Journal of NeuroVirology (1998) 4, 619–626

http://www.jneurovirol.com

Cocaine opens the blood-brain barrier to HIV-1 invasion

Ling Zhang[1], David Looney[3], Dennis Taub[4], Sulie L Chang[5], Dennis Way[6], Marlys H Witte[6], Michael C Graves[1] and Milan Fiala[2]

Departments of [1]Neurology and [2]Medicine, UCLA School of Medicine, Los Angeles, California 90095-1769; [3]Infectious Diseases 9-111F, VA San Diego Healthcare Center, San Diego, California 92161; [4]National Institute on Aging, Baltimore, Maryland 21224-2780; [5]Department of Biology, Seton Hall University, South Orange, New Jersey 07079-2694; and [6]Department of Surgery, University of Arizona, Tucson, Arizona 85724-5063, USA

Cocaine abuse has been associated with vasculitis and stroke, and is suspected to influence the progression of AIDS dementia. Cocaine may enhance HIV-1 neuroinvasion by actions directed at the blood-brain barrier. HIV-1 appears to penetrate the human brain microvascular endothelial cell barrier by a paracellular route breached by tumor necrosis factor-α (TNF-α). Cocaine's effects on the blood-brain barrier were investigated using human brain microvascular endothelial cells and peripheral blood monocytes. Cocaine (10^{-5} M and 10^{-6} M) increased molecular permeability of the barrier and viral invasion by the macrophage-tropic HIV-1_{JR-FL} into the brain chamber. Cocaine also augmented apoptosis of brain endothelial cells and monocytes, increased secretion of four chemokines (interleukin-8, interferon-inducible protein-10, macrophage inflammatory protein-1α, and monocyte chemoattractant protein-1) and the cytokine, TNF-α, by human monocytes. TNF-α enhanced invasion of the brain compartment by macrophage-tropic, lymphotropic, and bitropic HIV-1 strains. These data indicate that HIV-1 neuroinvasion can be increased by (a) cocaine's direct effects on brain microvascular endothelial cells and (b) paracrine effects of cocaine-induced pro-inflammatory cytokines and chemokines on the blood-brain barrier.

Keywords: cocaine; HIV-1; apoptosis; endothelial cells; tumor necrosis factor-α; macrophage inflammatory protein-1α; monocyte chemoattractant protein-1

Introduction

Immunological consequences of cocaine abuse on the blood-brain barrier (BBB) are poorly understood, but appear significant since cocaine abuse is associated with vasculitis (Mody *et al*, 1988; Daras *et al*, 1994), suggesting an enhancement in leukocyte migration through endothelial barriers. Cocaine abuse is also accompanied by changes detected by brain imaging, indicating a defect in the BBB (Holman *et al*, 1992; Strickland *et al*, 1993). Cocaine may induce brain damage by its toxicity to neurons, as shown *in vitro* by synergy with HIV-1 (Koutsilieri *et al*, 1997), and by enhancement of HIV-1 replication in mononuclear cells (Bagasra and Pomerantz, 1993). The importance of drug abuse in the AIDS epidemic is highlighted by a recent report noting the history of drug abuse in a majority of HIV-1 infected persons, including those with HIV-1 encephalitis who were autopsied by the Medical Examiner of New York City (Kibayashi *et al*, 1996). However, epidemiological studies of cocaine's role in AIDS dementia have produced highly variable results in different populations (Bacellar *et al*, 1994; Wellman, 1992; Handelsman *et al*, 1992). This discrepancy may, in part, be a reflection of the variability of cocaine's immunomodulating effects on induction of pro-inflammatory cytokines and chemokines (Fiala *et al*, 1996a).

Our previous work indicated that: (a) in cocaine-addicted individuals, cocaine infusion has divergent effects of secretion of pro-inflammatory cytokines, which appear to be heterogeneous for mononuclear cells from different donors, with inhibitory effects seen in macrophages and stimu-

Correspondence: M Fiala, M.D.
Received 7 April 1998; revised 26 June 1998; accepted 23 July 1998

latory effects in lymphocytes, and (b) tumor necrosis factor-α (TNF-α) increases HIV-1 penetration across a human BBB model (Fiala *et al*, 1996a). We have recently shown that *in vivo* cocaine infusion stimulates secretion of interferon-γ (IFN-γ), and probably TNF-α, in mononuclear cells of a majority of addicted subjects (Gan X-H *et al*, 1998 (manuscript submitted)), and have demonstrated that TNF-α does indeed increase HIV-1 penetration across a human BBB model (Fiala *et al*, 1997). We have also shown that cocaine enhances monocyte migration across human brain microvascular endothelial cells (BMVEC) in the BBB model. These effects of cocaine appear to be mediated by increased expression of cell adhesion molecules (Gan X-H *et al*, 1998 (manuscript submitted)). Taken together, the data suggest that cocaine may increase HIV-1 neuroinvasion by its effect on migration of virus-infected monoyctes, and by induction of TNF-α and other pro-inflammatory cytokines which can open the blood-brain barrier to cell-free virus. The data presented in this report demonstrate cocaine's stimulatory effects on apoptosis of brain endothelial cells and monocytes, and on induction of chemokines and pro-inflammatory cytokines, such as TNF-α, which can modify the integrity of the BBB and increase HIV-1 neuroinvasion.

Results

We have shown that cocaine can induce adhesion molecules, and enhance transendothelial migration of leukocytes (Gan X-H *et al*, 1998 (manuscript submitted)), which may disturb the function of the BBB. The *in vivo* effects of cocaine on the BBB could also be mediated by cocaine's direct actions on endothelial cells, and by paracrine responses to cytokines induced by cocaine. We have, therefore, investigated cocaine's effects on endothelial cell permeability and apoptosis as well as cocaine's induction of chemokines and cytokines.

Cocaine's enhancement of endothelial permeability
Experiments in the *in vitro* BBB model demonstrated that cocaine has direct effects on BMVEC permeability. Treatment of the BBB model with cocaine (10^{-5} M to 10^{-6} M) resulted in a significant dose-dependent increase in permeability to both inulin and dextran (Figure 1).

Cocaine's induction of apoptosis
Cocaine has previously been reported to induce apoptosis of thymocytes (Wu *et al*, 1997). We examined cocaine's induction of apoptosis in cultures of BMVEC and in monocytes using an enzyme-linked immunosorbent assay (ELISA) of generated nucleosomes. As shown in Figure 2, cocaine induced apoptosis in both cell types in a

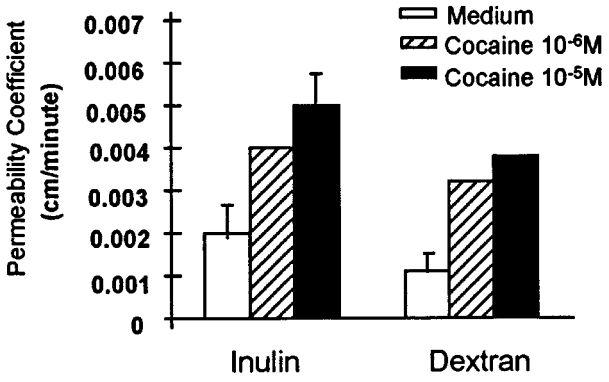

Figure 1 Effect of cocaine on permeability of the BBB model. Replicate models were treated for 4 h with cocaine at the indicated concentration in the medium of the upper chamber. After 4 h, the coefficient of permeability was determined using [^{14}C]-methylated inulin (M.W. 5000) or ^{14}C-labeled dextran (M.W. 70 000) (Sigma Chemical Company, St. Louis, MO, USA) as described in Materials and methods.

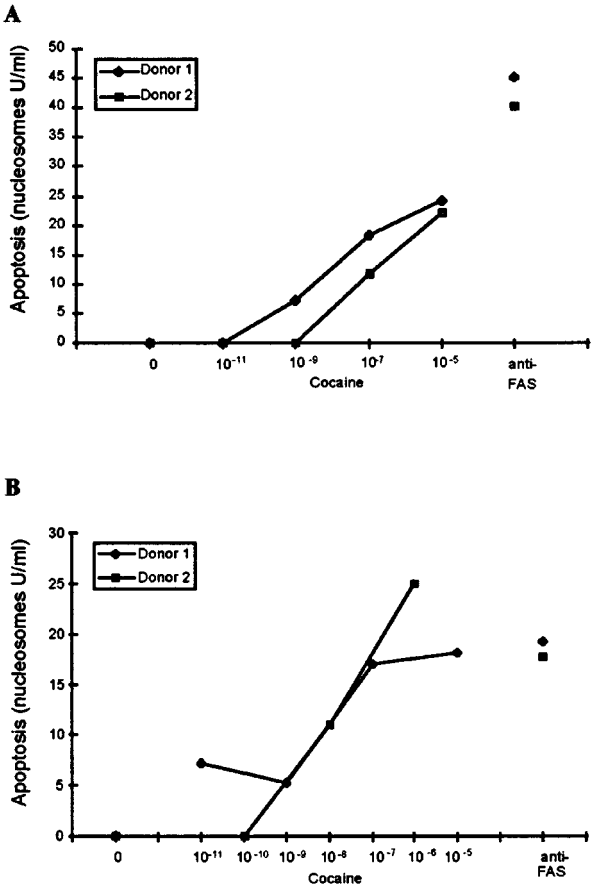

Figure 2 Cocaine's effects on induction of apoptosis in (**A**) brain endothelial cells, and (**B**) monocyte cultures. BMVEC or peripheral monocytes were incubated in presence of indicated concentrations of cocaine for 24 h at 37°C. Apoptosis was evaluated using a Nucleosome ELISA kit (Oncogene Research Products, Cambridge, MA 021420, USA) as described in Materials and methods.

dose-dependent fashion. The effect of 10^{-5} M cocaine on apoptosis of monocytes was comparable to that seen following an anti-FAS antibody response.

Cocaine's enhancement of chemokine secretion by monocytes

In preliminary experiments, we showed, in the BBB model, that the chemokines normally expressed and secreted by T-cells upon activation, RANTES, interleukin-8 (IL-8) and macrophage inflammatory protein-2 (MIP-2), strongly attract monocyte transmigration into the lower chamber of the model, and increase ionic permeability. Medium containing MIP-2 (50 ng/ml), a chemokine previously shown to cause a severe breach in the BBB (Bell *et al*, 1996), decreased electrical resistance in the model by 35%. We have now examined cocaine's induction of two C-X-C chemokines, IL-8 and interferon-inducible protein-10 (IP-10), and two C-C chemokines, macrophage inflammatory protein-1α (MIP-1α) and monocyte chemoattractant protein-1 (MCP-1), which were shown to be chemoactive in the murine central nervous system (Bell *et al*, 1996). Cocaine (10^{-9} M – 10^{-6} M) induced secretion of the two C-X-C chemokines, IP-10 and IL-8, and the two C-C chemokines, MIP-1α and MCP-1, into the supernatant of 48-h monocyte cultures, while higher cocaine concentrations (10^{-5} M – 10^{-4} M) inhibited secretion, so as to produce a bell-shaped dose-response profile (Figure 3). However, using monocytes from two other donors, cocaine was found to up-regulate MIP-1α, MCP-1, IP-10 and IL-8 secretion at the higher cocaine concentrations (10^{-4} M – 10^{-5} M) (data not shown).

Cocaine's enhancement of TNF-α secretion by monocytes

TNF-α plays a crucial role in AIDS dementia by stimulating HIV-1 expression in microglia (Chao *et al*, 1994) and macrophages (Poli and Fauci, 1992), reactivating HIV-1 infection in astrocytes (Fiala *et al*, 1996b), inducing astrocytosis (Wilt *et al*, 1995) and damaging the BBB. The effects of cocaine in HIV dementia may be mediated, in part, through TNF-α. Cocaine treatment of monocytes increased secretion of TNF-α in a dose-responsive fashion (10^{-5} M – 10^{-9} M) and potentiated the effects of LPS

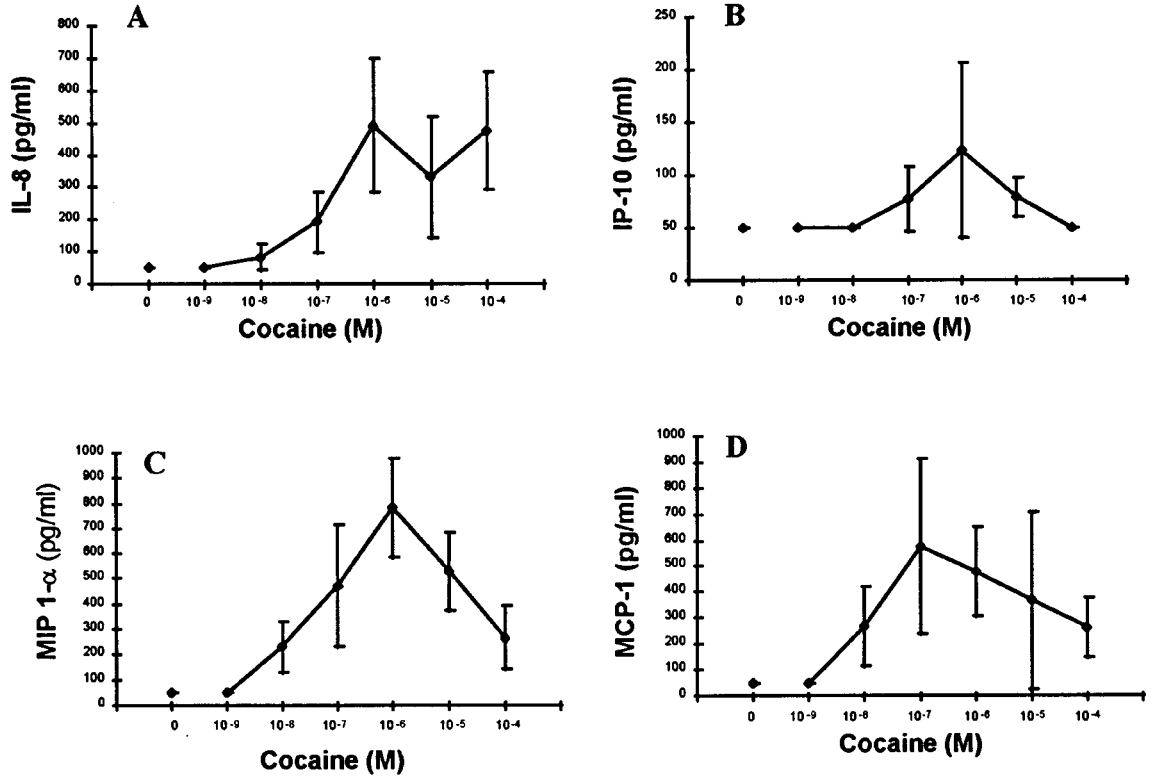

Figure 3 Cocaines effects on C-X-C chemokines (IL-8 and IP-10) and C-C chemokines (MIP-α and MCP-1) secretion by peripheral blood monocytes. Monocytes (400 000), suspended in RPMI 1640 with 10% autologous serum with or without cocaine at the indicated concentration, were incubated for 48 h. The supernatant medium was collected and stored frozen until the ELISA assay as described in Materials and methods. The results are expressed in pg/ml.

on monocyte TNF-α secretion twofold over baseline (Figure 4).

Enhancement of HIV-1 penetration across human BMVEC by TNF-α or cocaine

Previously, using the *in vitro* BBB model, we showed that the HIV-1_{JR-FL} strain penetrates the BMVEC/astrocyte bilayer via a paracellular route opened by TNF-α (Fiala *et al*, 1997). The effect of TNF-α is dose-dependent without an apparent threshold in the range of 1–100 ng/ml. The significance of this finding to human disease could be substantial if it is also found to occur with other HIV-1 strains. We, therefore, examined the effects of TNF-α on HIV-1 invasion in the BBB model using four different HIV-1 strains. Without treatment, the model functioned as a tight barrier against HIV-1, retaining approximately 99.5% of the viral RNA copies for 24 h.

TNF-α treatment enhanced the penetration of all strains tested: JR-FL, a monocytotropic strain, increased 5.6-fold; ADA, a monocytotropic strain, increased 2.2-fold; a 5.9-fold increase was seen with the 89.6, a dual-tropic strain; and IIIB, a lymphotropic strain, increased 3.8-fold (Table 1).

Cocaine (10^{-5} M) treatment alone enhanced penetration of the HIV_{JR-FL} strain into the brain chamber up to 8.4-fold when replicate BBB models were treated for 24 h or 48 h (Table 2).

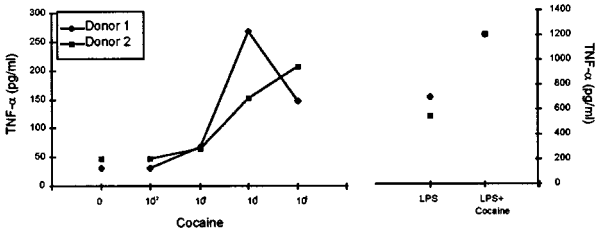

Figure 4 Effect of cocaine on TNF-α secretion by peripheral blood monocytes. Peripheral monocytes (400 000) from two donors were incubated in RPMI 1640 medium with 5% fetal calf serum for 24 h in the presence of various concentrations of cocaine or LPS. At the completion of the incubation, TNF-α concentration in the supernatant medium was determined by the ELISA assay as described in Materials and methods.

Discussion

Route of HIV-1 invasion across the human BMVEC/astrocyte barrier

Our experiments in the human BBB model are designed to experimentally study possible routes and molecular mechanisms of HIV-1 invasion across the BBB, and the role of factors, such as

Table 1 Effect of TNF-α on penetration of HIV-1 strains across the blood-brain barrier model.

HIV-1 strain	*Strain classification*	*Virus inoculum in upper chamber (RNA copies)*	*Virus penetration into lower chamber*[a] *without TNF-α (RNA copies)*	*with TNF-α*
JR-FL	Monocytotropic R5, R3, STRL33	1065 488	1574±127[b]	8856±1478[b]
ADA	Monocytotropic R5, R3, STRL33	1575 826	31 096	67 212
IIIB	Lymphotropic X4	783 361	10 144	38 920
89.6	Dual-tropic X4, R2b, R3, R5	1017 000	15 840±5059[b]	94 498±25 718[b]

[a]Replicate blood-brain barrier models were pre-treated with TNF-α or control medium for 4 h, when each HIV-1 strain was placed in the upper chamber at the indicated dose. After 24 h, an aliquot was removed from the lower chamber and the amount of virus was determined by the HIV-1 RNA assay. [b]Mean and s.e.m. of duplicate models.

Table 2 Effect of cocaine on penetration of macrophage-tropic HIV-1_{JR-FL} strain across the BBB model.

Virus inoculum in upper chamber (RNA copies)	*Pre-treatment of the upper chamber*[a]	*Virus penetration into lower chamber (RNA copies)* *24 h*	*48 h*
1065 488	Medium	1574+126[b]	31 948+1384[b]
1065 488	Cocaine (10^{-5} M)	13 176+4449[b]	74 398+8595[b]

[a]Replicate blood-brain barrier models were pre-treated with cocaine for 4 h, when HIV-1_{JR-FL} (1065 488 RNA copies) was placed in the upper chamber. After 24 and 48 h, an aliquot was removed from the lower chamber and the amount of virus was determined by the HIV-1 RNA assay. [b]Mean and s.e.m. of duplicate models

cocaine, in AIDS. Early after the primary HIV-1 infection, the individual's plasma contains enormous concentrations of cell-free HIV-1 (Piatak *et al*, 1993). HIV-1 RNA levels in the cerebrospinal fluid (CSF) appear to correlate with those in the plasma (Ellis *et al*, 1997; McArthur *et al*, 1997; Roberston *et al*, 1998). This relationship, however, was observed by Ellis *et al*. (1997) in subjects with CD4+ counts $>200/mm^3$, whereas McArther *et al* (1997) found this correlation in those with counts $<200/mm^3$, and Roberston *et al* (1998) noted this correlation in patients at various stages of HIV disease. The role of HIV-1-infected CD4+ lymphocytes in the pathogenesis of early HIV-1 encephalitis (Massari *et al*, 1990) is supported by early stage-specific lymphocytic pleocytosis (McArthur *et al*, 1989; Elovara *et al*, 1988) and perivascular lymphocytic infiltrates in the brain (Gray *et al*, 1992). The virus in the CSF of the subjects examined by Ellis *et al* (1997) was, however, cell-free virus. A previous study also noted that HIV-1 isolated from the CSF was found in the supernatant rather than in the cellular fraction (Spector *et al*, 1993) suggesting that the CSF/plasma relationship is attributable to cell-free virus, and might be indirectly related to lymphocytic pleocytosis.

As demonstrated previously and herein, cell-free macrophage-, lympho- and bi-tropic HIV-1 strains can penetrate across an *in vitro* endothelial barrier using a route opened by TNF-α. This response to TNF-α is specific to human endothelial cells, specifically tight brain microvascular endothelial cells. Other endothelial cells present a weaker barrier to HIV-1 penetration, and astrocytes present no significant barrier to penetration (Fiala *et al*, 1997). Most viral isolates in early infection are macrophage-tropic; yet our human BMVEC were not susceptible to infection with a macrophage-tropic HIV-1_{JR-FL} strain (Fiala *et al*, 1997). Other investigators have shown HIV-1 infection of human BMVEC, but only by lymphotropic strains (Moses *et al*, 1993). These observations would argue against HIV-1 infection of BMVEC as a mechanism of early neuroinvasion analogous to the simian immunodeficiency virus (SIV) infection of the simian brain endothelial cells (Edinger *et al*, 1997). Nevertheless, using a combination of *in situ* DNA polymerase reaction, reverse transcriptase-initiated *in situ* PCR and immunohistochemistry, HIV-1 mRNA expression was demonstrated within microvascular endothelial and choroid plexus cells in the brain from AIDS dementia patients (Bagasra *et al*, 1996), suggesting that the endothelial cells can be infected by lymphotropic strains in neuro-AIDS.

The paracellular route opened by TNF-α and possibly cocaine for cell-free plasma virus could be of considerable significance for HIV-1 neuroinvasion in early HIV-1 disease. Additionally, lymphocytes could be major participants in early neuroinvasion, indirectly mediating an opening in the BBB during lymphocyte migration, which could be augmented by cocaine's enhancement of the transendothelial migration of leukocytes (Gan X-H *et al*, 1998 (manuscript submitted)). In late HIV-1 disease, in patients with AIDS dementia, the 'Trojan horse' transport of HIV-1 by monocytes and T lymphocytes is believed to be the major route of neuroinvasion (Gendelman *et al*, 1997), which could also be augmented by cocaine. These different mechanisms may overlap in successive stages of AIDS, as reflected by a gradual increase in immune stimulation noted in the CSF (Gisslén *et al*, 1994).

Role of cocaine in cell-free HIV-1 invasion across the human BMVEC barrier

Similar to TNF-α, cocaine was shown to increase HIV-1 penetration across the BBB model. Cocaine's effects on the BBB appear complex including both direct, pro-apoptotic effects on endothelial cells, and indirect effects mediated by chemokines and cytokines. Although the relative significance of these molecular mechanisms for HIV-1 neuroinvasion is not established, TNF-α may play a critical role in cocaine's actions since it was shown to open the BBB for all macrophage-tropic, T-cell-tropic and bi-tropic HIV-1 strains. In addition, cocaine's direct effects on BMVEC may help to clear the way for HIV-1_{JR-FL} penetration.

Role of chemokines induced by cocaine and chemokine receptors in HIV-1 invasion

Cocaine stimulates human monocytes to secrete chemokines, some of which breach the BBB *in vivo*, as demonstrated by horseradish peroxidase staining (Bell *et al*, 1996). The potential role of chemokine receptors in retroviral neuroinvasion has recently produced considerable interest. SIV infection of simian brain endothelial cells was shown to be mediated via a chemokine receptor CCR5-dependent mechanism, which was inhibited by the CCR5 ligand, RANTES (Edinger *et al*, 1997). The C-C chemokines, RANTES, MIP-1α, and MIP-1β, were initially hailed as potential therapeutic agents for AIDS (Cocchi *et al*, 1995). Their role, however, appears to be cell-type dependent-inhibitory to primary strains in T lymphocytes and stimulatory in macrophages (Schmidtmayerova *et al*, 1996). The major physiological role for the chemokines is as participants in the inflammatory response, aiding leukocyte trafficking across the vascular wall. The part that chemokines play in HIV-1 neuroinvasion may, therefore, be a double-edged sword. Chemokines could block chemokine receptors, which are putative HIV co-receptors, on brain endothelial cells, but they could also open the BBB to HIV-1 invasion.

Cocaine is considered a significant co-factor in AIDS, but its mechanisms are poorly understood. Since cocaine was shown to induce monocytic secretion of two C-X-C and two C-C chemokines,

as well as TNF-α, and inasmuch as chemokines can breach the BBB *in vivo*, as shown by horseradish peroxidase staining (Bell *et al*, 1996), it seems likely that cocaine can have a significant impact on HIV-1 neuroinvasion.

Materials and methods

Chemicals
(−)-cocaine was provided by the Research Triangle Institute (Research Triangle Park, NC, USA) through the Research Technology Branch of the National Institute on Drug Abuse, and was found to be negative (less than 10 pg/ml) for endotoxin by the Limulus Lysate Test (Sigma, St. Louis, MO, USA).

Cell culture and peripheral blood monocytes
The human blood-brain barrier model was constructed using adult human brain microvascular endothelial cells and human astrocytes on opposite sides of a porous Cyclopore membrane coated with collagen I and fibronectin (Collaborative Biomedical Products, Bedford, MA, USA) and cultured in DME/F12 (Irvine Scientific, Santa Ana, CA, USA), with 10% fetal calf serum and endothelial cell growth supplement (Upstate Biotechnology, Lake Placid, NY, USA) as described previously. Human brain microvascular endothelial cells were prepared from adult cortical tissues obtained during surgery (Fiala *et al*, 1997).

Human monocytes were separated from the elutriated peripheral blood of leukopheresed healthy volunteers who had provided informed consent under a protocol approved by the Institutional Review Board. The mononuclear cells were subsequently passed through a continuous Percol gradient to enrich for $CD14^+$, $CD3^-$, $CD56^+$, $CD19^+$ mononuclear cells. The monocyte preparations were greater than 90% monocytes flow cytometry. Monocytes by were cultured in RPMI 1640 with 10% autologous serum with or without cocaine for 48 h for the chemokine assays, or for 24 h for the TNF-α assay. At the endpoints, the medium was separated by brief centrifugation and frozen at −70°C until the ELISA assay was performed.

Virus strains
HIV-1 strains were classified on the basis of their use of chemokine receptors as proposed recently (Berger *et al*, 1998). The following HIV-1 strains were used: HIV_{JR-FL}, a monocytotropic R5 (Deng *et al*, 1996), R3 (He *et al*, 1997) and STRL33 (Liao *et al*, 1997) strains (1.06×10^{11} RNA copies/ml); ADA, a monocytotropic R5 (Deng *et al*, 1996), R3 (He *et al*, 1997) and STRL33 (Liao *et al*, 1997) strains (1.26×10^{11} RNA copies/ml); 89.6, a dual-tropic X4, R2b, R3, R5 (Doranz *et al*, 1996),and STRL33 (Liao *et al*, 1997) strain (2×10^{11} RNA copies/ml); and IIIB, a T-cell tropic X4 virus (Feng *et al*, 1996) (1.56×10^{10} RNA copies/ml). HIV_{JR-FL} stock was prepared in human cord umbilical cells, as described previously (Fiala *et al*, 1997), whereas the other strains were grown in normal human donor PBMC at the UCSD Center for AIDS Research.

HIV-1 RNA assay
HIV-1 RNA was quantitated in supernatants diluted 1 : 4 with PBS, using the Amplicor HIV Monitor Test, performed according to the manufacturer's suggested procedure (Fiala *et al*, 1997).

Apoptosis assay
Apoptosis quantitation was performed using the Nucleosome ELISA kit (Oncogene Research Products, Cambridge, MA, USA) which quantitates mono- and oligo-nucleosomes in treated cells by DNA affinity-mediated capture of free nucleosomes, followed by ELISA detection of the histone component. As positive controls, cells treated with anti-FAS antibody or phorbol-12-myristate-13-acetate (10 μg/ml) (Calbiochem) with phytohemagglutinin A (50 ng/ml) were assayed. The extracts for ELISA testing were prepared by cell centrifugation at 1000 r.p.m. (200 × g), followed by resuspension of the cell pellet in lysis buffer. Sample diluent was added to the final concentration of 1×10^6 cells/ml, and the cells were incubated for 30 min on ice, centrifuged at 3000 r.p.m. (1500 × g) for 10 min at 4°C, and the resulting lysate was frozen at −20°C. The Nucleosome ELISA was performed according to the manufacturer's instructions, and the Nucleosome standards (10, 3.3, 1.1 and 0.366 U/ml) were run with each assay. The quantitation of nucleosomes was expressed in nucleosome units (One unit=number of nucleosomes from 444 UV-treated Daudi cells/ml).

Molecular permeability
Inulin permeability was measured by adding [^{14}C]-methylated inulin (M.W. 5000) or ^{14}C-labeled dextran (M.W. 70 000) (Sigma Chemical Company, St. Louis, MO, USA), to the upper chamber of the BBB model for 30 min. Radioactivity was determined by scintillation counting in the lower chamber. The permeability coefficient was calculated using the formula:

{V/(A*D)}*{ΔR/ΔT} where V=volume of the receptor chamber (1.0 cm^3), A=area of the cell monolayer (0.36 cm^2), D=initial amount of the marker molecule in the donor chamber, ΔR/ΔT=amount of the marker molecule in the receiver solution (ΔT=4 h).

ELISA for chemokines and TNF-α
Human MIP-1α, MCP-1, IL-8, IP-10 and TNF-α levels were determined using Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA), following the manufacturer's instructions.

Acknowledgements

This work was supported by NIH grant, DA 10442, and a subcontract of NS 26126 to MF, DA 07058 to SLC, and HL 48493 to MHW by the UCSD Center for AIDS Research, DAIDS 2 P30 AI36214.

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