldehyde was removed and the cells were washed three times with 1× phosphate-buffered saline, pH 7.4 (PBS). An aliquot of 300 μl of 1 μM Hoechst dye solution with 0.1% Triton X-100 was added to each well. The culture plate was wrapped in foil and incubated at room temperature for 30 min. The wells were then washed three times with 1× PBS. PBS was left in each well from the previous washing step. The cells were visualized using a fluorescence microscope with a ultraviolet (UV) filter with a 340-nm excitation and 510-nm barrier, using a 40× oil immersion lens. Five random areas were photographed and cells were counted for apoptotic (condensed and fragmented) nuclei.

Neuritic length
DRG cultures were prepared as described above and allowed to differentiate and grow processes for 72 h, following which nucleoside analogs were added at varying concentrations. Parallel controls with vehicle and blank were also used. Cultures were then incubated for an additional 72 h. The cells were then washed once in serum-free medium and fixed in 4% paraformaldehyde in PBS at room temperature for 30 min followed by three washes with PBS. After blocking for 1 h in PBS with 10% serum and 1% Triton-X100 (Sigma), the cells were incubated with a mouse polyclonal anti-β-tubulin III antibody (1:1000; Promega, Madison, WI) or a monoclonal antibody to microtubule-associated antigen (MAP-2; 1:200) (Chemicon, CA) diluted in PBS–1% serum at 4°C overnight. Following overnight incubation, unbound antibody was removed by three washes in PBS and cells were incubated with a rabbit anti-mouse antibody conjugated to a fluorescent probe for one hour at room temperature. The cells were then washed in PBS to remove the unbound secondary antibody. Photographs of the neurites were taken using a digital camera attached to a phase contrast, inverted Zeiss microscope. Length of each individual neurite was measured using Openlab software (Improvision, Lexington, MA), from which the total neurite length for each neuron was calculated. A minimum of 25 neurons per cover slip was measured in an unbiased manner by random focusing.

Statistical analysis
All experiments were performed in triplicate with a minimum of six experiments for each condition and reported as mean ± standard error. A one-way analysis of variance (ANOVA) was used with the non-parametric Kruskal-Wallis test and analyzed using GraphPad Prism 4.01 software (GraphPad Software, San Diego, CA) with P value $<.05$ used to delineate significance for analysis of all results.

Ultramicroscopic investigation
DRG cultures were prepared as described above and processes were allowed to extend for 72 h. After incubation for 72 h, varying concentrations of nucleoside analogs or HIV proteins were added and allowed to incubate for another 72 h before being fixed in a 4% paraformaldehydye/3% glutaraldehyde solution for 30 min. After fixation, the samples were processed for electron microscopy and negatively stained with uranyl acetate and lead citrate and examined with a Hitachi H600 electron microscope (Nath *et al*, 1995).

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