Neurology 2003;60:307-314

Oxidative stress in HIV demented patients and protection ex vivo with novel antioxidants

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Article Abstract

Objective: To determine the role of oxidative stress in mediating HIV dementia and to identify novel therapeutic compounds that may block this oxidative stress.

Methods: Brain tissue from patients with HIV encephalitis and macaques with simian immune deficiency virus encephalitis was immunostained for lipid peroxidation. Oxidized proteins in CSF of patients with various stages of HIV dementia were quantitated and we determined whether CSF from these patients could alter mitochondrial function. Several novel compounds with antioxidant effects were screened to determine their relative efficacy in protecting against CSF-induced neurotoxicity.

Results: Evidence for oxidative stress was present both in brain and in CSF. The presence of oxidized proteins in the CSF and CSF-induced progressive decrease in mitochondrial activity correlated with the severity of cognitive impairment, but only the group of patients with moderate to severe dementia

reached statistical significance. L-deprenyl, didox, imidate, diosgenin, and ebselen blocked the CSF-induced toxicity. No effect of trimidox, ruthenium red, or Quercetin was seen.

Conclusions: Increased oxidative stress is present in brain and CSF of HIV-infected patients. There is also an accumulation of toxic substances in the CSF that are capable of inducing oxidative stress. The authors have identified several novel compounds that are capable of blocking the CSF-induced toxicity, the therapeutic potential of which is worthy of further exploration.

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Much attention has been given recently to oxidative stress in the setting of HIV infection. Serum lactate levels are frequently elevated,[1] suggesting mitochondrial dysfunction; however, the role of oxidative stress in HIV pathogenesis remains uncertain. It also remains unknown whether oxidative stress plays a role in the pathogenesis of HIV dementia. Certainly oxidative stress seems to play an important role in the pathogenesis of other neurodegenerative diseases[2,3] and antioxidants may alter the course of progression of AD.

Recognition of dementia as an important consequence of HIV infection and the realization of its socioeconomic impact has led to the establishment of large multicenter research groups to evaluate the efficacy of antiretroviral or neuroprotective drugs in this patient population. However, the rationale for the usage of neuroprotective medications is based on their effects on neurotoxicity of HIV proteins or their efficacy in other neurodegenerative diseases thought to have similar underlying pathogenic mechanisms. Currently no biologic assays exist for monitoring the efficacy of these agents in HIV-infected patients. There is also a need to develop biologic ex vivo assays to screen drugs that may potentially be useful in treating patients with HIV dementia. It has become apparent that functional neuronal impairment without cell death may occur in patients with HIV infection. Several viral proteins, cytokines, and other neurotoxins implicated in the neuropathogenesis of HIV infection also cause mitochondrial impairment in neurons.[4] We thus determined whether oxidized proteins and lipids could be found in the brain and CSF of HIV-infected individuals and whether CSF from patients with HIV dementia can cause mitochondrial impairment in neuronal cultures. We also screened several novel compounds known to have antioxidant properties. Although structurally unrelated, most of these compounds alter the intracellular redox state via different mechanisms and actions. However, the potential role of most of these drugs as neuroprotective agents has not been studied before. We thus determined their relative efficacy in protecting against CSF-induced neurotoxicity.

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Tissue samples and immunostaining. Paraffin-embedded brain sections from basal ganglia and hippocampus of five adult patients with HIV encephalitis with dementia (two had severe dementia and three had moderate dementia), five with

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HIV infection without encephalitis (two patients had minor cognitive impairment, three were normal), and five with no HIV infection (three patients died of bacterial sepsis, one of cirrhosis, and one of pneumonia) were stained as described below. Postmortem intervals varied between 6 and 17 hours. All groups were age matched with a range of 29 to 57 years. All human tissue samples were obtained from the national neuro-AIDS tissue consortium. Brain tissue samples were obtained from three macaques infected with a chimeric strain of HIV and simian immune deficiency virus (SHIV) that developed encephalitis. An uninfected macaque was used as a control. Five micron thick, paraffin-embedded, formalin-fixed sections from the temporal lobe and basal ganglia were immunostained with a well-characterized monoclonal anti-4-hydroxynonenal (HNE)/protein complex antibody 1g4h7 (1:100).[5] A standard immunohistochemistry protocol was used as described previously.[6]

CSF samples. All CSF samples were centrifuged and cell free CSF was aliquoted and immediately frozen at -70 °C until further testing. Severity of dementia was categorized in the HIV-infected patients using the Memorial Sloan Kettering Scale (MSK). The patients were divided into three groups: no dementia (MSK = 0; n = 16), mild dementia (MSK = 0.5 or 1; n = 22), and moderate to severe dementia (MSK = 2 or 3; n = 8). CSF from patients with headaches or degenerative disc disease (n = 11) or MS (n = 9) were used as controls. The former group is referred to as normal controls. CD4 cell counts and details of antiretroviral therapy were available on all patients. CD4 cell counts (cells/mm^3; mean ± SEM) were as follows: nondemented group, 262 ± 48; mildly demented group, 164 ± 28; moderately to severely demented group, 125 ± 51. Despite a trend for decrease in CD4 cell counts with the severity of dementia, the differences between the groups were not significant. No significant differences in the CSF viral loads (mean ± SE) were present between the groups (no dementia, -832 ± 414; mild to moderate dementia, -1,015 ± 292; severe dementia, -4,882 ± 1558). Details of antiretroviral therapy are provided in the table. In each group, patients were stratified into two categories depending on exposure to antiretroviral therapy: either no prior exposure to antiretroviral drugs or on greater than two antiretroviral drugs for at least 3 months before spinal tap. Exposure to nucleoside analogs was comparable in all three groups of patients. Nearly half of the patients with no dementia or moderate to severe dementia were on no antiretroviral therapy.

View this table: ***Table*** *Characteristics of HIV-infected patients whose CSF was tested*
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Protein carbonyl detection. Protein carbonyl levels, an index of protein oxidation, were measured by Western blot as described.[7] The level of protein oxidation was determined by an Oxidized Protein Detection Kit (Oxyblot, ONCOR Cat# S7150-Kit). Equal amounts of protein (1.5 µg) from each CSF sample were loaded on the blot and the intensity of the bands was quantitated by densitometry. Samples were incubated for 20 minutes with 12% sodium dodecyl sulfate and 2,4-dinitrophenylhydrazine (DNPH) in 10% trifluoroacetic acid, vortexing every 5 minutes, and then neutralized with 6% ß-mercaptoethanol in Oxyblot neutralization solution. Samples were transferred to nitrocellulose paper by

dot-blotting technique. After the transfer, membranes were blocked with 3% BSA (in phosphate buffered saline [PBS] with 0.01% sodium azide and 0.2% Tween-20) overnight at 2 to 8 °C. The nitrocellulose membrane was exposed to a primary rabbit anti-DNPH protein antibody from ONCOR Oxyblot (1:150 working dilution) for 1 hour, and then to a secondary antibody (antirabbit immunoglobulin [Ig]G coupled to alkaline phosphatase [Sigma, St. Louis, MO]) diluted in the blocking solution 1:15,000 for 90 minutes at room temperature. Membranes were washed after every step in washing buffer (PBS with 0.01% sodium azide and 0.2% Tween-20). The nitrocellulose paper was then developed by a Sigmafast tablet, dissolved in 10 mL of distilled water, until the bands of oxidized proteins changed color. Blots were analyzed using computer-assisted scion imaging software.

Cultures of human brain cells. Brain specimens were obtained from human fetuses of 12 to 14 weeks gestational age with consent from women undergoing elective termination of pregnancy and approval by the University of Kentucky Institutional Review Board. Neuronal cultures were prepared as described previously.[8,9] Briefly, the cells were mechanically dissociated; suspended in Opti-MEM (Gibco, Gaithersburg, MD) with 5% heat-inactivated fetal bovine serum, 0.2% N2 supplement (Gibco, Gaithersburg, MD), and 1% antibiotic solution (penicillin G 10^4 units/mL, streptomycin 10 mg/mL, and amphotericin B 25 µg/mL); and plated in flat bottom 96-well plates. The cells were maintained in culture for at least 1 month before conducting the mitochondrial experiments.

Measurement of mitochondrial membrane potential activity. At the time of experimental treatment, the culture medium was replaced with Locke buffer containing (in mM) 154 NaCl, 5.6 KCl, 2.3 $CaCl_2$, 1 $MgCl_2$, 3.6 $NaHCO_3$, 5 glucose, and 5 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.2), and neurons were incubated with either known mitochondrial toxins (3-nitroproionic acid [3NP], staurosporine, or valinomycin) or CSF from each patient. Initially CSF samples (eight nondemented patients, and six each with either mild dementia, moderate to severe dementia, or MS, or normal controls) were analyzed by serial dilutions and we determined that 1:100 dilution was sufficient to show mitochondrial toxicity. No toxicity was noted in the headache or MS groups even at 1:3 dilution. Hence for the remainder of the study we used all CSF samples at 1:100 dilution in Locke buffer. CSF from patients with moderate to severe dementia was also analyzed with or without L-deprenyl (N,N-dialkylated amphetamine, N-methyl-N-propargylamphetamine; 1 µM), didox (3,4-dihydroxybenzohydroxanic acid; 100 µM), trimidox (3,4,5-tetrahydroxybenzohydroxamide; 100 µM), imidate (ethyl-3,4,5-trihydroxyenzenecarboximidate; 1 µM) (gifted by Molecules for Health, Richmond, VA), EUK-8 (500 µM) (gifted by Eukarion, Bedford, MA), diosgenin ([25R]-5-Spirosten-3ß-ol; 10 µM), ebselen (2-Phenyl-1,2-benzisoselenazol-3[2H]-one; 5 µM), or trolox (Vitamin E analogue; 10 µM). Unless otherwise indicated all chemicals were obtained from Sigma. CSF from each of the patients with moderate to severe HIV dementia was also heat treated at 56 °C or 100 °C for 60 minutes each and was similarly analyzed for effects on mitochondrial potential in cultured neurons. Each experiment was conducted in eight wells and at least three independent experiments were conducted with each CSF sample and each pharmacologic agent. To investigate the neuroprotective properties of the drugs, the cells were incubated with drugs at various concentrations followed by a 6-hour exposure to CSF in Locke buffer. To monitor effects on mitochondrial activity the cells were further incubated for 30 minutes at 37 °C in a 5% CO_2 incubator in the presence of JC-1 (10 µM) and then washed in Locke

solution.

JC-1 is a fluorescent dye that measures changes in mitochondrial membrane potential.[10] It exists as a green fluorescent monomer at low membrane potential. Once loaded into the mitochondria, JC-1 aggregates in regions of high potential, giving a red florescence. The resulting shift of the dye is used to detect changes in mitochondrial activity. Optical measurements were acquired with excitation at 485 nm and emission at 527 nm and 590 nm. The levels of fluorescence at both emission wavelengths were quantified and ratio of measurements was assessed. The data were calculated as mean ± SEM for mean optical measurements from three separate experiments and analyzed using analysis of variance (ANOVA) with Tukey-Kramer post hoc comparisons.

Cytochrome c assay. Neuronal cultures were plated on coverslips in 24-well plates and incubated for 30 minutes with the media containing Mitotracker Red (Molecular Probes, Eugene, OR) at a final concentration of 0.1 µL/mL to stain the mitochondria with red fluorescence. After 30 minutes, the medium was replaced with Locke buffer containing CSF (1:100) for 6 hrs. CSF from each of the patients in the mild dementia group and the moderate to severe dementia group was used for this assay. Each experiment was performed in triplicate wells. Staurosporine (1 µM), 3NP (2 mM), and valinomycin (10 nM) were used as positive controls. The cells were then fixed in 3% paraformaldehyde, permeabilized with 0.1% triton x100, and immunostained with monoclonal antisera to cytochrome *c* (Zymed, San Francisco, CA) at 1:1,000 dilution. Goat antimouse conjugated to fluorescein isothiocyanate (1:500) was used as a secondary antiserum. The coverslips were mounted onto slides with mounting media containing 0.1% Hoecht 33349 (Molecular Probes) to stain the nuclei with blue fluorescence. Colocalization of mitochondria and cytochrome *c* gave a yellow fluorescence whereas release of cytochrome *c* gave a green fluorescence. Nuclei showing condensation of chromatin were considered apoptotic and counted as a percentage of all nuclei. Approximately 500 cells were counted from each well. Mean and SEM were determined for each group and analyzed by ANOVA with Tukey-Kramer post hoc comparisons.

Mitochondrial oxidation assay. Human neuronal cell cultures were washed with Locke buffer and incubated with CSF 1:100 in Locke buffer for 6 hours, washed again, and then incubated with 150 nM Mitotracker Green (λex = 490 nm and λem = 516 nm) (Molecular Probes) and 500 nM Mitotracker Red (λex = 579 nm and λem = 599 nm) (Molecular Probes) at 37 °C for 30 minutes and then washed extensively with buffer. Mitotracker Green is a cell-permeant, mitochondrial-specific dye. It is nonfluorescent in aqueous solutions and becomes fluorescent only on sequestration and association with lipids within the mitochondria without requiring oxidation or reduction. The dye measures cellular mitochondria content and distribution. Mitotracker Red is also cell-permeable and sequesters in the mitochondria, but does not emit fluorescence unless it is oxidized, implying that actively responding mitochondria are required to obtain fluorescence. Ratio of red/green fluorescence was calculated and expressed as % of control. The data were calculated as mean + SEM for mean optical measurements from three experiments and analyzed by ANOVA (Tukey-Kramer post hoc comparisons).

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Detection of oxidized lipids in brain. Prominent staining for HNE was noted in neurons, glial cells, and perivascular cells of patients with HIV encephalitis (figure 1). Interestingly, even in patients who died of bacterial sepsis, only small numbers of cells immunostained for HNE, indicating that comparatively there are massive amounts of oxidative stress in patients with HIV encephalitis (see figure 1). Immune reactive cells for HNE were also present in all animals with SHIV encephalitis. No HNE-positive cells were present in the uninfected animal. Large numbers of neurons and glia were immunoreactive for HNE. Intense immunoreactivity was localized to cell bodies of glial cells and the perikaryon of the neurons. In one animal HNE immunoreactive blood vessels were also noted (figure 2).

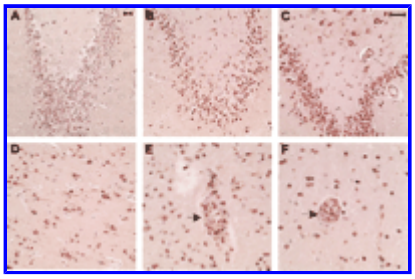

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Figure 1. Oxidative stress in brain and CSF. Immunolocalization of anti-4-hydroxynonenal (HNE) products in HIV encephalitis. Paraffin-embedded sections from HIV-infected patients were immunostained with polyclonal antisera to HNE. (A) Few cells show immunoreactivity in the dentate gyrus of a patient without HIV infection who died of bacterial sepsis. (B) Prominent staining for HNE is noted in the dentate gyrus. (C through F) Sections from patients with HIV encephalitis. (C) Additional cells are noted staining in the hilum of a dentate gyrus. (D) Multiple neurons and glial cells immunostaining for HNE in the temporal cortex. (E) Perivascular cells show prominent immunostaining for HNE. (F) Multinucleated giant cell immunostaining for HNE. Magnification in panels A and B is x100 and C through F is x200.

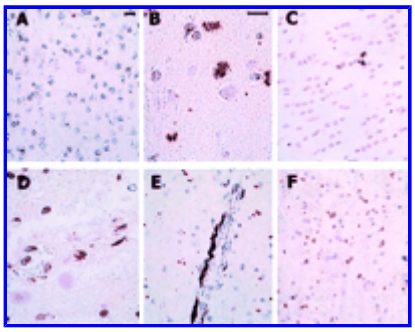

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Figure 2. Oxidative stress in brain and CSF. Immunolocalization of HNE products in simian immune deficiency virus (SHIV) encephalitis. Paraffin-embedded formalin-fixed sections from macaques with SHIV encephalitis were similarly immunostained for HNE. Diaminobenzidine was used as a chromogen. (A) Lack of HNE immunoreactivity in an uninfected macaque. (B) HNE-positive cells in the basal ganglia with SHIV encephalitis have neuronal and non-neuronal cell morphology. (C) HNE-positive cells in the perivascular region. (D) HNE-positive cells in the perivascular region of macaque SHIV encephalitis. (E) Prominent HNE immunoreactive product in the wall of a blood vessel and punctate staining in the surrounding region. (F) A focal area of a large number of HNE-positive cells. Magnification in panels A, C, E, and F is x400; B and D are x1,000.

Detection of protein carbonyl in CSF. Protein carbonyl formation could be detected in CSF samples

from all groups of patients and normal controls. As shown in figure 3, oxidized proteins were elevated in both mild dementia ($p < 0.001$) and moderate to severe dementia ($p < 0.01$) groups when compared to normal controls.

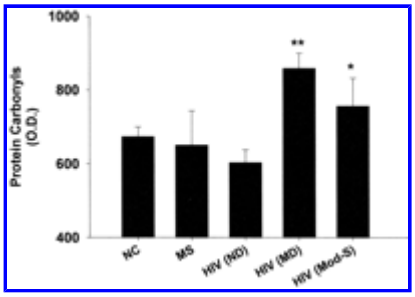

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Figure 3. Oxidative stress in brain and CSF. Protein oxidation in CSF of HIV-infected patients. CSF samples were analyzed by slot blot using antisera for protein carbonyl production. Increased protein carbonyl formation is noted in the CSF of patients with mild HIV dementia (MD) when compared to HIV-infected patients without dementia (ND) (**$p < 0.001$) and normal controls (NC) ($p < 0.05$). Patients with moderate to severe (Mod-S) HIV dementia also had elevated protein carbonyl levels compared to normal controls (*$p < 0.05$).

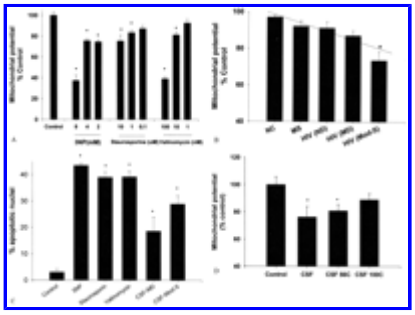

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Figure 4. Neurotoxic properties of CSF. (A) Effect of mitochondrial toxins on human fetal neurons. Cultures of human fetal neurons were treated with 3NP, staurosporine, or valinomycin and mitochondrial activity determined by JC-1 assay. A significant decrease in mitochondrial potential in a dose responsive manner was seen with each of the compounds. Data represent mean ± SEM. *$p < 0.05$. (B) Effect of CSF on mitochondrial membrane potential. Human fetal neurons in culture were treated with CSF and mitochondrial activity determined by JC-1 assay. A progressive decrease in mitochondrial membrane potential is seen with CSF from the groups of patients with HIV infection (HIV [ND] = no dementia; HIV [MD] = mild dementia; HIV [Mod-S] = moderate to severe dementia) when compared to patients with MS or normal controls (NC). Data represent mean ± SEM. *$p < 0.01$. (C) Induction of apoptosis by CSF and mitochondrial toxins. Significant neuronal apoptosis was induced by all three mitochondrial toxins (staurosporine, valinomycin, and 3NP) as well as CSF samples from patients with mild dementia or moderate to severe dementia. *$p < 0.05$ When compared to controls. Data represent mean ± SEM. (D) Effect of heat treatment of CSF on mitochondrial potential. Human fetal neurons were exposed to CSF from patients with moderate to severe HIV dementia following heat treatment at 56 °C or 100 °C. Partial reversal of toxicity is noted. Data represent mean ± SEM. *$p < 0.05$.

Effect of mitochondrial toxins on human fetal neurons. To characterize the susceptibility of human fetal neurons to mitochondrial injury, we initially treated cultures of human fetal neurons with three known

mitochondrial toxins—3NP, staurosporine, and valinomycin—each of which causes mitochondrial toxicity by a different mechanism (see below). Each of the compounds caused a significant decrease in mitochondrial potential in a dose responsive manner at 6 hours post incubation (figure 4A). The neurons were most susceptible to valinomycin followed by staurosporine and then 3NP.

Effect of CSF on mitochondrial potential activity, mitochondrial oxidation, and apoptosis. A progressive decrease in mitochondrial activity was noted in HIV-infected patients with the severity of cognitive impairment when compared to normal controls or patients with MS (figure 4B). However, only the HIV-infected patients with moderate to severe dementia reached statistical significance ($p < 0.01$) when compared to normal controls, patients with MS, or HIV-infected patients without dementia. No correlation of mitochondrial activity was found with CD4 cell counts or the presence or absence of antiretroviral therapy.

CSF from patients with severe HIV dementia was further analyzed for ability to induce oxidation of mitochondria in neurons. Significant increase in mitochondrial oxidation was noted with this group of patients (control vs CSF 100 ± 2.45 vs 108 ± 3.17; $p < 0.05$). 3NP was used as a positive control (108 ± 4.16; $p < 0.05$). Significant neuronal apoptosis was induced by the mitochondrial toxins and with CSF from the patients with HIV dementia. Induction of neuronal apoptosis was more prominent with CSF from patients with moderate to severe dementia compared to the mildly demented patients (figure 4C). Heat treatment of the CSF from patients with moderate to severe HIV dementia at 56 °C or 100 °C only partially reversed the neurotoxicity of the CSF, suggesting that the CSF contains both heat labile and heat resistant neurotoxic substances (figure 4D).

Effect of CSF on cytochrome c release. Prominent release of cytochrome *c* into the cytoplasm and the neurites was noted in the cultures treated with CSF from the moderate to severe dementia group, staurosporine, 3NP, or valinomycin. No release of cytochrome *c* was noted in the untreated cultures or those treated with normal CSF and only occasional neurons showed release of cytochrome *c* in cultures treated with CSF from mildly demented patients (figure 5).

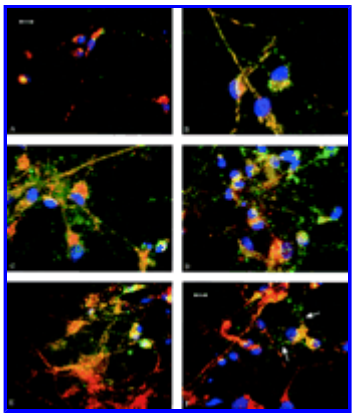

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Figure 5. Effect of CSF and mitochondrial toxins on cytochrome *c* release. Human fetal neuronal cultures were treated with CSF or known mitochondrial toxins and stained with Mitotracker (Red), anti-cytochrome *c* (green), and DAPI (blue). Arrows show condensed chromatin in nuclei. (A) Untreated neurons show colocalization of mitochondria and cytochrome *c*. (B, C, D) Cells treated with staurosporine, 3NP, and valinomycin show prominent green fluorescence in neurites indicating release of cytochrome *c*. (E) Cells treated with CSF from a patient with mild dementia show minimal cytochrome *c* release in the neurites. (F) Cells treated with CSF from a patient with severe dementia show increased cytochrome *c* release in neurites. Magnification is x600 for panel A and x1,000 for panels B through F.

Effect of antioxidants on CSF-induced mitochondrial toxicity. R(-)-deprenyl (1 to 1,000 μM), diosgenin (2, 10 μM), ebselen (1, 5 μM), ruthenium red (1 to 100 μM), quercetin (5 to 50 μM), and trolox (1 nM to 200 μM), when tested alone, did not produce any significant changes in mitochondrial potential in neurons. Didox (≤100 μM), trimidox (≤200 μM), and imidate (≤50 μM) were also nontoxic but at higher concentrations produced decreases in mitochondrial potential (data not shown).

We next determined whether these drugs could block the CSF-induced mitochondrial toxicity. These experiments were conducted only with CSF samples from the moderate to severe dementia group. L-deprenyl (1 μM), didox (100 μM), imidate (1 μM), diosgenin (10 μM), ruthenium red (100 μM), quercetin (25 μM), and ebselen (5 μM) blocked CSF-induced neurotoxicity. No effect of trimidox (100 μM) was seen (figure 6). Trolox (10 μM) was used as a positive control that blocked CSF-induced neurotoxicity completely, suggesting that the neurotoxicity is predominantly via induction of oxidative stress.

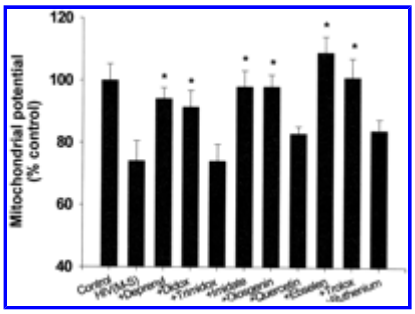

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Figure 6. Effect of novel antioxidants on CSF-induced mitochondrial dysfunction. Human fetal neurons were exposed to L-deprenyl (1 μM), didox (100 μM), imidate (1 μM), diosgenin (10 μM), Euk8 (500 μM), ebselen (5 μM), trimidox (100 μM), or trolox (10 μM) followed by CSF from HIV-infected patients with moderate to severe dementia. All pharmaceutical agents except trimidox were able to block the CSF neurotoxicity. Data represent mean ± SEM. *$p < 0.05$.

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We demonstrate the presence of oxidative stress in brain and CSF of patients with HIV dementia and that circulating toxins in CSF can further induce mitochondrial damage. This mitochondrial damage can lead to release of cytochrome *c* and initiate a cascade of events leading to apoptosis. Polyunsaturated fatty acids, which make up the brain's membrane phospholipids, are especially vulnerable to free radical attack because their double bonds allow easy removal of an H atom from an allylic carbon. Oxidation of polyunsaturated fatty acids results in the production of multiple aldehydes with different carbon chain lengths including HNE.[11] HNE forms adducts with proteins by covalent bonding to histidine, lysine, and cysteine residues.[12] HNE is toxic to neurons and astrocytes by causing disruption of a variety of cell surface receptors and transport mechanisms.[2] Here, using immunohistochemical techniques, we demonstrate that increased levels of HNE were present in macaques with SHIV

encephalitis. Factors that lead to the production of HNE in HIV-infected patients still need to be characterized; however, we have previously shown that the HIV envelope protein gp120 can cause lipid peroxidation as measured by thiobarbituric acid reactive substances.[12]

Protein carbonyl analysis is used as a general assay for oxidative damage to proteins. Carbonyl derivatives are formed by reactive oxygen species mediated oxidation of side chains of some amino acid residues. Carbonyl groups also may be introduced into proteins by glycoxidation and lipid peroxidation products.[13] The oxidation of proteins by free radicals may be responsible for damaging enzymes critical in neuronal function.[14] Once the proteins are oxidized they are cleaved by proteases[14] and thus would likely have access to the CSF. We found an increase in protein carbonyls in the CSF of patients with severe dementia. We have previously shown that HIV proteins gp120 and Tat can cause free radical production.[12,15] Previous studies also show that levels of quinolinic acid and viral proteins are elevated in the CSF of patients with HIV dementia, both of which cause neurotoxicity via mitochondrial dysfunction,[4] and Tat protein causes increased levels of calcium in mitochondria in neurons.[15] Tat also causes increased formation of protein carbonyls when injected into rat striatum.[16]

To determine whether CSF may contain neurotoxic substances, we developed a functional assay for mitochondrial function using cultured human fetal neurons. We initially determined the effects of known mitochondrial toxins using this assay. 3NP inhibits succinate dehydrogenase,[17] staurosporine is a potent inhibitor of calcium dependent protein kinase,[18] and valinomycin is a cyclodepsipeptide potassium ionophore that uncouples oxidative phosphorylation.[19] All three compounds showed significant changes in mitochondrial potential in a dose responsive manner. Consistent with the above observations on detection of protein carbonyls in CSF we also found that CSF from HIV-infected patients with moderate to severe dementia causes significant mitochondrial dysfunction in neurons as shown by induction of changes in mitochondrial potential, oxidative stress in mitochondria, release of cytochrome *c*, and induction of apoptosis. These observations are also consistent with a previous study showing that CSF from patients with HIV dementia caused increases in intracellular calcium in astrocytes.[20,21] The absence of such a response in CSF obtained from patients with MS suggests that neuroinflammatory responses alone are likely not sufficient to cause mitochondrial dysfunction.

Nucleoside analogs, particularly 3'-azido-3'-deoxythymidine (AZT), may also cause mitochondrial dysfunction. However, maximal effects of AZT are on liver and muscle.[22] In one study, no effect of AZT or (2R,cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one (3TC) was seen on neurons, whereas other nucleoside analogs—2',3'-dideoxyinosine (ddI), 2',3'-dideoxycytosine (ddC), and 2',3'-didehydro-3'-deoxythymidine (d4T)—led to a dose-dependent inhibition of neurite regeneration.[23] In our study, two patients in the moderately to severely demented group were on AZT; one of them was also on ddC. None of the patients in this group was on d4T or ddI. In fact, five of eight patients in this group were on no antiretroviral therapy. Hence nucleoside analogs alone cannot explain the changes in mitochondrial potential in this group of patients. The nature of the neurotoxic substances in the CSF remains unknown; however, the neurotoxic components are likely complex because heat treatment of the CSF only partially reversed the neurotoxicity, indicating that both heat labile and heat resistant neurotoxic substances must be present. Potential substances could thus include viral proteins

such as Tat and gp120, various cytokines, quinolinic acid, glutamate, and other uncharacterized substances.[4] The mean viral load in the CSF of patients with HIV dementia was higher than in the nondemented patients; however, owing to small sample sizes, the differences were not significant.

In this study, although we found oxidized protein in mildly demented patients, mitochondrial toxins were present only in the later stages of dementia. This may reflect the buffering capacity of the brain and CSF in the earlier stages of dementia, which may be overwhelmed in later stages, leading to detection of mitochondrial toxins in CSF.

We evaluated the ability of several novel antioxidant compounds to block CSF-induced neurotoxicity. L-deprenyl has been shown to prevent accumulation of reactive oxygen species in cells exposed to glutamate.[24] Recently it was shown to produce improved memory and motor performance in HIV-demented patients.[25] Based on these observations, the AIDS Clinical Trials Group is initiating a multicenter double blind study to determine the efficacy of L-deprenyl in patients with HIV dementia. Observations from the current study show that L-deprenyl can effectively block CSF-induced neurotoxicity at easily obtainable pharmacologic levels and at high concentrations it did not induce any neurotoxicity.

Didox, trimidox, and imidate inhibit cellular enzyme ribonucleotide reductase and thus inhibit retroviral replication.[26] These compounds are one- to two-log-fold more effective than hydroxyurea, but lack the hematopoetic toxicity of hydroxyurea. Besides, these compounds also have free radical scavenging activity and iron chelating activity.[27] We found that didox and imidate could inhibit CSF-induced neurotoxicity whereas trimidox did not. However, at high concentrations, these drugs induced neurotoxicity themselves. These observations suggest that didox and imidate may be worthy of further exploration owing to their dual antiretroviral and neuroprotective properties.

Ebselen is a selenium-containing organic compound that mimics the action of glutathione peroxidase.[28] Diosgenin is a plant-derived steroid with anti-inflammatory effects but its neuroprotective properties have not been studied before.[29,30] These compounds completely blocked the neurotoxicity of CSF from patients with moderate to severe HIV dementia. In contrast, no protection was noted with ruthenium red or quercetin, even though previous studies have shown that ruthenium red protected against HIV protein neurotoxicity[15] and quercetin has antioxidant properties mediated via protein kinase C interactions.[31] In comparison, of the above compounds, only imidate, L-deprenyl, and diosgenin protected against 3NP neurotoxicity. These differences likely represent different mechanisms in causing mitochondrial dysfunction.

We describe a simple neuronal mitochondrial assay that may be useful in monitoring patients with HIV dementia and in identifying potential neuroprotective compounds for this patient population. Using this assay we show that HIV–CSF-induced neurotoxicity responds to a wide variety of antioxidants, providing strong rationale for the use of such agents in this patient population.

Acknowledgments

Supported by grants from the NIH to A.N., J.G., H.E., D.A.B., and J.R.B. G.S. is a member of the NEAD Consortium (University of Rochester, Columbia University, Johns Hopkins University, and Northwestern University), and 26 HIV-infected subjects enrolled by G.S. in this study are coparticipants of the NEAD cohort.

Supported in part by P20RR015592.

The authors thank Dr. Tomasz Cholewo from Lexmark International, Inc. for technical assistance.

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Received July 31, 2002. Accepted in final form September 26, 2002.

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