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Peripheral nerve-derived HIV-1 is predominantly CCR5-dependent and causes neuronal degeneration and neuroinflammation

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Abstract

HIV-related peripheral neuropathy is a major neurological complication of HIV infection, although little is known about its pathogenesis. We amplified HIV-1 C2V3 envelope sequences from peroneal nerves obtained from HIV/AIDS patients. Sequence analysis and infectious recombinant viruses containing peripheral nerve-derived C2V3 sequences indicated a predominance of CCR5-dependent and macrophage-tropic HIV-1, although dual tropic viruses using both CCR5 and CXCR4 were identified. The neuropathogenic effects of recombinant HIV-1 clones were investigated using a novel dorsal root ganglion culture system that was comprised of sensory neurons, macrophages and Schwann cells from transgenic rats expressing human CD4 and CCR5 on monocytoid cells. Despite restricted viral replication, HIV-1 infection caused a reduction in the percentage of neurons with neuritic processes together with significant neurite retraction, which was accompanied by induction of IL-1β and TNF-α expression, depending on the individual virus. Our results suggest that HIV-1 infection of the peripheral nervous system causes axonal degeneration, possibly through the induction of pro-inflammatory cytokines.
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Introduction

Peripheral neuropathy has become the chief neurological syndrome observed among persons infected with human immunodeficiency virus type-1 (HIV-1) in the developed world (Brinley et al., 2001). Of these peripheral neuropathies, HIV-associated sensory neuropathies (HIV-SN) are the most common forms recognized, affecting approximately 30% of both adults and children with acquired immunodeficiency syndrome (AIDS) (Araujo et al., 2000; Keswani et al., 2002; Tagliati et al., 1999). Furthermore, Morgello et al. (2004) have recently demonstrated HIV-SN

prevalence rates of up to 52%, including patients who receive highly active antiretroviral therapy (HAART). HIV-SN includes both 'distal sensory polyneuropathy' (DSP) and 'antiretroviral toxic neuropathy' (ATN), conditions which frequently overlap (Keswani et al., 2002). HIV-SN is predominately defined by disabling pain with parathesiae, gait instability and autonomic dysfunction (reviewed in Pardo et al., 2001). In addition, sub-clinical nerve damage may also occur in HIV-1-infected patients in the absence of symptoms of HIV-SN (Gastaut et al., 1989; Gulevich et al., 1992; Tagliati et al., 1999).

The principal pathological feature of HIV-SN is defined by distal degeneration of long axons in a 'dying back' pattern (Pardo et al., 2001). Prominent loss of the small, unmyelinated nociceptive sensory neurons occurs although reduced density of small and large myelinated axonal fibers has also been observed (Araujo et al., 2000; Griffin and

McArthur, 1998). Demyelination, if present, is not segmental and may be the result of axonal degeneration. The precise mechanism(s) underlying the neuropathogenesis of HIV-SN remains unclear. Both the 'indirect' neurotoxicity of products secreted within the nerve by activated macrophages and the 'direct' effect of viral proteins on dorsal root ganglion (DRG) neurons have been suggested. In support of the former, prominent infiltration of peripheral nerve by both inflammatory macrophages and T-cells has been demonstrated in HIV/AIDS patients compared to uninfected controls (de la Monte et al., 1988; Pardo et al., 2001; Rizzuto et al., 1995). Furthermore, in one study, the severity of peripheral neuropathy was correlated with the degree of inflammatory cell infiltration (Griffin and McArthur, 1998). Lymphocyte and macrophage infiltrates are also seen within the DRG of AIDS patients, with concomitant presence of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1 (IL-1) and interleukin-6 (IL-6) (Nagano et al., 1996; Rizzuto et al., 1995; Shapshak et al., 1995; Yoshioka et al., 1994).

Several groups have demonstrated the presence of HIV-1 transcripts and proteins in peripheral nerves and DRG (Brannagan et al., 1997; Cornford et al., 1992; Gherardi et al., 1989; Grafe and Wiley, 1989; Nagano et al., 1996; Rizzuto et al., 1995; Vital et al., 1992; Yoshioka et al., 1994). However, it is generally held that HIV-1 replication in peripheral nerves is sparse, and is restricted to the macrophage cell population. While successful isolation of whole virus from ex vivo peripheral nerve tissue of HIV/AIDS patients has been reported (Chaunu et al., 1989; Cornblath et al., 1987; de la Monte et al., 1988; Ho et al., 1985), little is known about the nature of the virus isolated from peripheral nerves and whether it differs between HIV/AIDS patients with or without HIV-SN. In this study, we have characterized the co-receptor usage and cell tropism of HIV-1 derived from peroneal nerve tissue samples from HIV/AIDS patients both with and without HIV-SN. Recombinant HIV-1 expressing envelope sequences from peripheral nerve-derived virus were constructed, and their potential neurotoxic effects were investigated in vitro using a novel DRG mixed cell culture system from rats transgenic for human CD4 and human CCR5 (Keppler et al., 2002). Lastly, the induction of host immune responses following exposure to nerve-derived HIV-1 in both the in vitro cultures and in the peroneal nerve tissue samples was investigated by real time RT-PCR analysis.

Results

Detection of HIV-1 genomes in peripheral nerve

Although HIV-1 antigens have been detected in peripheral nerve previously (Rizzuto et al., 1995), we investigated the presence of HIV-1 provirus in peripheral nerve tissue from twelve HIV/AIDS patients. Successful extraction of genomic DNA from all 12 peroneal nerve samples was demonstrated by PCR amplification of the GAPDH gene (data not shown). HIV-1-specific amplicons corresponding to a 445-base pair fragment of the proviral C2V3 region of the *env* gene were detected in seven (HIV-SN, $n = 4$; HIV-NSN, $n = 3$) of 12 patient samples (58%), using established protocols (Chesebro et al., 1992; Power et al., 1994; Van Marle et al., 2002). Having demonstrated the presence of HIV-1 provirus in peripheral nerves, we next investigated whether the nerve was a site of viral transcription. Total RNA was extracted from 10 out of the 12 nerve samples, and was used as a template for cDNA synthesis. The generation of cDNA was confirmed by PCR amplification of the GAPDH gene (data not shown). In contrast to the HIV-1 provirus analysis, HIV-1 C2V3 sequences derived from RNA were detected only in one of ten nerve samples (patient 417, with HIV-SN). The detection of HIV-1 provirus in over half of the samples studied demonstrates the presence of HIV-1-infected cells within the peripheral nerve of HIV/AIDS patients both with and without sensory neuropathy. However, the low level detection of HIV-1 RNA suggested that there was limited viral replication at this site.

Analysis of HIV-1 env sequences

Studies of HIV-1 infection of the central nervous system have demonstrated that polymorphisms within the HIV-1 *env* gene distinguish patients with and without HIV-1 associated dementia (Kuiken et al., 1995; Power et al., 1994). Since HIV-1 provirus was detected in the peripheral nerve of patients both with and without HIV-SN, we sequenced the amplified C2V3 region of the HIV-1 *env* gene and investigated the phylogenetic relationships of virus sequences from patients with and without sensory neuropathy (Fig. 1A). As the nerve-derived C2V3 sequences were obtained from clade B infected HIV/AIDS patients, we chose to root the neighbor-joining tree against the distantly related HIV-1 clade D strain NDK, permitting comparisons to be made between the virus sequences obtained from patients with and without sensory neuropathy. Although these sequences were obtained from a single clone per patient, they were identical to that obtained by direct sequencing of the C2V3 PCR amplicon. To assess the molecular diversity within the nerve from individual patients, we sequenced a minimum of three extra clones per patient in individual PCR reactions. In three individuals, the sequence of the *env* amplicon in 2 out of 3 clones was identical to that obtained by direct sequencing, while the third clone differed by only one amino acid (99% homology). For the remaining individuals, all clones demonstrated identical sequences to that obtained by direct sequencing (data not shown), suggesting limited molecular diversity within the C2V3 *env* region from the nerve of individual patients. The macrophage-tropic and CCR5-dependent brain-derived JRFL and SF162 strains were included in the analysis as control viruses isolated from
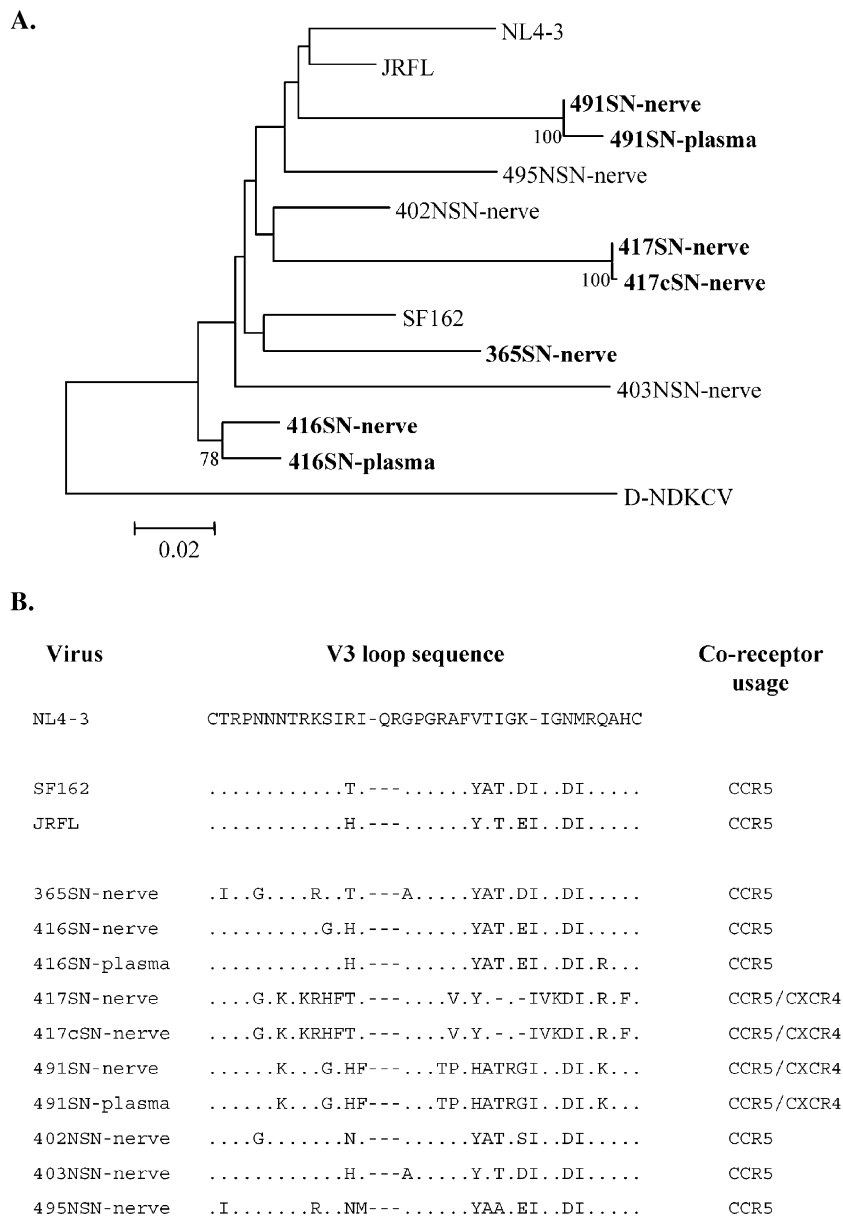
Fig. 1. Analysis of HIV-1 envelope sequences of peripheral nerve-C2V3 recombinant HIV-1 clones. (A) Phylogenetic neighbor-joining tree (using the Kimura 2-parameter model) based the C2V3 sequences obtained from peripheral nerve and plasma of HIV/AIDS patients. The tree is rooted against the C2V3 sequence of the HIV-1 clade D strain NDK, and bootstrap values greater than 70 are indicated (bootstrap analysis of 1000 replicates). Bold type indicates patients with HIV-SN. (B) V3 loop amino acid sequences of HIV-1 derived from the nerve tissue or plasma of HIV/AIDS patients aligned relative to the HIV-1 NL4-3 strain. Predicted co-receptor usage was analyzed as described in Materials and methods.

the nervous system, while NL4-3 was included as a prototypic CXCR4-dependent isolate. The C2V3 nucleotide sequences from the three patients without sensory neuropathy (495NSN-nerve, 402NSN-nerve and 403NSN-nerve) were more closely related to sequences from patients with neuropathy than they were to each other, indicating that there was no clustering of the C2V3 region on the basis of clinical diagnosis of HIV-SN. Similar results were obtained when the inferred amino acid sequences were analyzed (data not shown). HIV-1 proviral (417SN-nerve) and viral (417cSN-nerve) C2V3 sequences were similar for patient 417, with

the only difference being a four amino acid deletion in the C2 region for 417SN-nerve (data not shown).

For patients 491 and 416, viral RNA was also extracted from plasma samples obtained 3 and 8 months prior to the autopsy. Sequence analysis revealed a 99% homology between 491SN-nerve and 491SN-plasma samples (sequences differed by 2 out of 141 amino acids) and a 95% sequence homology between 416SN-nerve and 416SN-plasma (sequences differed by 7 out of 144 amino acids).

Having shown the presence of HIV-1 provirus in the peripheral nerve of HIV/AIDS patients, we then examined

the potential co-receptor usage of these viruses. HIV-1 co-receptor usage was initially analyzed by evaluating the charge and amino acid composition of the V3 loop from the amplified C2V3 region (Fig. 1B), using methods previously described (Briggs et al., 2000). The V3 loop sequences of two known macrophage-tropic and CCR5-dependent isolates (JRFL and SF162) were included. Using this approach, the predicted co-receptor usage for these isolates matched their known phenotypes. Of the seven peripheral-nerve derived HIV-1 sequences, five (365SN, 416SN, 402NSN, 403NSN and 495NSN) were predicted to be CCR5-dependent, while two (417SN and 491SN) were predicted to use both CCR5 and CXCR4 as a co-receptor. Where matched plasma samples were available, similar virus phenotypes were predicted. In addition, the V3 loop sequences of nerve-derived HIV-1 provirus and HIV-1 virus for patient 417

(417SN and 417cSN, respectively) were identical and predicted dual tropism for CCR5 and CXCR4.

Investigation of cell tropism and co-receptor usage

To investigate HIV-1 co-receptor usage, pseudotyped viruses that contained the firefly luciferase gene and expressed the nerve-derived C2V3 region were constructed from three predicted CCR5-using strains (NL-365, NL-402 and NL-416) and one predicted dual tropic strain (NL-417c). NL4-3 and NL-J1 were included as controls for HIV-1 isolates that utilize either CXCR4 or CCR5, respectively. NL-J1 is a recombinant strain that expresses the envelope region of the HIV-1 JRFL strain in a NL4-3 backbone (Zhang et al., 2003). All three predicted CCR5-using viruses infected HeLa-CD4 cells expressing CCR5 (Fig. 2A, open
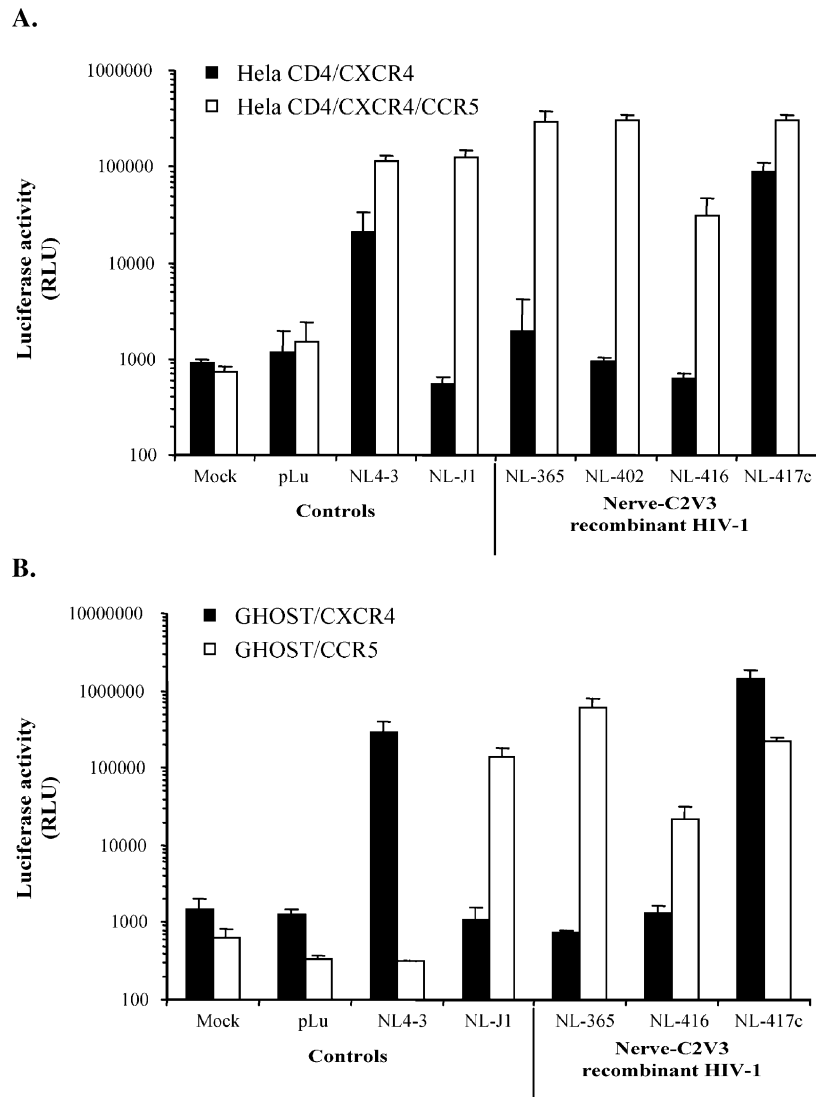

Fig. 2. Both CCR5-dependent and CXCR4-dependent HIV-1 are present in the peripheral nerve tissue of HIV/AIDS patients. (A) HeLa-CD4/CXCR4 and HeLa-CD4/CXCR4/CCR5 or (B) GHOST/CXCR4 and GHOST/CCR5 cells were infected with pseudotyped HIV-1 expressing the nerve-C2V3 region and the firefly luciferase gene. As controls, target cells were also infected with supernatants from non-transfected 293-T cells (mock) and 293-T cells transfected with pNL-Luc-E⁻R⁻ alone (pLu). Results are presented as the mean relative light units (RLU) and standard deviation of triplicate samples.

bars), however, no infection was observed in HeLa-CD4 cells that expressed only CXCR4 (closed bars), clearly demonstrating their dependence on CCR5 for infection. In contrast, NL-417c-infected HeLa-CD4 cells expressing CXCR4 alone or expressing both CXCR4 and CCR5. These results confirmed that the biological phenotype for co-receptor usage of peripheral nerve-derived HIV-1 matched that predicted by sequence analysis of the V3 loop (Fig. 1B). However, as the two HeLa-CD4 cell lines both constitutively expressed CXCR4, it was not possible to determine whether NL-417c used only CXCR4 or in fact was dual tropic (for both CXCR4 and CCR5). To address this question, we tested the ability of these pseudotyped viruses to infect the GHOST cell lines expressing either CXCR4 or CCR5 alone (Fig. 2B). As expected, NL4-3 and NL-J1 were shown to infect GHOST cells expressing either CXCR4 or CCR5 respectively. Again, NL-365 and NL-416 only infected cells that expressed CCR5. In contrast, NL-417c infected both GHOST/CXCR4 and GHOST/CCR5 cells, suggesting that as predicted by sequence analysis this virus was dual tropic for either CXCR4 or CCR5.

Although CXCR4 and CCR5 are the major co-receptors for HIV-1, other chemokine receptors have also been demonstrated to mediate HIV-1 infection (Berger et al., 1999). Therefore, we extended the co-receptor usage study to include a cell line expressing CCR3. In addition to expressing distinct chemokine receptors, the GHOST cell lines are also stably transfected with a construct expressing the humanized green fluorescent protein (hGFP) under the control of the HIV-2 LTR. Therefore, HIV-1 infection of these cells can be detected via induction of the hGFP gene. Recombinant HIV-1 strains expressing the nerve-derived C2V3 region in the NL4-3 backbone (herein to be known as nerve-C2V3 recombinant HIV-1) were generated from four HIV/AIDS patients (#365, #402, #416, #417c) and these recombinant HIV-1 clones were shown to be replication-competent in activated human PBMC (Fig. 3A). Mock infection of the GHOST cell lines did not result in hGFP expression, while infection with NL-J1 induced hGFP expression in the GHOST/CCR5 cell line only (Fig. 3B). Similarly, infection with the NL-365, NL-402 and NL-416 strains resulted in hGFP expression in only the GHOST/CCR5 cells (data not shown). Infection of GHOST/CXCR4 cells, and to a lesser extent GHOST/CCR2b, was seen with both NL4-3 and NL-417c strains, whereas the latter also induced hGFP expression in GHOST/CCR5 cells. In summary, all four recombinant HIV-1 strains expressing the C2V3 region derived from peripheral nerve utilized CCR5 for infection. For three of these viruses (NL-365, NL-402 and NL-416), CCR5 appeared to be the primary co-receptor used. However, NL-417c was also able to utilize CXCR4 and, to a lesser extent, CCR2b (Fig. 3C).

To investigate the cell tropism of nerve-derived HIV-1, human MDM cultures were exposed to recombinant infectious HIV-1 clones expressing the nerve-derived C2V3. NL-365, NL-402 and NL-416 demonstrated sus-

tained viral replication in human MDM (Fig. 3A). In contrast, NL-417c failed to replicate in human MDM, with culture supernatant HIV-1 p24 values similar to that of the background (mock treated controls). Surprisingly, NL4-3 also replicated in MDM cultures at early time points, although by day 12 post-infection HIV-1 p24 values in culture supernatant were also approaching background levels. Furthermore, NL4-3 failed to replicate in MDM from two other individuals (data not shown), suggesting that the replication observed in Fig. 3A was donor-specific. Nonetheless, NL4-3 is generally considered to be a CXCR4-dependent T-tropic isolate, although its replication in macrophages has also been previously documented (Valentin et al., 2000).

Infection of DRG cultures from human CD4 and CCR5 transgenic rats

The in vitro DRG cultures consisted of a mixed population of cells containing neurons (MAP-2+, Fig. 4A), macrophages (ED-1+, Fig. 4D) and Schwann cells (GFAP+, Fig. 4G). MAP-2 staining of neurons clearly labeled not only the cell body, but also the neuritic process (Fig. 4A, arrowed). Human CCR5 expression in the DRG cultures was restricted to macrophages, as evidenced by co-localization of CCR5 with Iba-1-expressing cells (Figs. 4E and F, arrowed cells and inset). Similarly, human CD4 expression in the DRG cultures was also restricted to macrophages, confirmed by co-localization of CD4 with Iba-1-expressing cells (Figs. 4H and I, arrowed cells). DRG cultures were infected with the nerve-C2V3 recombinant HIV-1 clones (NL-365, NL-402, NL-416 and NL-417c) or the brain-derived SF162 isolate. NL-J1 and NL4-3 were also included as controls for CCR5-using and CXCR4-using viruses respectively. Viral nucleocapsid protein (p24) expression could be detected in macrophages (Figs. 4B and C, arrowed cells and inset (representative image from SF162-infected cultures)), but p24 was not detected in the supernatants from infected cultures (data not shown). The presence of HIV-1 RNA and provirus at day 1 and day 4 post-infection was assessed using the nested PCR approach described earlier. Viral RNA corresponding to the C2V3 region of the *env* gene was detected in cultures 1-day post-infection, irrespective of whether the virus used CCR5 or CXCR4 as a co-receptor (Fig. 4J). For all clones used, HIV-1 provirus was detected at day 4 post-infection, suggesting the persistence of virus-infected cells in the DRG cultures. However, at this time point, viral RNA was undetectable. The inability to detect viral RNA at day 4 post-infection was likely not due to inadequate RNA extraction and cDNA preparation, as the house keeping gene β-actin could be amplified from the same samples. In addition, viral RNA specific for the HIV-1 gene *nef* was also undetectable at day 4 post-infection (data not shown). Taken together, these results demon-
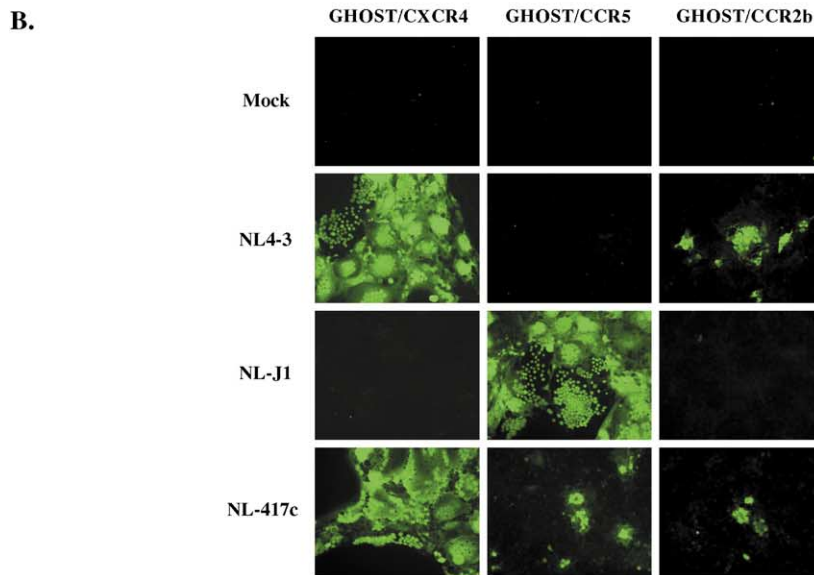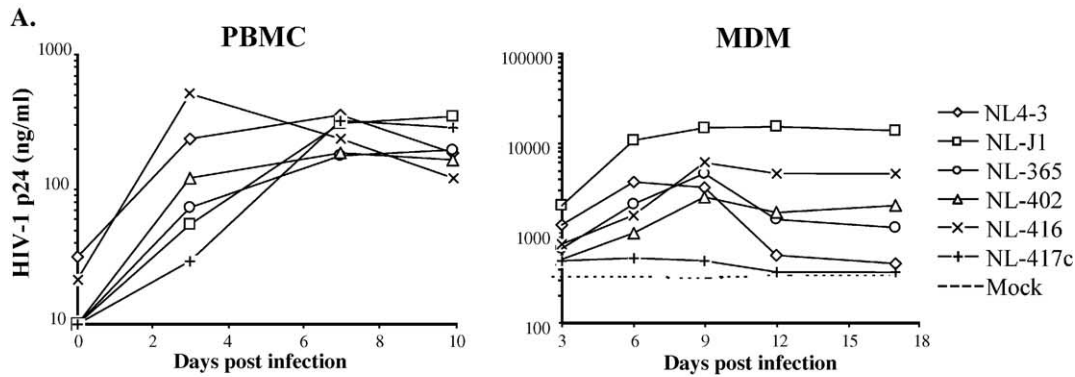
Fig. 3. Cell tropism and co-receptor usage of nerve-C2V3 recombinant HIV-1. (A) Supernatants from 293-T cells transfected with different HIV-1 molecular clones expressing nerve-C2V3 HIV-1 were used to infect activated human PBMC. NL4-3 and NL-J1 (a recombinant strain that expresses the envelope region of the JRFL strain in a NL4-3 backbone) were included as controls for CXCR4-dependent or CCR5-dependent strains, respectively. Human MDM were infected with PBMC-expanded recombinant HIV-1 (virus input titre of 5 ng p24 protein per culture). As a control, MDM were infected with culture supernatants from activated PBMC that had not been exposed to HIV-1 (mock). Virus protein levels in culture supernatants were quantified using the HIV-1 p24 Antigen Capture Assay Kit at the indicated time points. Values are the mean of duplicate wells. (B) GHOST cell lines were infected with the recombinant HIV-1 strains as described in Materials and methods. Infection of the different cell lines was evaluated by the induction of green fluorescent protein expression 3 days post-infection. (C) Summary of the co-receptor usage of the different nerve-C2V3 recombinant HIV-1 as indicated by infection of the GHOST cell lines. (Original magnification ×200).

strate that both CXCR4-using and CCR5-using HIV-1 are capable of infecting the mixed cell DRG cultures. However, the detection of viral RNA at day 1 post-infection but not at day 4 suggests that there is limited viral replication within these cultures.

Morphological effects of HIV-1 infection on DRG neurons

To assess the DRG culture system as a potential model for evaluating neuropathogenic effects of HIV-1 infection in the peripheral nervous system, a series of morphological
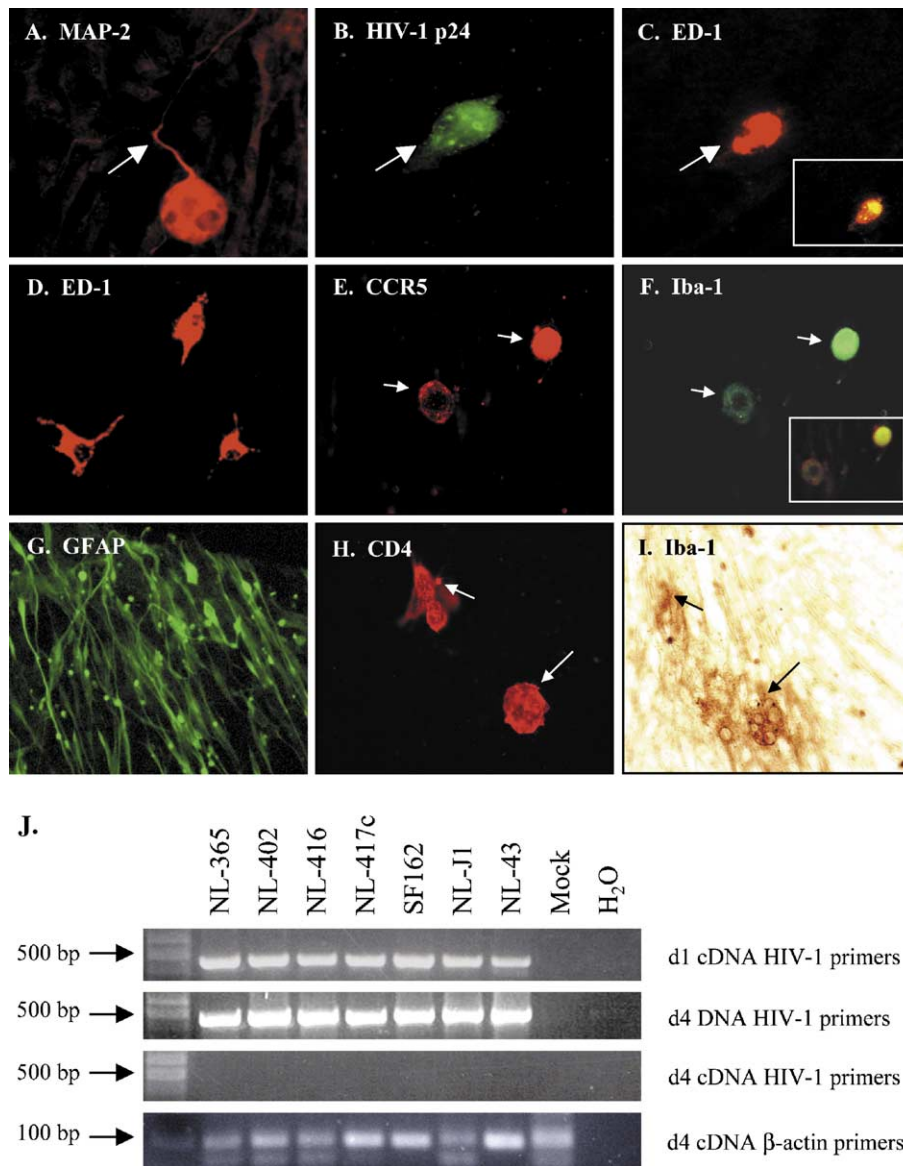
Fig. 4. DRG cultures from rats transgenic for human CD4 and CCR5 harbor HIV-1 provirus after infection with different HIV-1 strains. DRG cultures consist of (A) neurons (MAP-2+) with neurite processes (arrowed), (D) macrophages (ED-1+) and (G) Schwann cells (GFAP+). (B and C) Following HIV-1 infection, HIV-1 protein was detected in ED-1-expressing macrophages (representative images from SF162-infected cultures). Inset represents merged HIV-1 p24 and ED-1 images. (E) Human CCR5 and (H) human CD4 expression co-localized with Iba-1-expressing macrophages (F and I). Inset represents merged CCR5 and Iba-1 images. (J) The presence of HIV-1 viral RNA and proviral DNA was investigated using nested PCR to amplify the C2V3 region of the envelope gene (detailed in methods section). Envelope specific amplicons (455 base pairs) were detected in cDNA samples at day 1 post-infection (but not at day 4) and in DNA samples at day 4 post-infection. Mock represents DRG cultures infected with supernatants from activated PBMC that had not been exposed to HIV-1. (Original magnification ×400).

examinations were performed on DRG cultures infected with the nerve-C2V3 HIV-1 recombinant clones. These studies comprised evaluation of neuronal soma numbers, quantifying the percentage of neurons with neurite out-growths, measuring neurite length and calculating the area of the neuron soma (Fig. 5). To standardize the results between experiments, neuronal soma numbers were expressed as a percentage of the mock treated control for that experiment. Total neuronal soma numbers were not different in DRG cultures infected with the nerve-C2V3 recombinant HIV-1 compared to mock-infected controls

(Fig. 5A). In contrast, a reduction in the average percentage of neurons with neuritic processes was observed in cultures infected with the nerve-C2V3 recombinant HIV-1 compared to the mock-infected controls (Fig. 5B). This reduction reached statistical significance for two of the recombinant strains, NL-402 and NL-417c, and was borderline significant for NL-365 ($P = 0.06$). Neurite lengths in DRG cultures infected with the different nerve-C2V3 recombinant HIV-1 clones were significantly less than the mean value of the mock-infected controls (Fig. 5C). Mean neurite lengths were 410, 311, 312, 343 and 339 μm for mock, NL-365,
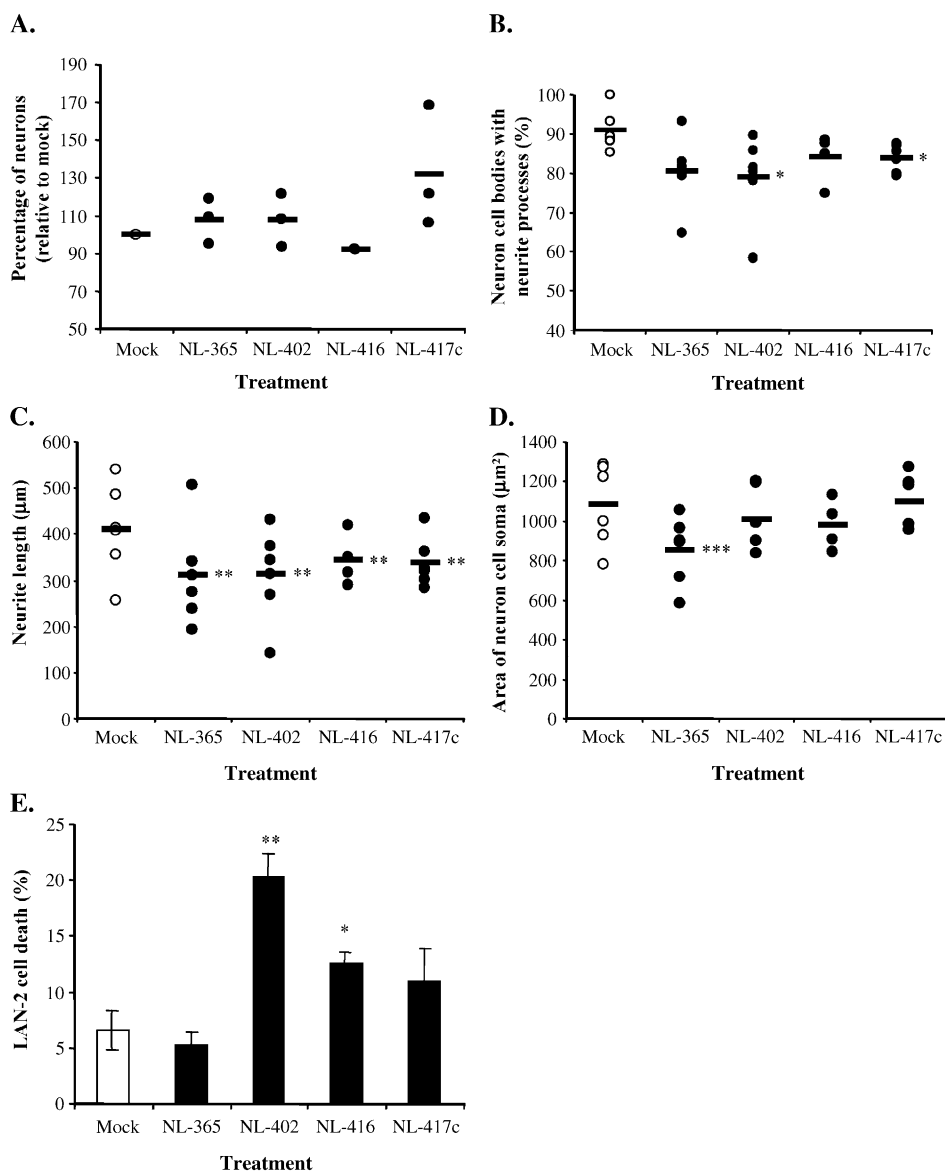
Fig. 5. DRG cultures infected with nerve-C2V3 recombinant HIV-1 clones exhibit neurite retraction and fewer processes. (A) Neuronal loss was not observed in HIV-1-infected cultures compared to uninfected controls. (B) Neurons with neurite processes were reduced in HIV-1-infected cultures in a viral strain-dependent manner compared to uninfected controls. (C) Neurite length was diminished in HIV-1-infected cultures compared to uninfected controls. (D) Neuronal soma area was reduced in HIV-1-infected cultures depending on the viral strain. Circles represent (A) average of duplicate wells from three independent experiments, or (B, C and D) individual wells from three independent experiments, with horizontal bars representing the mean value from three experiments. (E) Supernatants from human CD4 and CCR5 transgenic rat cultured macrophages infected with nerve-C2V3 recombinant HIV-1-induced cell death in LAN-2 cells. Data represent mean values and standard errors of the mean. *$P < 0.05$, **$P < 0.005$, Student's t test (versus mock); ***$P < 0.001$ Mann–Whitney U test (versus mock).

NL-402, NL-416 and NL-417c infected cultures, respectively, demonstrating a reduction in neurite length, ranging from 16% to 24% of the mock-infected control, depending on the virus strain. The majority of viruses containing the nerve-C2V3 fragment had minimal effects on neuronal soma size (Fig. 5D), with the exception of NL-365, which induced a significant reduction in the average neuronal soma size to 79% of the mock control (853 and 1083 μm^2 for NL-365 and mock treated DRG cultures, respectively). Thus, infection of DRG cultures with nerve-C2V3 recombi-

nant HIV-1 clones resulted in several morphological changes in neurons, including a reduction in the percentage of neurons with neurite outgrowth (Fig. 5B), neurite retraction (Fig. 5C) and, to a lesser extent, a reduction in neuronal soma area (Fig. 5D).

Host response in DRG cultures following HIV-1 infection

To investigate possible mechanisms underlying the morphological changes described above, real time RT-

PCR analysis of pro-inflammatory gene expression was performed on the DRG cultures. Compared to mock-treated controls, exposure to three out of the four different nerve-C2V3 recombinant HIV-1 clones enhanced IL-1β expression in the DRG cultures at day 1 post-infection (Fig. 6A). NL-365 and NL-416 induced approximately a two-fold increase in expression while NL-417c induced a three-fold
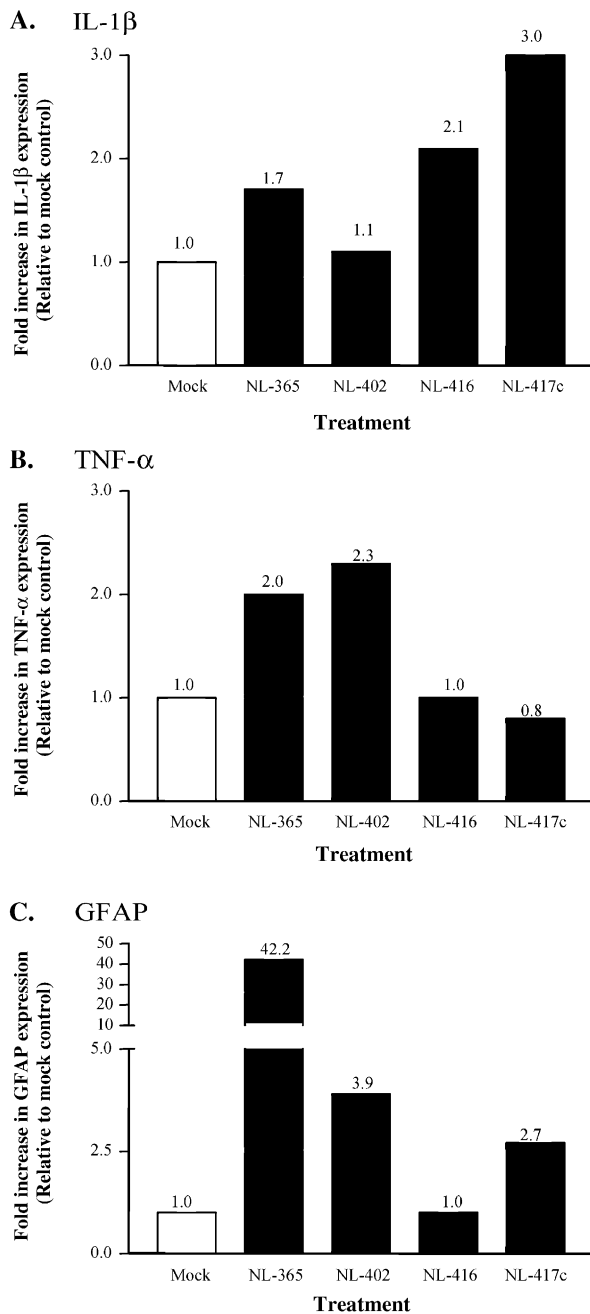
increase in expression. Exposure of the DRG cultures to the different infectious HIV-1 clones had less effect on TNF-α expression, with only two of the four viruses inducing increased expression relative to the mock control (an approximate two-fold increase for both NL-365 and NL-402 at day 1 post-infection; Fig. 6B). The effect of HIV-1 exposure on Schwann cells of the DRG cultures was investigated by analyzing GFAP expression. Compared to mock-treated controls, three out of the four different HIV-1 strains enhanced GFAP expression in the DRG cultures at day 1 post-infection (Fig. 6C). The ability of these viruses to induce GFAP expression varied: NL-417c induced approximately a three-fold increase in expression; NL-402 induced a four-fold increase in expression while NL-365 induced over a forty-fold increase in expression relative to the mock control. Taken together, these results demonstrate that HIV-1 infection of DRG cultures caused host immune activation depending on the individual viral clone.

Given that induction of pro-inflammatory cytokines was observed in DRG cultures infected with nerve-C2V3 recombinant HIV-1, we investigated whether soluble factors released by HIV-1-infected macrophages might mediate neuronal toxicity. Macrophages from human CD4 and CCR5 transgenic rats were infected with different nerve-C2V3 recombinant HIV-1 clones, and cell culture supernatants were harvested 4 days post-infection. Application of the supernatant to human LAN-2 cholinergic neuronal cells resulted in significant cell death that was dependent on the individual virus (Fig. 5E). HIV-1 proviral DNA was detected in the macrophages, but HIV-1 p24 protein was not detected in the supernatants (data not shown). These latter findings imply that release of host factors from macrophages following HIV-1 infection with nerve-C2V3 recombinant HIV-1 clones may mediate neuronal toxicity, rather than the virus itself.

Host response in peroneal nerve tissue from HIV-SN and HIV-NSN patients

Since nerve-C2V3 recombinant HIV-1 induced expression of inflammatory cytokines in the in vitro DRG culture model, we next examined whether these cytokines were expressed in the peroneal nerve tissue of HIV/AIDS patients. IL-1β and IL-6 were detected in nerve tissue from both groups (Figs. 7A and B), although TNF-α mRNA was not detected in nerve tissue. The frequency of IL-1β detection was slightly greater in HIV-SN compared to HIV-NSN patients, with IL-1β transcripts detected in four out of five (80%) HIV-SN samples compared to four out of seven (57%) HIV-NSN samples (Fig. 7A). In contrast, the frequency of IL-6 expression appeared to be similar, with IL-6 transcripts found in three out of four (75%) HIV-SN samples compared to six out of seven (86%) HIV-NSN samples. Thus, induction of the cytokines IL-1β and IL-6 was evident in peripheral nerves of patients with or without HIV-SN (Fig. 7C).

Fig. 6. Expression of mRNA for (A) IL-1β, (B) TNF-α and (C) GFAP in DRG cultures 24 h post-infection with different nerve-C2V3 recombinant HIV-1 strains. Mock represents DRG cultures infected with supernatants from activated PBMC that had not been exposed to HIV-1. Values were normalized against the GAPDH mRNA level and are given as the fold increase in expression relative to mock-infected cultures.
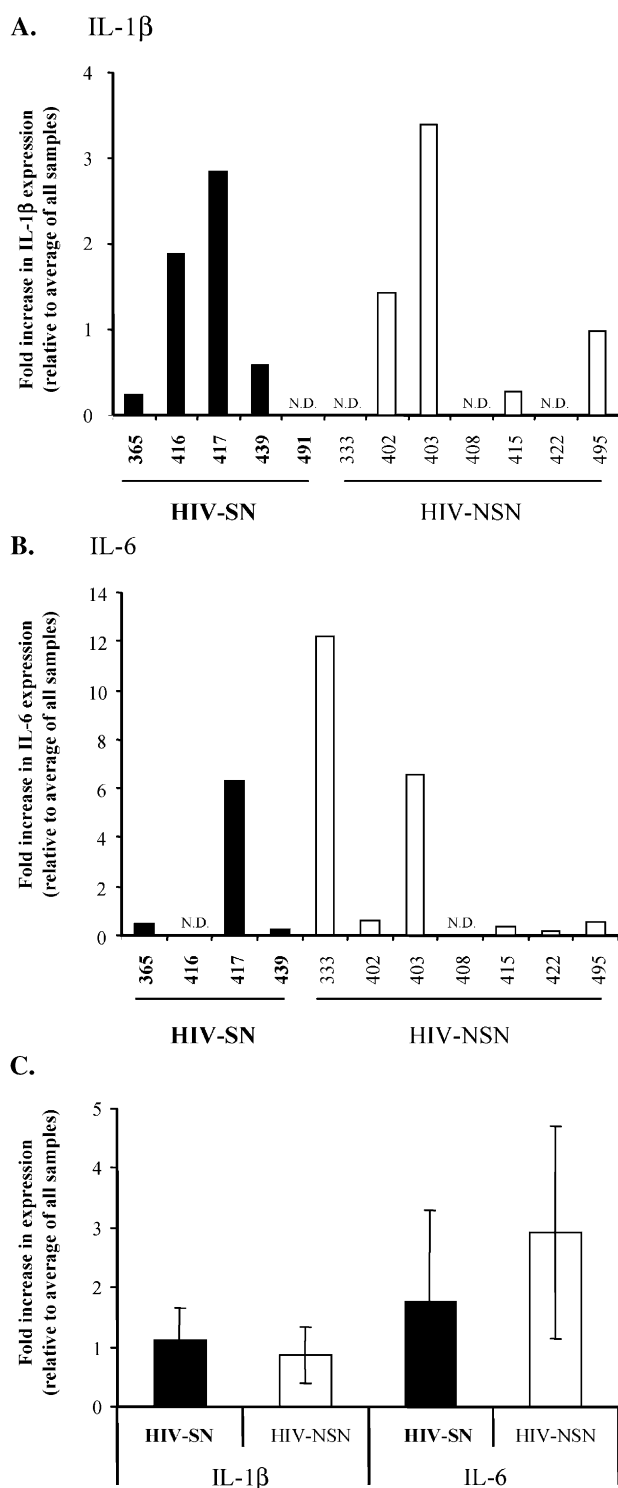
A. IL-1β

B. IL-6

C.

Fig. 7. Expression of mRNA for (A) IL-1β and (B) IL-6 in peripheral nerve tissue from HIV/AIDS patients with and without peripheral neuropathy (HIV-SN and HIV-NSN, respectively). N.D. denotes specific transcript was not detected. (C) Pooled data from HIV-SN or HIV-NSN patients. Data represent mean values and standard errors of the mean. In samples where no specific transcripts were detected, the fold increase was assigned zero. (A, B and C) Data were normalized against the GAPDH mRNA level and fold increase in mRNA expression levels is given for each patient relative to the average value across all patients.

Discussion

While HIV-SN affects nearly a third of all HIV/AIDS patients, little is known about the extent or type of virus infecting the peripheral nervous system. The results presented herein demonstrate HIV-1 infection of peripheral nerves occurs in HIV/AIDS patients both with and without clinically evident neuropathy. Although HIV-1 genome was present in over half of the peripheral nerve samples analyzed, active viral replication was limited. All but two of the patients studied had prior exposure to some type of antiretroviral therapy and thus, it is likely that the restricted viral replication may have been due to virological suppression. Plasma HIV-1 RNA levels were not available for these patients because all of the samples were collected from autopsies performed in the pre-HAART era. Despite limited viral replication, nerve-C2V3 recombinant HIV-1 clones induced neurite degeneration, and to a lesser extent, a reduction in neuronal soma size in a DRG culture system. In addition, HIV-1 infection of DRG cultures induced expression of host pro-inflammatory cytokines, which were also detected in the peripheral nerve tissue from several HIV/AIDS patients. Thus, the present studies indicate that viral infection of peripheral nerve (despite low levels of replication) caused neuropathogenic effects in a viral sequence-dependent manner.

The present results also demonstrate that the majority of viruses detected in peripheral nerve were CCR5-dependent and exhibited macrophage tropism. These results reflect HIV-1 infection of the central nervous system, where the majority of viruses found in the brains of AIDS patients are CCR5-dependent and macrophage-tropic (Albright et al., 1999; Gorry et al., 2001; Ohagen et al., 2003; Power et al., 1998). Although our results in general support the concept that HIV-1 infection in the peripheral nerve is restricted to the resident and/or infiltrating macrophage population, we also demonstrated the presence of viruses that engaged both CXCR4 and CCR5. Recombinant HIV-1 expressing the C2V3 region from one of these patients (#417) replicated in PBMC but not in MDM, suggesting that in some individuals HIV-1 infection of peripheral nerve may also include infiltrating T-cells as we did not see any evidence of productive infection of Schwann cells.

We have developed a mixed cell in vitro culture system to investigate the neuropathogenic effects of HIV-1 infection on the peripheral nervous system. These cultures consisted of the cell types found in the DRG of HIV-1-infected patients, namely neurons, Schwann cells and macrophages. In contrast to other in vitro models that studied the effects of recombinant HIV-1 proteins on rat sensory neurons (Keswani et al., 2003), we believe that the transgenic rat model described herein allows us to investigate potential neurotoxic factors released from HIV-1-infected macrophages, which are present in the peripheral nerve of HIV-1-infected individuals, thereby recapitulating in vivo HIV-1 infection of the peripheral nervous system. Of interest, detection of

HIV-1 provirus in DRG cultures infected with the NL4-3 strain was unexpected, given that primary macrophages from these human CD4 and CCR5 transgenic rats had previously been shown to be refractory to entry with this isolate using a luciferase reporter assay (Keppler et al., 2002). Although not expressing human CXCR4, it is possible that the expression of rat CXCR4 in conjunction with human CD4 might allow for entry of CXCR4-dependent HIV-1 into rat macrophages. Indeed, it has previously been shown that transfection of rat CXCR4 into the human CD4+ glioma cell lines U373MG and U87MG rendered them permissive to infection with the HIV-1 NL4-3 isolate (Pleskoff et al., 1997). Flow cytometric analysis revealed a two-fold increase in the expression of both human CD4 and human CCR5 on monocytes from the transgenic rats described herein compared to human monocytes (data not shown). Thus, this higher level of CD4 expression may help to explain why the NL4-3 strain could unexpectedly infect macrophages derived from these transgenic animals. In addition, by using a sensitive nested PCR approach, we may have detected NL4-3 provirus in the macrophages of the DRG cultures that may have otherwise been missed using a luciferase reporter assay. While viral antigen (p24) was observed in both DRG macrophages and bone marrow-derived macrophages by immunofluorescence, secreted p24 was not detected in supernatants from the same cultures. This dichotomy may reflect a lack of sensitivity in ELISA used herein or alternatively a failure to release viral antigens from these cells. Indeed, it has been reported that HIV-1 infection of human fetal DRG cultures resulted in the expression of HIV-1 proviral DNA and structural proteins in the absence of assembly and release of mature virus (Kunsch and Wigdahl, 1991). Nonetheless, the limited viral production resembles our findings in autopsied nerves in HIV/AIDS patients, and moreover did not preclude damage to DRG neurons and their processes following infection. Thus, highly productive HIV-1 infection in the peripheral nerve may not be essential for the development of nerve disease. Indeed, we have recently demonstrated that neuronal cell death induced by supernatants from HIV-1-infected macrophages was independent of the replicative ability of the virus (Zhang et al., 2003).

The neuropathogenic effects of HIV-1 manifested herein as neurite retraction, which may represent an in vitro correlate of the 'dying back' axonal pathology observed in HIV-SN. The results presented here complement a recent study demonstrating recombinant HIV-1 gp120 induced neuritic degeneration in mixed in vitro cultures of rat DRG neurons and Schwann cells (Keswani et al., 2003). In this latter model, presumed gp120 ligation of CXCR4 on Schwann cells induced their production of RANTES, which in turn induced TNF-α production by DRG neurons to mediate neurotoxicity in an autocrine manner. In support, we observed activation of Schwann cells (as evident by increased GFAP mRNA expression) and up-regulation of TNF-α expression

following infection with several recombinant nerve-C2V3 HIV-1 clones. Our study extends the findings of Keswani et al. (2003) by indicating that, in addition to recombinant gp120 protein, live HIV-1 virus also induces neurite degeneration in DRG neurons in a CCR5-dependent manner. The finding that nerve-C2V3 recombinant HIV-1 clones from both HIV-SN (#365, 416 and 417c) and HIV-NSN (#402) induced neurite retraction raises the possibility that perhaps HIV-1 proteins other than the envelope may be involved in the development of peripheral neuropathy. In addition to the HIV-1 envelope protein, HIV-1 Nef (Trillo-Pazos et al., 2000), Vpr (Patel et al., 2000) and Tat (Zauli et al., 2001) proteins also demonstrate neurotoxicity.

Several studies of the central nervous system have led to the suggestion that HIV-1 neurotoxicity is mediated in part through the pro-inflammatory cytokine IL-1β. In vivo and in vitro studies in non-transgenic rats have led to the hypothesis that IL-1β produced by neurons in response to gp120 ligation of CXCR4 acts in an autocrine fashion to sensitize neurons to excitotoxicity mediated cell death (Bagetta et al., 1999; Corasaniti et al., 2001a, 2001b). In addition to neuronal-derived IL-1β, activated and/or HIV-1-infected macrophages may also serve as a potential source for IL-1β production. In our study, we show that neurite degeneration is associated with up-regulation of IL-1β expression in DRG cultures following infection with HIV-1. We also show that soluble host factors produced by nerve-C2V3 HIV-1-infected macrophages promoted neuronal cell death. Furthermore, we demonstrated the expression of IL-1β in peroneal nerve samples from HIV/AIDS patients. Of note, viral clones that caused cytokine regulation in the DRG in vitro model did not in all cases correlate to increased detection in vivo (e.g., NL-365); this may reflect the fact that for the in vitro data, cytokine expression is calculated relative to a mock-treated uninfected control, while for the in vivo data, cytokine expression is calculated relative to the average of all the HIV-1-infected samples. Thus, we would still expect the level of cytokine expression in the nerve tissue of patient 365 to be greater than that from uninfected individuals. Using immunohistochemistry, previous studies have demonstrated the presence of IL-6 in DRG (Yoshioka et al., 1994) from HIV/AIDS patients, similar to our findings of increased IL-6 mRNA in the peripheral nerves of HIV/AIDS patients. The exact role of IL-6 in pathophysiological processes of the nervous system is unclear. In the central nervous system, IL-6 plays a role in the promotion of neuronal survival (Hama et al., 1991) and protection against neuronal damage (Akenaya et al., 1995; Hirota et al., 1996; Ikeda et al., 1996). In contrast, direct injection of recombinant IL-6 into the sciatic nerve of rats induced inflammation and demyelination (Deretzi et al., 1999), while intrathecally administered IL-6 elicited touch evoked allodynia (DeLeo et al., 1996). Our in vitro DRG model may provide a valuable tool in elucidating the role of these

different cytokines in peripheral nerve degeneration, as there are few models of HIV-SN. Very recently, our group has developed an in vivo model of lentivirus infection in which feline immunodeficiency virus (FIV) was shown to cause sensory neuropathy (Kennedy et al., 2004). Of interest to the present study, peripheral nerve axonal injury and inflammation occurs in the FIV model, but similar to observations in autopsied human samples, there is not a significant loss in DRG neurons (Power and Zochodne, unpublished data). Thus, studies of HIV-SN can be extended in several infection models to investigate the neurotoxic effects of select anti-retroviral therapies and to test the efficacy of potential neuroprotective agents.

Materials and methods

Patient samples

Peroneal nerve tissue was obtained from twelve male HIV/AIDS patients at autopsy, five with HIV-SN (neuropathic signs of reduced ankle reflexes and distal sensory loss) and seven with no evidence of neuropathic signs or symptoms (HIV-NSN). Both age and the last CD4 count prior to autopsy were similar in the two groups, with a median age of 39 and 48.5 years and a median CD4 T-cell count of 24 and 27 cells/μl for the HIV-SN and HIV-NSN, respectively. All HIV-SN patients had exposure to antiretroviral therapy: three patients received AZT and ddI while two patients received AZT and ddC. Five of the seven HIV-NSN patients had exposure to antiretroviral therapy: four patients received AZT alone, while one patient received AZT, ddI, ddC and d4T. Unfortunately, plasma HIV-1 RNA levels were not available.

Cell cultures

293T cells (American Type Culture Collection), HeLa-CD4/CXCR4 (clone 1022) cells (Chesebro et al., 1991), HeLa-CD4/CXCR4/CCR5 cells (Platt et al., 1998), the human osteosarcoma cell lines GHOST CCR2b, GHOST Hi-CCR5 and GHOST CXCR4 and human cholinergic neuronal (LAN-2) cells were cultured as previously described (Zhang et al., 2003). The GHOST cell lines (catalogue numbers 3681, 3944 and 3685 respectively) were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAD, NIH. Human peripheral blood mononuclear cells (PBMC) were isolated from whole blood of healthy donors using Histopaque (Sigma-Aldrich, Oakville, Canada). Human monocyte-derived macrophages (MDM) were generated as follows: human monocytes were isolated from PBMC by adherence on poly-orthinate coated tissue culture plates, after which non-adherent cells were removed and the adherent cell population cultured in RPMI 1640 (Sigma-Aldrich) medium containing 10% FBS (Sigma-Aldrich), 10% L929 cell-conditioned medium, as a

source of macrophage colony-stimulating factor-1 (Tsutsui et al., 2004), and 1% penicillin-streptomycin (Sigma-Aldrich). Cultures were washed every 2–3 days until the cells exhibited macrophage morphology (Power et al., 1998), demonstrating greater than 98% purity for macrophages as assessed by CD68 immunoreactivity (unpublished observations). Rat bone marrow-derived macrophages were isolated from the pelvis and femurs of adult Sprague–Dawley rats transgenic for human CD4 and CCR5 (Keppler et al., 2002) using culture methods previously described (Tsutsui et al., 2004).

DNA/RNA isolation, PCR and sequencing

Genomic DNA was isolated from peroneal nerve biopsies via a phenol/chloroform extraction and ethanol precipitation procedure. Genomic RNA was isolated from nerve tissue using TRIzol (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. Total RNA was isolated, dissolved in diethylpyrocarbonate (DEPC)-treated water and used for the synthesis of cDNA. The HIV-1 C2V3 envelope region was amplified from genomic DNA or cDNA using a nested PCR protocol as previously described (Power et al., 1994). PCR fragments corresponding to the amplified C2V3 region (445 base pairs) were isolated from agarose gel and cloned into the pSL1180 vector (Amersham Biosciences Inc, Baie d'Urfe, Canada) using the StuI and NheI sites. All reagents were obtained from New England BioLabs Ltd. (Mississauga, Canada) and used following the manufacturer's specifications. Sequencing reactions and DNA phylogenetic analysis was performed as previously reported (Van Marle et al., 2002). HIV-1 co-receptor usage was investigated by analyzing the V3 loop charge and amino acid residues as previously described (Briggs et al., 2000). Using this procedure, viral isolates can be predicted to be (i) CCR5-dependent; (ii) CCR5/CXCR4 dual tropic; or (iii) CXCR4-dependent.

Construction of recombinant infectious HIV-1 clones

Recombinant HIV-1 clones containing the C2V3 region of nerve-derived envelope sequences (herein to be known as nerve-C2V3 recombinant HIV-1) were constructed in the genomic backbone of the molecular clone pNL4-3 as previously described (Zhang et al., 2001). To generate infectious virus, 293-T cells were transfected with 2 μg of plasmid DNA of the recombinant HIV-1 clones using FuGENE6 (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's protocol. Culture supernatants were harvested 3 days post-transfection and cleared of cellular debris by low-speed centrifugation. Virus stocks were expanded by co-culture of the 293-T cell transfection supernatants with PBMC, stimulated 3 days previously with 5 μg/ml of concanavalin A, and maintained in RPMI 1640 medium with 10% FBS and 100 IU/ml interleukin-2 (IL-2). Culture supernatants were removed at

days 3, 7 and 10 post-infection, cleared of cellular debris by low-speed centrifugation and viral protein levels were quantified using the HIV-1 p24 Antigen Capture Assay Kit (AIDS Vaccine Program, National Cancer Institute, Frederick, MD.). Viral stocks were stored at −80 °C until required.

Chemokine co-receptor utilization

The chemokine co-receptor utilization of the recombinant HIV-1 strains was investigated using the previously described luciferase reporter virus infection assay (Jian and Zhao, 2003). Using FuGENE6 reagent, the molecular clones of the recombinant HIV-1 strains were co-transfected into 293-T cells along with a plasmid expressing the firefly luciferase gene within an *env*-inactivated HIV-1 clone (pNL-Luc-E$^-$R$^-$; obtained through the AIDS Research and Reference Reagent Program, catalogue number 3418). Culture supernatants were collected 3 days post-transfection, cleared of cell debris by low-speed centrifugation and used to infect cell lines expressing different chemokine receptors. Infection of the target cells by the pseudotyped virus led to luciferase expression, which was quantified in cell lysates 2 days post-infection using the Luciferase Assay Kit (BD Biosciences, Mississauga, Canada) following the manufacturer's instructions. In addition to expressing distinct chemokine receptors, the GHOST cell lines are also stably transfected with a construct expressing humanized green fluorescent protein (hGFP) under the control of the HIV-2 LTR (HIV-1 infection of these cells can be detected through induction of the hGFP gene). The GHOST cell lines were seeded overnight in 16-well chamber slides (Nalgene Nunc International, Naperville, IL), infected with the recombinant HIV-1 strains and cultured for 3 days, following which cells were fixed with 4% formalin and washed with PBS. Slides were mounted and induction of hGFP expression analyzed via fluorescent microscopy using a Zeiss Axioskop 2 upright microscope (Oberkochen, Germany).

Dorsal root ganglia (DRG) cultures

DRGs were harvested from previously described adult Sprague–Dawley rats transgenic for human CD4 and CCR5 (Keppler et al., 2002). DRG were cleared of connective tissue, dissected into small pieces and digested for 90 min at 37 °C in DMEM (Sigma-Aldrich) containing 1 mg/ml collagenase (Sigma-Aldrich), 0.5% trypsin (Gibco BRL, Burlington, Canada) and 0.1 mg/ml DnaseI (Roche Diagnostics Corporation). Cells were harvested via centrifugation, re-suspended in DRG medium (DMEM containing 10% FBS, 5% horse serum (Gibco BRL), 5% L929 cell-conditioned medium, 1% penicillin-streptomycin, 2 mM L-glutamine and 1% N-2 supplement (Gibco BRL)), and plated in either 8-well chamber slides or 24-well tissue culture plates (Nalgene Nunc International), pre-coated with

a 1:2 dilution of Matrigel (BD Biosciences) in DMEM. Culture medium was replaced after 24 h, and then every 2–3 days for the next week. DRG cultures were infected with standardized viral inputs (HIV-1 p24 5 ng per culture) for 6 h, following which the cultures were washed to remove input virus and cultured in DRG medium for a further 4 days, with medium replenished at day 2. As the recombinant HIV-1 clones were grown in activated PBMC, DRG cultures were treated with supernatant from activated but uninfected PBMC (mock) as controls.

Immunocytochemistry

At day 4 post-infection, DRG cultures were washed with PBS, fixed with 95% ethanol and blocked overnight at 4 °C with PBS containing 50% normal goat serum (NGS). After removal of the blocking reagent the cells were incubated overnight at 4°C with either mouse anti-MAP-2 (clone HM-2, 1:1000 dilution, Sigma), mouse anti-ED-1 (1:200 dilution, Chemicon International), rabbit anti-Iba-1 (1:500 dilution, Wako Chemicals), rabbit anti-GFAP (1:400 dilution, Dako), mouse anti-human CD4 (1:100 dilution, Dako), mouse anti-human CCR5 (clone 2D7, 1:100 dilution, BD Biosciences) or HIV-1$_{SF2}$ p24 rabbit antiserum (1:1000 dilution, obtained through the AIDS Research and Reference Reagent Program, catalogue number 4250). Following primary antibody staining, the cells were washed in PBS and incubated with either Cy3-conjugated goat anti-mouse (1:2000 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA) or Alex Fluor-488-conjugated goat anti-rabbit (1:2000 dilution, Molecular Probes) secondary antibodies. In addition, cultures labeled with rabbit anti-Iba-1 were incubated with a horseradish peroxidase-conjugated secondary antibody, and the cells were developed with 3,3′-diaminobenzidine as the substrate. All antibody dilutions were made with PBS containing 10% NGS and 0.1% Triton X-100 (Sigma). Slides were mounted with Gelvatol and viewed using a Zeiss Axioskop 2 upright fluorescent microscope. For the DRG morphological studies, digital images of MAP-2+ neurons from 12 fields of view per well were captured using the Advanced Spot system (Diagnostic Instruments, Sterling Heights, MI) and quantitative analysis of neuron cell soma size and neurite lengths were performed using the Scion Image program (Scion, Frederick, MD).

Real time RT-PCR

24 h post-infection, DRG cultures were washed with PBS, lysed in TRIzol and genomic DNA and RNA isolated in accordance with the manufacturer's guidelines. Total RNA was isolated, dissolved in DEPC-treated water and used for the synthesis of cDNA. The primers used in the real time PCR were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Silva et al., 2003); GFAP (Latour et al., 2003); IL-1β, forward primer 5′-CCA AAG AAG AAG ATG GAA AAG CG-3′ and reverse primer 5′-GGT

GCT GAT GTA CCA GTT GGG-3′ (Tm 58 °C); TNF-α, forward primer 5′-CTA TCT GGG AGG GGT CTT CC-3′ and reverse primer 5′-GGT TGA GGG TGT CTG AAG GA-3′ (Tm 58 °C); IL-6, forward primer 5′-ACC CCT GAC CCA ACC ACA AAT-3′ and reverse primer 5′-AGC TGC GCA GAA TGA GAT GAG-3′ (Tm 55 °C); β-actin forward primer 5′-GGA TGC AGA AGG AGA TCA CTG-3′ and reverse primer 5′-CGA TCC ACA CGG AGT ACT TG-3′ (Tm 56 °C). Semiquantitative analysis was performed by monitoring in real time the increase in the fluorescence of the SYBR-green dye (Molecular Probes) on a Bio-Rad i-Cycler (Bio-Rad Laboratories, Hercules, CA). Proper amplification was confirmed by performing melting-curve analysis. Real-time fluorescence measurements were performed and a threshold cycle value for each gene of interest was determined, as previously reported (Power et al., 2003). All data were normalized against the GAPDH or β-actin mRNA level and expressed as fold increases relative to the mock control.

In vitro indirect neurotoxicity assay

Bone marrow-derived macrophages from rats transgenic for human CD4 and CCR5 were infected with nerve-C2V3 recombinant viruses (HIV-1 p24 5 ng per culture). Day four cell culture supernatants were cleared of cellular debris by low speed centrifugation and applied to differentiated human cholinergic neuronal (LAN-2) cells, as previously described (Zhang et al., 2003). After 24 h of exposure, LAN-2 cell death was quantified via trypan blue exclusion.

Statistical analysis

Statistical analysis was performed using GraphPad InStat version 3.01 software. The effect of HIV-1 infection on neurite length and neuronal soma size was examined using z score analysis. z scores were calculated for each experiment relative to the mock control group. In this way, the z score denotes how far and in which way the neurite lengths and neuronal soma size for the virus treated cultures deviate from the mean values of the mock controls, and is expressed in units of the standard deviation for the mock control values. Statistical analysis was performed on the pooled z scores from three independent experiments.

Acknowledgments

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