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Differential effects of HIV infected macrophages on dorsal root ganglia neurons and axons

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Abstract

Human immunodeficiency virus-associated distal-symmetric neuropathy (HIV-DSP) is the most common neurological complication of HIV infection. The pathophysiology of HIV-DSP is poorly understood and no treatment is available for this entity. The dorsal root ganglia (DRG) are the principal sites of neuronal damage and are associated with reactive mononuclear phagocytes as well as HIV-infected macrophages. To determine the role of HIV-infected macrophages in the pathogenesis of HIV-DSP, we developed a technique for culturing human DRG's. When the dissociated DRG neurons were exposed to supernatants from macrophages infected with CXCR4 or CCR5 tropic HIV-1 strains axonal retraction was observed without neuronal cell death but there was mitochondrial dysfunction in the neuronal cell body. Even though CXCR4 and CCR5 were expressed on the DRG neurons, the effects were independent of these receptors. Antioxidants rescued the neuronal cell body but not the axon from the toxic effects of the culture supernatants. Further, peripheral nerves of HIV-infected patients obtained at autopsy did not show evidence of increased oxidative stress. These observations suggest a differential effect on the axon and cell body. Different mechanisms of injury may be operative in these two structures.

Keywords

neuropathy; dorsal root ganglia; macrophages; neurotoxicity; axonal retraction; mitochondria; chemokine; AIDS

Introduction

Human immunodeficiency virus (HIV)-associated polyneuropathy has become the most common neurological complication of HIV infection (24). More than half of the individuals with advanced HIV infection display signs of a neuropathy (19,31). Of all forms of acquired

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immunodeficiency syndrome (AIDS)-associated neuropathies, distal-symmetric polyneuropathy (DSP) is the most frequent subtype, occurring especially in the later stages of AIDS (24). It manifests as a painful sensory neuropathy and is an important cause of morbidity in this population. Indeed, the use of antiretroviral agents can in themselves cause a peripheral neuropathy that may be clinically indistinguishable from HIV-DSP(5). Despite its importance, it remains perhaps the most understudied complication of HIV infection.

The pathogenesis of HIV-DSP remains unclear. The most characteristic pathological feature is the distal degeneration of long axons (24) accompanied by macrophage infiltration (1,24) and either the absence or modest loss of neurons in the sensory dorsal root ganglia (DRG) (8, 11,27). Furthermore, there is degeneration within the centrally directed extension of sensory DRG neurons (27). These findings have led to the hypothesis that the primary pathology could be at the level of the sensory neurons in the DRG, leading secondarily to a dying back process with axonal degeneration.

The presence of HIV-infected perivascular macrophages has been shown in the DRG of patients with and without DSP. These studies demonstrated the presence of HIV proviral DNA, mRNA, p24 antigen in these cells (2,20,29,38). Another consistent neuropathological abnormality in the DRG appears to be the presence of activated macrophages, which express MHC antigens and pro-inflammatory cytokines (20,24,38). HIV-DSP correlates with the degree of macrophage activation (13). Taken together, this has led to the hypothesis that activated macrophages play an important role in the pathogenesis of peripheral neuropathy. Two possible neuropathogenetic mechanisms have been proposed; the direct effect of HIV or HIV proteins on DRGs and the indirect neurotoxicity of products secreted by activated macrophages.

HIV-infection of the central nervous system is mainly CCR5-dependent and macrophage-tropic (15,23). Nonetheless neurological symptoms such as HIV-DSP typically occur late in the course of disease progression, when there is an emergence of CXCR4-tropic and dual tropic HIV isolates that use both CCR5 and CXCR4 receptors. Macrophages can support efficient replication by a subset of primary CXCR4 viruses (32), but it is not known whether late-emerging CXCR4 isolates are macrophage tropic and mediate at least some of their pathogenetic effects by infecting macrophages. Reactive macrophages in the DRG may release pro-inflammatory cytokines, reactive oxygen species and excitotoxins, which could result in damage to axons and nerve cells.

We reasoned that since HIV infects only humans, the best model to study the mechanisms of HIV mediated neuropathy would be to use human DRG cultures and HIV-infected human macrophages. We thus developed techniques to culture human DRG and exposed them to supernatants from macrophages infected with CXCR4 or CCR5 tropic strains of HIV. We monitored the cell bodies and neurites for morphological and functional changes, including indicators of oxidative stress. We also determined the role of chemokine receptors in mediating these changes.

Materials and Methods

Characterization of HIV infection and macrophages in DRG

Paraffin and free-floating sections from lumbar DRGs obtained at autopsy from three patients with history of HIV-associated neuropathy and three controls were processed for immunocytochemistry. The distribution of macrophage infiltration in the DRG were assessed by immunostaining of paraffin sections with anti-CD68 antibodies (Dako Inc., Carpinteria, CA). Double immunostaining with anti-HIV-p24 and anti-GFAP antibodies (Dako Inc., Carpinteria, CA) allowed evaluation of productive infection and the topographic association

with Schwann cells stained with anti-GFAP antibodies. Double immunostained sections were visualized by confocal microscopy (Zeiss LSM 510 Meta, Germany).

Preparation and culture of Dorsal Root Ganglion cells

DRGs were obtained from human fetuses of 6–16 weeks gestational age with consent from women undergoing elective termination of pregnancy and approval by the Johns Hopkins University Institutional Review Board. DRGs were mechanically dissected under aseptic conditions from the intervertebral foramina by using a dissecting microscope (Nikon SMZ660), following which they were treated with 2.5% trypsin/deoxyribonuclease I (Invitrogen, Carlsbad, CA) solution for 10 min at 37°C, centrifuged for 6 min at 1000 rpm and resuspended in DRG culture medium (DMEM/F12 (Gibco, Rockville, MD) with 10% heat-inactivated fetal bovine serum (FBS; Sigma, St Louis, MO), 100 U/ml of penicillin G, 100 µg/ml of streptomycin, 250 ng/ml of amphotericin B (Gibco, Rockville, MD), 50 µg/ml of gentamycin (Quality Biological, Inc., Gaithersburg, MD), 15 nM nerve growth factor-beta (Sigma, St Louis, MO), 25 µg/ml insulin (Sigma, St Louis, MO), 1 µg/ml linoleic acid-albumin bovine serum albumin (Sigma, St Louis, MO) and 1× B27 (Gibco, Rockville, MD). Dissociated cells (50,000 cells/well) were directly plated onto poly-D lysine-coated plates and incubated in a humidified chamber with 5% CO₂ atmosphere at 37 °C. The mixed neural cell population contained about 5–10% neurons, 15–30% Schwann cells and 60–80% fibroblast cells as determined by immunostaining with antisera to βIII-tubulin, glial fibrillary acidic protein (GFAP) or anti-fibroblast surface antibody. DRG cultures were maintained for at least 5 days and monitored every day for viability by light microscopy before conducting the experiments below. Experiments were done when the supporting cell layer consisting of Schwann cells and fibroblasts was almost confluent. With this as a criterion, there were no significant differences between the cultures as estimated by light microscopy.

Culture and infection of THP-1 cells and Monocyte-derived Macrophages

THP-1, a human monocytic leukemia cell line (34) was obtained from American Type Culture Collection (Rockville, MD). Cells were grown in suspension in DMEM medium (Gibco, Rockville, MD) containing 10% FBS, 100 U/ml of penicillin G, 100 µg/ml of streptomycin, 250 ng/ml of amphotericin B and cultured in 75 cm² tissue flasks (Corning, NY) in a humidified 5% CO₂ atmosphere at 37°C. 5×10^6 cells were infected with 1 ml of cell-free inoculum of HIV-1 _{IIIB} (p24=10ng/ml) (25,28), a CXCR4 tropic strain, for 1 h at 37°C in a water bath. Cells were washed to remove unabsorbed virus and fresh medium was added. HIV-p24 antigen was determined in tissue culture supernatants by an enzyme-linked immunosorbent assay (ELISA) kit (Beckman Coulter, Inc., Fullerton, CA) every 3 to 5 days over 4 weeks. HIV viral load was determined using a Roche Amplicor version 1.5 kit. To obtain serum-free supernatant, the medium was replaced by serum-free DMEM for 16 h. Cell viability of infected THP-1 cells was evaluated by trypan blue exclusion at the time of collection of the supernatant, and was at least 90%. After centrifugation, the serum-free supernatant was harvested and passed through a 0.2 µm membrane filter (Corning, NY) to remove any cell debris. The culture supernatant collected on day 14 (p24=245 ng/ml; viral load = 8,770,720 copies/ml) was used for further experiments. It was stored in liquid nitrogen until the time of experimental treatment and thawed immediately before use. Uninfected THP-1 cells were cultured in a similar manner and used as a control.

Monocyte derived macrophages (MDM) were isolated from human peripheral blood mononuclear cells (PBMC) of healthy volunteers (New York Blood Center, NY) by using a ficoll-hypaque gradient. After isolation, 1×10^8 cells were seeded into a T75 flask in RPMI 1640 (Gibco, Rockville, MD) supplemented with 20% FBS, 10% pooled human serum from HIV-seronegative donors (Gemini Bio-Products, Woodland, CA), 2 mM L-glutamine, 100 U/ml of penicillin and 100 µg/ml of streptomycin (Sigma, St Louis, MO). After a confluent

monolayer was established, MDM were inoculated with cell-free HIV-1_{Ba-L} (p24=12 ng/ml) (9), a macrophage tropic strain, for 1 h, then washed and cultured in RPMI 1640 supplemented with 20% FBS, 2 mM L-glutamine, 100 U/ml of penicillin and 100 µg/ml of streptomycin. Culture supernatants were monitored for p24 antigen by ELISA (Beckman Coulter, Inc., Fullerton, CA) at selected time points, collected and stored in liquid nitrogen. To obtain serum free supernatant the medium in the MDM cultures was replaced with RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml of penicillin and 100 µg/ml of streptomycin for 16 to 18 hours. The culture supernatant collected on day 14 (p24=275 ng/ml; viral load = 35,367,800 copies/ml) was used for further experiments. Uninfected MDM from the same donor were treated in a similar manner and supernatants were used as a control. Both viral strains were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1_{III_B} from Dr. Robert Gallo and HIV-1_{Ba-L} from Dr. Suzanne Gartner, Dr. Mikulas Popovic and Dr. Robert Gallo.

Determination of Toxicity

At the time of experimental treatment, the DRG culture medium was replaced with Locke's buffer containing 154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl₂, 1 mM MgCl₂, 3.6 mM NaHCO₃, 5 mM glucose, and 5 N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid (pH 7.2). The cells were incubated with either different dilutions of the supernatant from infected or uninfected THP-1 cells or MDM and, depending on the experiment, with polyclonal antisera to CXCR4 at 1:100 (v/v) (catalog number PX 172A, Cell Sciences, Inc. Canton, MA), polyclonal antisera to CCR5 antisera at 1:100 (v/v) (catalog number PX164A, Cell Sciences, Inc. Canton, MA) or 10 µM trolox (alpha-tocopherol). At these concentrations the antisera block the effects of SDF-1 and RANTES on changes in intracellular calcium in neurons (data not shown). The concentration of trolox was chosen according to previously published experiments (35). After 24 h of incubation, the cells were fixed in 4% paraformaldehyde, immunostained with a monoclonal antibody to β III tubulin (Promega, Madison, WI) and labeled with Hoechst 33342-trihydrochloride trihydrate (15 µg/ml, Molecular Probes, Eugene, OR). Alexa Fluor-568 conjugated to goat anti-mouse (Molecular Probes, Eugene, OR) was used as secondary antiserum. Total neuritic length was measured in micrometers using a Nikon fluorescence microscope (OpenLab software, Improvision, Lexington, MA). For this purpose, random pictures of the slide were taken and every neurite, for which the beginning and the end point could be clearly identified, was measured. Each experimental condition was done in triplicate wells and repeated three to six times in cultures from different fetuses. With this method about 15 neurons per cover slip per condition were analyzed.

The DNA binding dye Hoechst 33342 was used to measure apoptotic cell death. Neurons were differentiated from other cell types based on positive β III-tubulin immunostaining and morphological characteristics. Pyknotic or fragmented chromatin was considered apoptotic. Nuclei were visualized under epifluorescence illumination (λ_{ex} = 340 nm and barrier filter 510 nm) with a 40× objective. About 200 neurons were analyzed for each experimental condition and each experiment was repeated three to six times. Neurons were counted and the percentage of apoptotic neurons was calculated. In each of the assays, to eliminate any investigator bias, the investigator was blinded to the treatment conditions.

Reactive oxygen species

DRG cultures were plated on 35 mm or 24-well glass bottom tissue culture plates and cultured for at least 7 days. After this time cells were exposed for 24 h to either different dilutions of the supernatant of infected or uninfected THP-1 cells or MDM and depending on the experiment 10 µM trolox. Hydrogen peroxide (H₂O₂) was measured using dihydrorhodamine 123 (DHR, Molecular Probes, Eugene, OR), which is specifically oxidized by H₂O₂ into 2 molecules of rhodamine. Neuronal cultures were incubated with DHR at a final concentration

of 10 μ M for 30 min at 37°C and then washed with Locke's buffer. The fluorescence emission pattern of DHR-labeled neurons was immediately observed under confocal microscopy (Zeiss LSM 410 or 510 Meta, Germany) (λ_{ex} = 488 nm and λ_{em} = 529 nm). To measure the DHR intensity, random pictures of the cultures were taken with the confocal microscope. Single neuronal cell bodies were traced and the was area analyzed (Image J software, NIH, USA). Each experiment was performed in triplicate and repeated 3 to 7 times.

Mitochondrial membrane potential

JC-1 is a fluorescent dye that accumulates in the mitochondrial matrix depending upon the membrane potential. In mitochondria with low potential, JC-1 shows green fluorescence, whereas at hyperpolarized potentials JC-1 monomers exceed a critical concentration and form J-aggregates which cause a shift in the fluorescence maximum, resulting in a red emission peak.

DRG cells on 35 mm dishes or 24-well glass bottom tissue culture plates were cultured for at least 5 days before use in the experimental treatments described above. After 24 h of exposure to culture supernatants, the cells were incubated for 30 min with the JC-1 dye (10 μ M) at 37°C and then washed with Locke's buffer. Optical measurements were acquired immediately under confocal microscopy (Zeiss LSM 510 Meta, Germany) in both the green and red regions of the spectrum. Stacks of images were collected at 0.2 μ m in the plane of the optical axis by using a 40 $\times$ oil immersion objective. Random pictures of the cultures were taken with the confocal microscope and the intensity was measured at both wavelengths by tracing the neuronal cell body and separately 2 to 3 cm of the proximal axon in the appropriate stack and the ratio of the intensity at both wavelengths was calculated (Image J software, NIH, USA). Each experiment was performed in triplicate and repeated 3 to 4 times.

Nitrosylated proteins by slot blot and Western blotting

Frozen sciatic nerve tissue was obtained from the National Neuro-AIDS Tissue Consortium (NNTC)/Manhattan HIV Brain Bank. Tissue was obtained from HIV-seropositive subjects with (n=5) or without clinical signs of a neuropathy (n=6) or HIV-seronegative control subjects without neuropathy (n=6). Control subjects were screened by chart reviews to ensure that they were neurologically normal by clinical examination and metabolic panels and complete blood cell counts established that they did not have any other cause of neuropathy. All patients were confirmed to be HIV-seronegative by analysis of a postmortem serum sample. The diagnosis of a neuropathy in the HIV-seropositive individuals was made ante-mortem by a detailed neurological examination.

A small tissue sample was cut from the nerve, and each sample was immediately homogenized with ice cold RIPA buffer (1% SDS, 1% triton X100, 150 mM NaCl, 1 mM sodium phosphate, 2 mM EDTA, sodium deoxycholate, protease inhibitor (Sigma, St. Louis, MO) and sonicated on ice. After high-speed centrifugation tissue extracts were stored at -20°C and thawed immediately before use. For immunochemical detection of 3-nitrotyrosine residues (3-NT) in sciatic nerve homogenates, a slot blot technique was used. After removing the detergents from the homogenates with a commercial kit (OrgoSol™, Detergent-Out™, Genotech, St. Louis MO), the protein concentration was measured using the BCA kit (Pierce, Rockford, IL) following manufacture's instructions. A total of 70 μ g of homogenate protein was loaded onto nitrocellulose in a slot-blot microfiltration unit (Bio-Rad Laboratories, Hercules, CA). For 3-NT determination, a monoclonal antibody (Upstate, Lake Placid, NY) was used. Images were scanned and the area under the curve was analyzed (ImageJ software, NIH, USA).

Characterization of the CXCR4 and CCR5 distribution in human DRG neurons

To characterize the CXCR4 and CCR5 distribution, the DRG cultures were fixed in 4% paraformaldehyde, blocked with 1× PBS containing 10% normal goat serum, and double immunostained with monoclonal anti-βIII tubulin antibody (Promega, Madison, WI) and either polyclonal antisera to CXCR4 at a dilution of 1:100 (v/v) (Cell Sciences, Inc. Canton, MA) or polyclonal antisera to CCR5 at a dilution of 1:100 (v/v) (Cell Sciences, Inc. Canton, MA). Following incubation with the primary antibody overnight at 4°C the cells were washed and incubated with Alexa Fluor-568 conjugated to goat anti-mouse (Molecular Probes, Eugene, OR) and Alexa Fluor-488 conjugated to goat anti-rabbit (Molecular Probes, Eugene, OR). Slides were mounted with Gel/Mount (Biomedica Inc., Foster City, CA) and viewed using a confocal microscope (Zeiss LSM 510 Meta, Germany). Each receptor staining was done in triplicate and repeated 3 times. 100 neurons were analyzed for each antisera in each experiment and the receptor distribution was expressed as a percentage of total cells.

Statistical analysis

Statistical analysis was performed using GraphPad InStat version 3.06 and GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego, CA). The results from each set of experiments were averaged and standardized. The data were expressed as the mean ± SEM. Comparisons between experimental groups (infected versus matched uninfected control supernatant) were performed with Student's t test for paired observations or ANOVA. Categorical variables were calculated with the Fisher's test. Differences were considered statistically significant for p values < 0.05.

Results

HIV infection in human DRG cells from patients with history of HIV peripheral neuropathy

We assessed DRG from HIV patients who had a history of AIDS and peripheral neuropathy and found marked perineuronal as well as interstitial infiltration of CD68- and HLA class II-positive cells, consistent with the presence of macrophages (figure 1A). Many of the immunostained cells exhibited macrophage morphology and the immunostaining with anti-HLA-DR confirmed their profile of activation. Double immunostaining with anti-HIV-p24 and anti-GFAP demonstrated productive HIV infection within cells with macrophage morphology localized in both perineuronal and interstitial compartments (figure 1B). No evidence of p24 immunostaining was seen in the Schwann cells.

Supernatants from HIV-1_{IIIIB}- and HIV-1_{Ba-L}-infected cells induce neuritic retraction in DRG neurons

To address the pathophysiology of HIV-DSP we established human DRG cultures and determined whether supernatants from HIV-1_{IIIIB}- and HIV-1_{Ba-L}-infected monocytic cells could induce neuritic retraction in human DRG neurons. We incubated human fetal DRG cells with the culture supernatants at a final dilution of 1:10, 1:100 and 1:1000 for 24 h in Locke's buffer. Reduction of the mean total neuritic length was consistently observed at dilutions of 1:10 and 1:100 (figure 2). We observed nearly similar amounts of neurite shortening (35–45%) with supernatants from HIV-1_{IIIIB}-infected cells and HIV-1_{Ba-L}-infected MDM. Interestingly, there was no significant difference between the effects at a 1:10 or 1:100 dilutions of the supernatants with either viral strain. Culture supernatants at 1:1000 dilution, however, failed to cause any neurite shortening (figure 2).

We observed a differential susceptibility of the neurons. Neurons which were completely integrated in a neuronal network appeared to have better preserved neurite length, whereas neurons growing at the edge of a neuronal network or as single non integrated neurons like the

ones illustrated in figure 2, showed neurite retraction after exposure to supernatants from HIV-infected cells. Neurite retraction was often accompanied by morphological changes, such as segmentation. It also appeared that there were more “single nonintegrated neurons” in cultures that had been exposed to the supernatants from HIV-1 infected cells, suggesting, that they may have lost their contact to the other neurons due to neurite degeneration. The results shown in figure 2I include all neurons without differentiation.

We next determined whether neurite trimming was accompanied by significant neuronal cell death. The general neuronal apoptotic rate depended on the fetus from which the cells were derived and varied between 0.5 and 10% with no significant difference between neurons exposed to supernatants from HIV-infected monocytic cells versus supernatants from uninfected cells. There was one exception, however. In neurons from one fetus, supernatants from HIV-1 _{III_B} and HIV-1 _{Ba-L} infected cells at dilutions 1:100 caused significant neuronal cell death (HIV-1 _{III_B} 12.7% and HIV-1 _{Ba-L} 17.1%) compared to the matched uninfected control (THP-1, 3% and MDM, 2.5%, $p < 0.05$).

Supernatants from HIV-1_{III_B}- and HIV-1_{Ba-L}-infected cells induce neuronal mitochondrial toxicity in DRG cultures

Reactive oxygen species (ROS) production was measured by DHR labeling of neurons in the presence of supernatants from HIV infected macrophages. Dose-independent, but significantly higher amounts of ROS in the neuronal cell body were observed with supernatants from both HIV-1 _{III_B}- and HIV-1 _{Ba-L}-infected cells (figure 3A). The DHR intensity in cells treated with infected macrophages conditioned media was 35–45% higher compared to the controls. Mitochondrial membrane potential was also measured using a JC-1 assay. Human fetal DRG cultures were exposed to supernatants from infected and uninfected monocytic cells at dilutions of 1:10 and 1:100 for 24 h in Locke’s buffer. A decrease in mitochondrial membrane depolarization occurred after exposure to the supernatants from HIV-infected monocytic cells. A small (<20%) but significant change in mitochondrial membrane potential was observed with supernatants from both HIV-1 _{III_B}- and HIV-1 _{Ba-L}-infected cells. Consistent with observations above, there was no significant difference between the 1:10 and 1:100 dilutions with either HIV strain (figure 3B). This suggests that the small changes in mitochondrial membrane potential may be sufficient to trigger ROS formation.

Effect of trolox on HIV-1_{III_B}- and HIV-1_{Ba-L}-induced neurotoxicity

We analyzed whether trolox (10 μ M) could prevent the accumulation of ROS in human neuronal DRG cultures. When tested alone, trolox, did not have a significant influence on the DHR intensity in the neuronal cell body suggesting that the culture conditions alone did not induce oxidative stress. When incubated together with supernatants from HIV-1 _{III_B}- and HIV-1 _{Ba-L}-infected cells at a dilution of 1:10, trolox prevented the oxidative stress (figure 4A).

To determine whether blocking ROS accumulation could also prevent axonal retraction in the neurons, we tested trolox together with the supernatants of HIV-1 _{III_B}- and HIV-1 _{Ba-L}-infected monocytic cells at a dilution 1:10 for 24 h, immunostained the neurons with anti- β III tubulin and measured the axonal length. Trolox failed to prevent neurite trimming (figure 4B).

Mitochondrial dysfunction in axons

Since trolox did not prevent axonal retraction, we compared the mitochondrial function in the neuronal cell body and neurites following exposure to supernatants from HIV-infected monocytic cells using the JC-1 assay. Interestingly, while significant changes in the mitochondrial membrane potential were noted in the neuronal cell body, similar changes did not occur in the neurites (figure 5).

To explore these observations further, we compared the amount of nitrosylated proteins in sciatic nerve obtained at autopsy from HIV-infected individuals with peripheral neuropathy with the amounts from neuropathy-free HIV-infected subjects and HIV-seronegative controls. The three groups did not differ in age and gender but differed in racial distribution (table 1). There was no significant difference in the amount of nitrosylated proteins between the control subjects and the HIV-seropositive individuals without neuropathy. Only small amounts of 3-NT-modified proteins were observed in HIV-infected subjects with neuropathy, while 4/6 patients with HIV infection without a peripheral neuropathy and 3/6 HIV-seronegative subjects had strong bands on the slot blot, indicative of 3-NT production (figure 6A). The HIV-infected subjects with neuropathy had significantly lower amounts of 3-NT compared to the HIV-seronegative subjects (figure 6B).

Characterization of chemokine receptor distribution in DRG neurons and prevention of axonal retraction by antisera to the chemokine receptors

Immunostaining of the DRG cultures with antisera to CXCR4 and CCR5 chemokine receptors showed that these receptors were expressed on all neurons in similar amounts (figure 7 A and B). Nonetheless, neutralizing antibodies to CXCR4 or CCR5 did not ameliorate neurite retraction (figure 7C) mediated by the supernatants from HIV-1_{IIIB} and HIV-1_{Ba-L} infected macrophages, suggesting that viral proteins and/or host factors can cause neurotoxicity independent of these chemokine receptors.

Discussion

In this study, we found that the DRG of HIV infected patients was infiltrated with HIV infected and activated macrophages; therefore, we have developed a biologically relevant human *in vitro* model of HIV-DSP. We established techniques for culturing mixed human DRG neurons with Schwann cells and then exposed them to supernatants from HIV-infected macrophages. We demonstrated that the supernatants of macrophages infected with CXCR4 and CCR5 tropic HIV-1 strains with comparable levels of HIV-p24 induced neuronal retraction in human DRG neurons and oxidative stress in the neuronal cell body. Humans in contrast to inbred rodent models are genetically heterogeneous and genetic factors may contribute to the development of HIV-DSP (4). We noticed that occasional DRG cultures were exquisitely sensitive to toxicity induced by the supernatants from HIV infected macrophages. Although technical factors cannot be entirely excluded, it is possible that host genetic factors may be a contributory factor.

Previous studies in which the HIV *env* was sequenced from the peripheral nerve showed a predominance of CCR5-dependent and macrophage tropic HIV-1, although dual tropic viruses were also identified (13). Most laboratory-adapted CXCR4 viruses, such as HIV-1_{IIIB}, do not replicate efficiently in macrophages (37). Nevertheless, macrophages can support efficient replication by a subset of primary CXCR4 viruses (32,36). We thus compared the neurotoxic potential of extracellular products released from HIV infected monocytic cells infected with either a CCR5 or CXCR4 tropic strain. We found that both viral strains produced similar amounts of toxicity in several different assays of neuronal injury. In each of these assays, similar amounts of toxicity were observed with dilutions of 1:10 and 1:100 of the culture supernatants while no toxicity was observed at 1:1000 dilution, suggesting that the toxic substance(s) was present at low concentrations despite very high viral loads in the supernatants.

Chemokine receptors CXCR4 and CCR5 are also expressed on neurons and glial cells and their respective endogenous ligands SDF-1 and RANTES can mediate neurotoxicity by interaction of these receptors (14,30). Further, the HIV envelope protein gp120 may also interact with these receptors to cause neurotoxicity in DRG neurons (14,22). By immunostaining we found that both CXCR4 and CCR5 were expressed on the human DRG neurons in nearly equal amounts. We were unable to block the neurotoxic effects of supernatants, however, from HIV-infected

monocytic cells with neutralizing antisera to these receptors suggesting that either substances other than these ligands may be responsible for the neurotoxic effects in this experimental system or that gp120 may cause neurotoxicity by mechanisms independent of its interactions with chemokine receptors. Although we have not yet identified the precise substance responsible for the neurotoxic effects on the DRG neurons, previous studies with brain derived neurons have implicated several other cytokines, and viral proteins in mediating these effects (reviewed in (17)). Immunopathological studies in DRG from patients with DSP have shown activated macrophages with concomitant immunostaining for pro-inflammatory cytokines such as TNF- α , interferon- γ and IL-6 (20,38) as well as evidence for upregulation of nitric-oxide (NO) (20). Experimental studies show that HIV infected macrophages also release glutamate (12) and excitatory amino acid receptors may be expressed on DRG neurons (3,16). However, HIV infected macrophages may also release other neurotoxic substances such as Ntox (10) and HIV proteins such as Tat (21). It has not been clear, however, which of these factors contribute to neuronal pathology in the DRG during HIV-infection.

Macrophages are the prime mediators of oxidative stress although human mononuclear cells may have little capacity to synthesize nitric oxide (6). Oxidative stress can also be induced by several HIV viral proteins and cytokines implicated in neuronal injury (33). Hence, we monitored the production of oxidative stress in the DRG neurons following exposure to supernatants from HIV-infected macrophages. We observed that in the neuronal cell body of the DRG neurons ROS were produced and mitochondrial membrane potential was reduced, which was prevented by the use of trolox as an antioxidant. Trolox has been shown to prevent mitochondrial toxicity in neuronal cultures exposed to the CSF from HIV-infected individuals (35). In contrast, the neurites did not show any changes in mitochondrial potential and trolox did not prevent neuritic retraction. We further measured 3-NT levels in the sciatic nerve of HIV-infected patients as a measure of oxidative stress. The HIV-infected patients with peripheral neuropathies did not show evidence of increased 3-NT proteins, instead, higher levels were noted in asymptomatic patients. Although these observations do not rule out the possibility that oxidative damage may occur at nerve terminals, it does raise the possibility that axonal injury may occur independent of oxidative stress. Similar observations have been made in patients with HIV dementia, where indices of oxidative stress are increased early in the disease and diminished in patients with severe HIV dementia (35). These findings suggest that the injury to the neuronal cell body and the axon may be differentially regulated. This is supported by observations that the signaling pathways mediating axonal degeneration might be distinct from those mediating neuronal apoptosis and dying back axonal degeneration may occur as a result of a caspase-independent pathways (7,18,26). But importantly, our observations suggest that antioxidants may not be sufficient to prevent peripheral neuropathy in HIV-infected patients.

Taken together we have shown that the supernatants of macrophages infected with either CCR5 or CXCR4 tropic HIV-1 strains mediate axonal retraction without causing neuronal cell death, but can cause mitochondrial dysfunction in the neuronal cell body in human DRG neuronal cultures. The toxic effect of the supernatants on the axons was not prevented by antioxidants while they protected the cell body. Further, peripheral nerves of HIV infected patients did not show evidence of increased oxidative stress. These observations suggest a differential effect on the axon and cell body and that different mechanisms of injury may be operative in these two structures. Further, even though CXCR4 and CCR5 were expressed on the DRG neurons, these effects were independent of these receptors, suggesting that factors other than these chemokine receptors should be considered in the neuropathogenesis of HIV-DSP.

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Figure 1. HIV infection in human DRG cells from patients with history of HIV-peripheral neuropathy

A) Macrophage infiltration of the DRG visualized with anti-CD68 immunostaining and counterstained with Cresyl-violet

B) Distribution of HIV-p24 antigen (red) in the DRG as visualized by confocal microscopy. The HIV-p24 was localized in the perineuronal compartment and within cells with macrophage morphology. Schwann cells and their processes (green) visualized with anti-GFAP antibodies lack any co-localization with HIV-p24.

Figure 2. Effect of supernatants from HIV infected monocytic cells on neurite length of DRG neurons

Supernatants from HIV-1_{III_B} THP-1 infected cells and HIV-1_{Ba_L} infected MDM induce neuritic retraction in human DRG neurons. Confocal images of anti-βIII tubulin immunostained DRG cultures treated for 24 hours with supernatants from uninfected THP-1 cells (A, 1:10 dilution; C, 1:100 dilution), HIV-1_{III_B} (B, 1:10 dilution; D, 1:100 dilution), uninfected MDM (E, 1:10 dilution; G, 1:100 dilution) and HIV-1_{Ba_L} supernatant (F, 1:10 dilution; H, 1:100 dilution). (I) Reduction of the total neuritic length per axon is shown as

standardized data. * Supernatant from infected monocytic cells versus the matched control, $p < 0.05$.

Figure 3. Effect of supernatants from HIV infected monocytic cells on mitochondrial function of DRG cell bodies

Mitochondrial free radical production and membrane potential was measured in human DRG cell bodies following treatment with supernatants from THP-1 cells infected with HIV-1_{III_B} and MDM infected with HIV-1_{Ba-L}.

A) Increase in DHR intensity is shown as standardized data. * Supernatant from infected monocytic cells versus the matched control, $p < 0.05$. Each data bar represents approximately 100 analyzed neurons

B) Mitochondrial membrane potential (red/green ratio) is shown as standardized data. * Supernatant from infected monocytic cells versus the matched control, $p < 0.05$. Each data bar represents at least 150 analyzed neurons.

Figure 4. Effect of trolox on HIV-1_{III_B} and HIV-1_{Ba-L} induced neurotoxicity

A) Trolox blocked oxidative stress in the neuronal cell body. DHR intensity is shown as standardized data. * Supernatant from infected monocytic cells versus the matched control, $p < 0.05$. Each data bar represents approximately 100 analyzed neurons. B) Trolox failed to prevent neuritic trimming in human DRG neurons. Reduction of the total neuritic length per axon is shown as standardized data. * Supernatant from infected monocytic cells versus the matched control, $p < 0.05$. There was no significant difference in the neuritic length of neurons treated with a 1:10 dilution of the supernatants from HIV-infected cells and trolox compared to the supernatants alone.

Figure 5. Effect of supernatants from HIV-infected monocytic cells on axonal mitochondrial potential

Supernatants from HIV-1_{III B} THP-1 infected cells and HIV-1_{Ba-L} infected MDM had no significant effect on the mitochondrial membrane potential in axons of neurons derived from human dorsal root ganglia. Each data bar represents approximately 150 analyzed axons.

Figure 6. Detection of 3NT modified proteins in peripheral nerve of HIV infected patients

A) Sciatic nerve extracts were analyzed by slot blot by using an antisera to 3-NT. The left row represents a negative (BSA) and a positive control (nitrosylated BSA), the second row shows 5 sciatic homogenate samples from HIV-seropositive individuals with neuropathy. The slots in the third row were loaded with samples from HIV-seropositive subjects without neuropathy and the right row with samples from the HIV-seronegative controls

B) The bar graph represents the mean area ± SEM. ** $p < 0.05$ compared to the HIV-negative controls.

Figure 7. Chemokine receptor expression and effect of chemokine receptor antibodies on neuritic retraction induced by HIV-infected monocytic cells

A) Human DRG neurons were (i) immunostained for CXCR4 showing red fluorescence. (ii) Hoechst 33342 stain was used to visualize the nuclei. (iii) Immunostaining with anti- β III-tubulin antisera shows green fluorescence. (iv) Shows the merged photomicrograph of all three panels

B) Human DRG neurons were (i) immunostained for CCR5 showing red fluorescence. (ii) Hoechst 33342 stain was used to visualize the nuclei. (iii) Immunostaining with anti- β III-tubulin antisera shows green fluorescence. (iv) Shows the merged photomicrograph of all three panels

C) Anti-CXCR4 and anti-CCR5 failed to prevent neurite retraction induced by supernatants from THP-1 cells infected with HIV-1_{III_B} or MDM infected with HIV-1_{Ba-L}. Reduction of the total neurite length per axon is shown as standardized data. * Supernatant from infected monocytic cells versus the matched control, $p < 0.05$.

Table 1

Demographic characteristics of the subjects who were compared for 3-NT protein residues

	HIV+ SUBJECTS WITH PNP (N=5)	HIV+ SUBJECTS WITHOUT PNP (N=6)	CONTROL SUBJECTS (N=6)	P
Age in years (mean/SD)	47.2 ± 15	45.2 ± 4.8	49.7 ± 8.7	0.74
Male	60%	50%	67%	
Race (AA/W/Hispanic)	3/2/0	2/1/3	1/0/5	

AA African-American; W White