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Neurotoxicity of CSF from HIV-infected humans

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Approximately 15–20% of individuals infected with the human immunodeficiency virus will develop severe neurological disease. This may be due in part to virus-induced release of a number of putative neurotoxins. However, there is little information to predict which individuals will progress to dementia or the precise mechanisms that drive pathogenesis. In an effort to identify early markers of neurological disease progression we used an *in vitro* bioassay with rat cortical neurons to test for the presence of toxins in CSF from 40 HIV-infected humans with mild, minimal or no neurological disease. A subset of HIV-infected individuals was found to have significant toxic activity in CSF indicating that toxic factors may be circulating prior to the development of dementia. The toxicity was concentration dependent and due to a factor with a molecular mass of less than 30 kDa. Only a small proportion of the cell death appeared to be due to apoptosis. Neuronal toxicity was associated with a gradual accumulation of intracellular calcium in a subset of cortical neurons over a period of 1–2 h and in the absence of a significant acute response. Individuals with both high viral burden and high CSF toxicity were significantly more likely to have neurological symptoms. These initial analyses indicate that toxic factors are present in the CSF of HIV-infected patients that could serve as useful markers of neurological disease progression and provide insights into pathogenic mechanisms *in vivo*.

Keywords: AIDS; brain; tissue culture; excitotoxicity

Introduction

The development of neurological disease in individuals infected with human immunodeficiency virus (HIV) is the culmination of a long history of viral interactions with the brain which slowly kill selected cortical and subcortical neurons (Wiley *et al*, 1991; Ketzler *et al*, 1990; Weis *et al*, 1993; Gray *et al*, 1991). Data from both human and animal studies suggest that the neural disease, is progressive and that it may begin in the asymptomatic stage, long before the development of AIDS (Lendhardt *et al*, 1988; Elovaara *et al*, 1990; Meeker *et al*, 1997). Exposure of the brain to virus appears to be a necessary but not sufficient condition for the development of brain disease, since HIV rapidly penetrates the brain of most individuals but results in severe neurological disease in only a small subset of infected individuals. Efforts to understand the specific viral interactions with the brain that lead to significant neurological disease have focused on the impact of viral burden and the secretion of potentially cytotoxic molecules by microglia/macrophages and astrocytes (Epstein *et al*, 1997). Plasma viral load has been correlated with systemic disease progression. Recent studies have also suggested a correlation between CSF viral load and neurological disease. Viral RNA titers in CSF from individuals at various stages of disease have correlated with neurological disease status in some studies (McArthur *et al*, 1997; Robertson *et al*, 1998; Ellis *et al*, 1997; Brew *et al*, 1997) but not others (Bossi *et al*, 1998; Conrad *et al*, 1995). However, the significant correlations were only found in individuals with encephalitis or neurocognitive impairment. No predictive value has been found at earlier disease stages. Consequently, the precise role of the virus in the development of brain disease is not clear. Alternative variables that are more closely related to the neurotoxic event might provide better early markers of neuropathogenesis leading to AIDS dementia.

Since HIV does not appear to directly infect and kill neurons, the development of neurological disease probably depends in part on interactions of HIV with microglia/macrophages and astrocytes in the brain. *In vitro* studies have identified many potential neurotoxic factors of microglia/macro-

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phage origin which could be toxic to neurons (Lipton, 1992a; Giulian *et al*, 1996; Heyes *et al*, 1991a,b; Dawson *et al*, 1993). These *in vitro* observations are consistent with the correlation between microglial densities in brains of HIV-infected individuals and the development of dementia (Glass *et al*, 1995). In addition, early activation of microglia in the brain of HIV-infected individuals could provide the opportunity for early toxin production and the initiation of gradual neurodegenerative changes. The leading candidates for soluble neurotoxic factors include viral proteins (gp120, tat) (New *et al*, 1997; Dreyer *et al*, 1990; Lipton *et al*, 1991; Epstein *et al*, 1997; Nath and Geiger, 1998), quinolinate (Heyes *et al*, 1991a,b), platlet activating factor (PAF) (Gelbard *et al*, 1994a), tumor necrosis factor-α (TNF-α) (Gelbard *et al*, 1994b), interleukins (Yeung *et al*, 1995; Holliday *et al*, 1995), nitric oxide (Dawson *et al*, 1993; Adamson *et al*, 1996) and NTox (Giulian *et al*, 1996). Many of these factors are elevated in the CSF of HIV-infected individuals and could potentially diffuse through the brain to promote the widespread changes seen in HIV encephalitis. Thus the accumulation of toxins in the CSF may at least partially parallel toxic activity within the brain parenchyma. However, little has been done to evaluate whether substances in the CSF of some HIV-infected individuals are cytotoxic at endogenous concentrations and whether this cytotoxic activity might parallel the development of neurological disease. Measures of cytotoxic activity in the CSF of HIV infected individuals could provide significant diagnostic and mechanistic insights into evolving pathogenesis. To test this possibility, we used a cytotoxicity assay in primary cultures of rat cortex to characterize CSF from HIV(+) and HIV(−) patients. A subset of HIV(+) patients was found to have significant cytotoxic activity in CSF at various stages of disease suggesting that the assay may provide a useful index of evolving CNS pathogenesis.

Results

Effects of HIV(+) CSF on cell death and LDH accumulation in cortical cultures

A total of 54 human CSF samples [40 HIV(+) and 14 HIV(−)] were screened for toxic activity using rat cortical cultures as a test system. All CSF samples were cell-free and filtered through an ultrafiltration membrane with a 30 000 Da cutoff to provide a virus-free preparation. HIV(+) patients represented a wide range of disease based on 1993 CDC criteria including nine at stage 0, seven at stage 1, 18 at stage 2 and six patients with no HIV-associated disease. Mild to moderate neurological disease was seen in two patients, minimal neurological symptoms in nine and no neurological symptoms in 29 patients. Initial cytotoxicity screening studies using a 1:5 dilution of CSF in the culture medium indicated that HIV(+) CSF from a subset of patients induced cell death *in vitro*. One HIV(−) CSF sample also showed a small level of cytotoxic activity relative to aCSF internal controls. To evaluate the relative potency of the HIV(+) and the HIV(−) CSF, a concentration-effect curve for cell death (ethidium homodimer stain) was run on CSF samples using the most cytotoxic HIV(−) CSF as well as HIV(+) CSF with high, medium and low levels of cytotoxic activity based on the initial screen. The results of this analysis, summarized in Figure 1, illustrate that the HIV(+) CSF was more potent than the HIV(−) CSF with peak cytotoxic activity achieved at a dilution of 1:10–1:30. The most toxic HIV(−) CSF sample, on the other hand (HIV(−)c), began showing signs of activity at a dilution of 1:5–1:10. Repeated HIV(+) samples run at CSF dilutions of 1:10–1:30 had an average of 67.7 ± 11.4 (s.e.m.) dead cells/mm^2 relative to 23.9 ± 7.0 dead cells/mm^2 for the HIV(−) CSF indicating an average within sample variability of 16.8% and 29.0%, respectively. Given these values a change of $\pm 57\%$ can conservatively be detected. All subsequent tests were run at dilutions of 1:20 to maximize the separation between the HIV(+) and HIV(−) CSF. Under these conditions, basal cell death in cultures treated with the internal non-toxic aCSF control averaged 37.7 ± 9.4 cells/mm^2. This value represents approximately 2.5% of the total neuronal population. A summary of the cytotoxic activity of

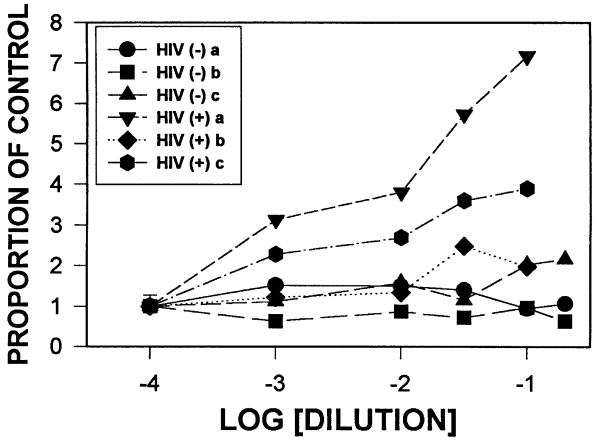

Figure 1 Proportional increase in cell death based on ethidium homodimer staining relative to artificial CSF controls for CSF samples collected from three HIV-infected (+) individuals (HIV(+)a,b,c) or three uninfected (HIV(−)a,b,c) individuals. HIV-infected CSF samples were chosen from those that gave reliable increases in toxicity in initial screening assays. These HIV(+) samples showed high, medium and low levels of toxic activity but were always greater than respective controls. Toxicity began to appear at a CSF dilution of 1:1000 in the most active sample. One control sample showed a significant toxic activity at a dilution of 1:10–1:5. All tests were run in dissociated cortical cultures.

all CSF samples relative to basal cell death is provided in Figure 2. Addition of a 1:20 dilution of HIV(+) CSF into the medium of rat cortical cultures resulted in a wide range of cytotoxic activity. HIV(−) CSF fell within a much narrower range of values with a mean increase in cell death of 55.0±9.5%. To define significant elevations in cytotoxic activity within the population of HIV(+) CSF samples, a 95% confidence limit was calculated from the HIV(−) samples (mean±1.96 s.d. units). The cutoff value is illustrated by a horizontal line at 92.2%. A subset of HIV(+) CSF (13/40 or 32.5%) showed cytotoxic activity in the CSF beyond the 95% confidence limit. No HIV(−) CSF sample was significant.

A parallel analysis of lactate dehydrogenase (LDH) activity in the medium on the day after addition of the CSF, resulted in a similar but less sensitive profile of cytotoxicity (Figure 3). Cultures treated with HIV(−) CSF showed a negligible average net increase in LDH activity of 0.0027±0.0014 units/ml (Uml). Using a 95% confidence limit calculated from the HIV(−) results, a total of 5/40 HIV(+) CSF samples (12.5%) showed significant cytotoxic activity. Within the HIV(+) group, CSF-treated cultures showing significant cell death by ethidium homodimer staining also had a large mean increase in LDH activity of 11.68±4.02% relative to an increase of 0.43±2.04% for CSF-treated cultures that did not have significant cell death. A few HIV(+) CSF samples were less cytotoxic or produced less LDH activity than the control HIV(−) CSF. However, these were in the range of values seen with application of artificial CSF (−5.1±4.2 [*n*=40] relative to HIV(−) CSF).

The ability to detect LDH activity in cultures treated with CSF, provided the opportunity to examine the temporal characteristics of the cytotoxicity. The accumulation of LDH activity at 1, 4, 12 and 24 h within the same cultures after addition of toxic HIV(+) CSF is illustrated in Figure 4. HIV(+) CSF induced a rise in LDH activity within the first 2 h which continued to increase slowly over the following 22 h. A gradual rise in LDH activity was seen in the HIV(−), and control (aCSF) groups. The control groups did not differ from one another. LDH activity was significantly increased in the HIV(+) CSF group at 1 and 24 h. An additional experiment was done to evaluate the accumulation of LDH activity in cultures treated with HIV(+) CSF in the absence of Mg^{2+}, a condition that enhances activity at the NMDA glutamate receptor. Both HIV(+) groups showed significantly higher LDH activity than the HIV(−) control. Culturing in Mg^{2+}-free medium produced a modest additional increase in the mean LDH accumulation relative to HIV(+) CSF in the absence of Mg^{2+} (Figure 5).

LDH activity in CSF in vivo

To rule out the possibility that LDH in CSF might have contributed to the LDH activity in the cultures and to test the possibility that LDH activity in CSF might provide a useful marker for *in vivo* cytotoxic activity, we measured the LDH activity in undiluted CSF from all fresh CSF samples (*n*=31). A low level of LDH activity could be measured from most CSF samples. However, no significant differences were seen between the HIV(+) and the HIV(−) CSF. In addition, the mean total LDH activity of 0.0110 U/ml in raw CSF was too low to account for any of the LDH

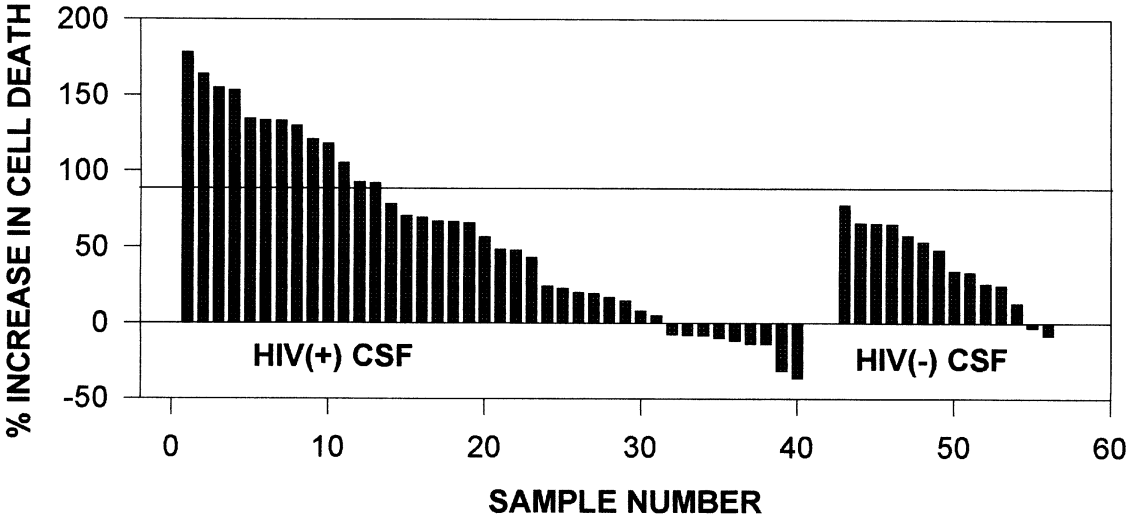

Figure 2 Toxicity profile of all HIV(+) and HIV(−) CSF samples. A subset of HIV(+) CSF showed a level of ethidium homodimer staining significantly greater than the HIV(−) controls based on the 95% confidence limit of the controls illustrated by the horizontal line (mean+1.96 s.d.). All CSF samples were tested on dissociated rat cortical cultures at a dilution of 1:20 for 28 h.

activity seen in the cultures (mean=0.0401 U/ml) where the CSF is diluted 20-fold.

The active toxins have a molecular mass of less than 30 kDa

Since ultrafiltration of the CSF through a 30 000 molecular weight cutoff filter could have removed viral particles and large proteins with significant toxic activity, we directly compared the activity of ultrafiltered CSF to raw unfiltered CSF. Ten HIV(+) CSF samples were chosen that reflected the entire range of toxic activities. As illustrated in pairwise comparisons in Figure 6, all of the toxic activity could be accounted for by the 30 kDa ultrafiltrate.

Apoptosis versus necrosis

The cell death triggered by exposure to HIV(+) CSF could be due to necrotic or apoptotic

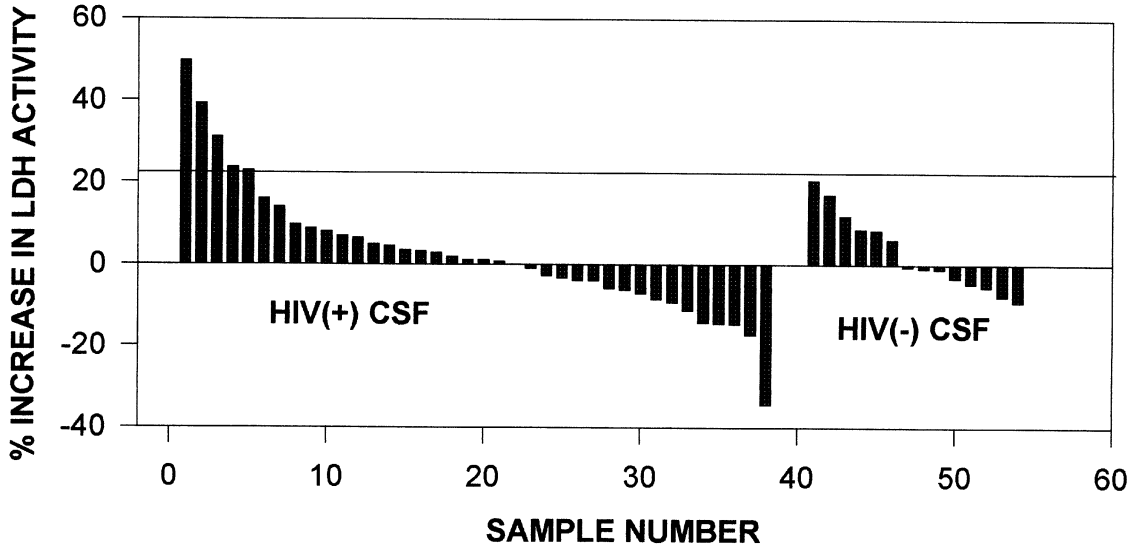

Figure 3 Toxicity profile of all HIV(+) and HIV(−) CSF samples. A subset of HIV(+) CSF showed a level of LDH accumulation significantly greater than the HIV(−) controls based on the 95% confidence limit of the controls illustrated by the horizontal line (mean+1.96 s.d.). All CSF samples were tested on dissociated rat cortical cultures at a dilution of 1 : 20 for 28 h.

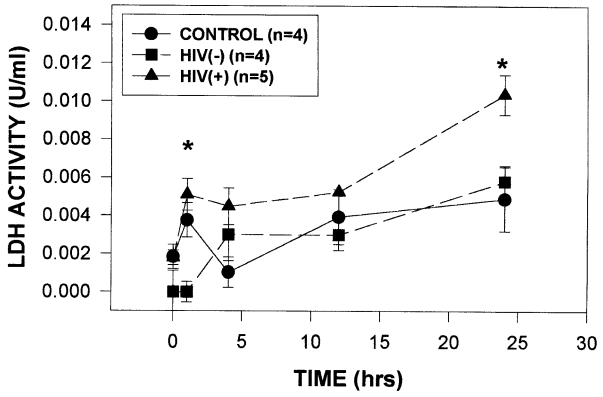

Figure 4 Time course of LDH accumulation after exposure to HIV(+) human CSF previously shown to have high levels of toxicity, HIV(−) CSF or artificial CSF controls. LDH activity in dissociated cortical cultures increased quickly (by 1 h) after exposure to toxic CSF. Controls and HIV(−) CSF showed a gradual accumulation of LDH activity over time that was consistently lower than the toxic CSF. All CSF was run at a dilution of 1 : 20. $^*P<0.02$.

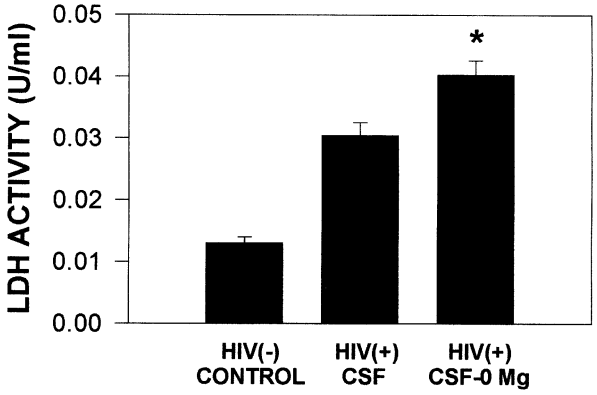

Figure 5 Enhanced accumulation of LDH activity in dissociated cortical cultures treated with toxic HIV(+) CSF in magnesium-free (0 Mg) medium. The accumulation of LDH activity (absorbance units/min) seen with toxic HIV(+) CSF ($n=7$) was further increased in the absence of magnesium ($^*t=-3.32$, $P=0.006$, $n=7$ each group). CSF was assayed at a dilution of 1 : 20 for 24 h in culture. Both HIV(+) CSF conditions produced significantly more LDH activity than the HIV(−) controls ($P<0.007$). Basal LDH accumulation under the same conditions was $0.0053 \pm .0004$ absorbance units/min. Bars represent the mean ± s.e.m.

mechanisms. To contrast the contribution of these two processes, cultures were incubated overnight with toxic HIV(+) CSF or HIV(−) CSF. After treatment, dead cells were labeled with ethidium homodimer and counted. The cells were then fixed and the fragmented DNA was end-labeled with biotinylated deoxynucleotides using terminal deoxynucleotidyl transferase (TUNEL procedure). An average of 37.4 ± 8.6 (n=5) apoptotic cells were seen in cultures treated with HIV(−) CSF relative to 30.4 ± 6.4 (n=9) for cultures treated with HIV(+) CSF. Corresponding ethidium staining of dead cells gave values of 56.6 ± 10.7 and 75.6 ± 12.8 for HIV(−) and HIV(+) CSF, respectively. Basal cell death was 47.0 ± 6.9 in this assay. In cultures with the highest level of cell death (104.5 ± 14.7; HIV(+) CSF, n=5), the corresponding apoptosis was only 24.7 ± 9.3 indicating that as more cells die, the ratio of apoptotic to dead cells decreases.

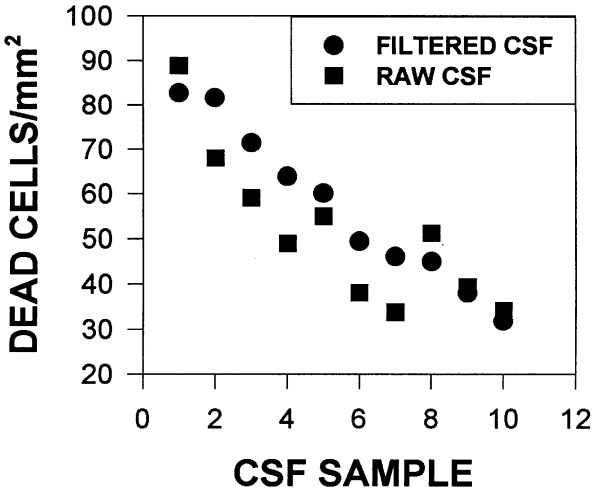

Figure 6 Comparison of the toxic activity (dead cells/mm^2) of 10 raw and ultrafiltered CSF samples representing the full range of toxic activities. Pairwise comparisons of cell death in dissociated cortical cultures due to raw CSF *versus* an ultrafiltrate (30 000 molecular weight cutoff) of the same sample indicated that all of the toxic activity in each sample could be accounted for by the low molecular weight components in the ultrafiltrate.

Intracellular calcium accumulation in neurons

Since accumulation of intracellular Ca^{2+} plays a crucial role in triggering excitotoxic cell death, we evaluated the ability of HIV(+) CSF to disrupt Ca^{2+} homeostasis in the cortical neurons. The mean calcium accumulations within rat cortical neurons treated with HIV(+) CSF (n=18), HIV(−) CSF (n=18) and aCSF (n=7) are summarized in Figure 7. Addition of HIV(+) CSF to neurons preloaded with the Ca^{2+} indicator dye Fluo-3 induced two very small acute increases in intracellular Ca^{2+} at 12 and 60 s post-stimulation followed by recovery to basal levels by 2 min. Between 10 and 30 min after the addition of the CSF, intracellular Ca^{2+} again began to rise and, by 1 h, reached a level more than tenfold greater than the initial acute peak responses. Neither the HIV(−) CSF or the aCSF induced a change in intracellular Ca^{2+} within the first 60 min. Of the 18 neurons tested in three experiments with HIV(+) CSF, 13 showed fluorescence increases in excess of 50% over basal and 11/18 showed increases in excess of 100%. None of the neurons tested with HIV(−)

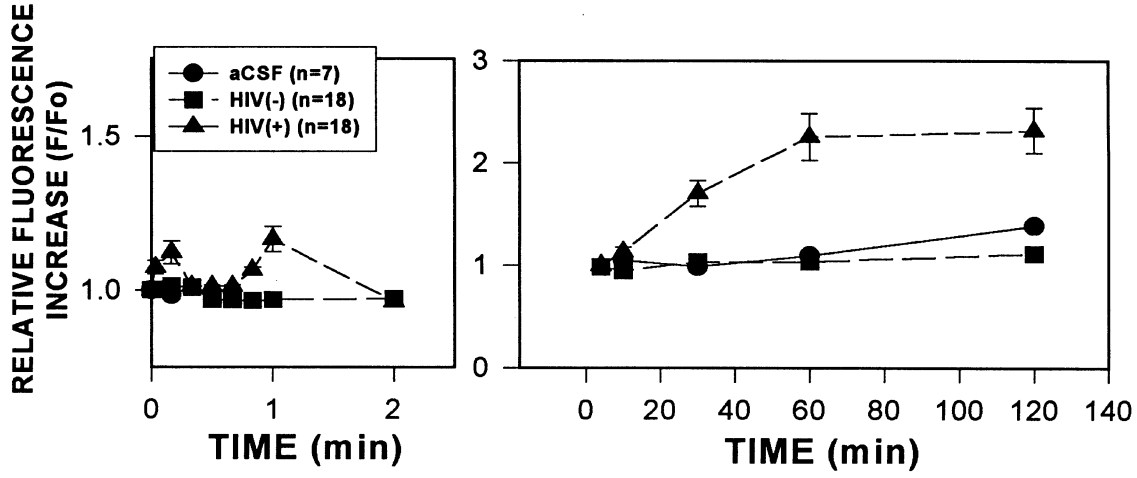

Figure 7 Mean increase in intracellular calcium in neurons from rat cortical punches treated with a 1 : 20 dilution of HIV(+) CSF. The HIV(+) CSF was selected from a sample previously shown to have toxic activity in the ethidium homodimer assay. Cells were preloaded with the fluorescent calcium indicator dye Fluo-3 AM 40 min prior to testing. Very small peaks of calcium fluorescence were seen due to the HIV(+) CSF (n=18 cells) at 10 s (P=0.289) and 60 s (P=0.0001) which recover to baseline by 2 min. All time points 30 min and beyond are significantly greater than the HIV(−) CSF controls ($P<0.0001$).

CSF (0/18) or aCSF (0/7) showed fluorescence increases in excess of 50% over basal. An example of the Ca^{2+} accumulation in a small cluster of cortical neurons treated with HIV(+) or HIV(−) CSF is illustrated in Figure 8. Of the five neurons treated with the HIV(+) CSF, four showed small acute increases. One large neuron (arrow) showed a dramatic gradual accumulation of intracellular Ca^{2+} culminating in extreme swelling and death by 120 min. No such responses have been observed in similar neurons exposed to HIV(−) CSF (open arrow, arrowhead) which show only a small accumulation of Ca^{2+} beginning at 60 min.

Relationship of CSF toxicity to viral load and clinical status

Although comparisons of clinical data with *in vitro* toxicity must be interpreted cautiously in the absence of longitudinal data, several trends are notable. The patients with toxic CSF (Tox(+)) were equally divided between asymptomatic, symptomatic and AIDS (n=4 each) suggesting that toxic factors appear in the CSF at all disease stages. The Tox(−) group had 13 AIDS patients, five asymptomatic and three symptomatic. The relative toxicity of the CSF did not correlate with the development of immunodeficiency. AIDS patients had an average toxicity score (per cent increase in cell death) of 130.8 ± 14.8 *versus* 140.1 ± 8.7 in asymptomatic patients. Correlation of the relative toxicity of CSF, plasma viral RNA and CSF viral RNA with a dichotomous ranking of neurological function (normal–stage 0 *versus* stage 0.5–1 based on Price and Sidtis (1990) is summarized in Table 1. No significant positive relationships were seen for any of these variables. However, using an additive model to assess the potential combined contributions of viral burden and toxic factors substantially increased the correlation. A combination of high CSF toxicity and high viral burden was the best predictor of neurological disease (r=0.559). The combined relationship of plasma virus with CSF toxicity showed a significant but weaker correlation.

Table 1 Relationship between CSF toxicity, viral burden and neurological status.

	Correlation	*Probability*
Plasma viral RNA	0.241	P=0.128
CSF viral RNA	0.270	P=0.093
CSF Toxicity	−0.145	P=0.345
Toxicity+Plasma viral RNA	0.340	P=0.034
Toxicity+CSF viral RNA	0.559	P=0.0002

The correlation of CSF toxicity (% increase in cell death), CSF viral RNA and plasma viral RNA with neurological disease status was calculated for the 40 HIV(+) individuals in this study. Neurological disease ranged from normal to mild (stage 1) and was dichotomously classified as normal or minimal-mild (stages 0.51–1). The additive effects of CSF virus or plasma virus with CSF toxicity were correlated with neurological disease status to evaluate the possible independent contribution of viremia and toxin accumulation to disease progression. The combined influence of CSF virus and high toxicity resulted in the highest correlation.

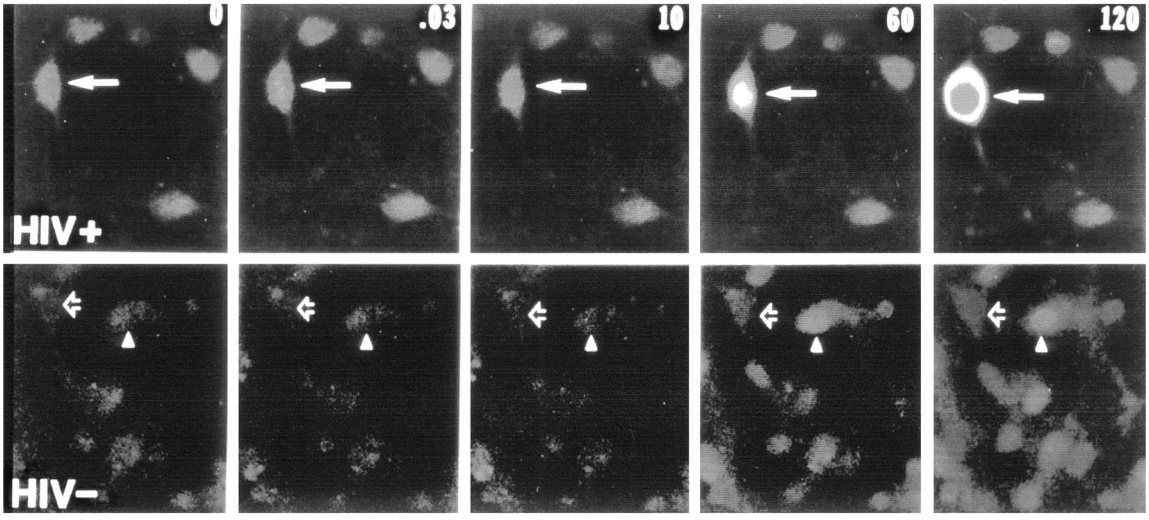

Figure 8 Example of the individual neuronal responses to toxic CSF illustrating the selective vulnerability of some neurons. Frames illustrate cell fluorescence prior to CSF application (0 min) and 0.03, 10, 60 and 120 min after application. One large cortical neuron (arrow) showed a very small acute increase at 0.03 min followed by a dramatic accumulation of calcium and swelling by 120 min. Other cells in the same field showed small or negligible increases in calcium. Large cortical neurons treated with HIV(−) CSF showed small increases in calcium (open arrow, arrowhead) after 60 and 120 min but rarely reached levels seen in responsive neurons treated with HIV(+) CSF. All CSF samples were tested on punch-cultured cortical neurons at a dilution of 1 : 20 in a HEPES buffered artificial CSF solution.

Discussion

Most evidence indicates that severe neurological disease is the result of interactions of HIV with microglia and astrocytes in the brain that lead to the secretion of a number of possible neurotoxic factors (Epstein *et al*, 1997). Early infiltration of macrophages (Lane *et al*, 1996) and interactions of the virus with microglia and astrocytes may initiate a persistent inflammatory cascade with release of putative neurotoxins. Since this process evolves gradually, early markers of the neurotoxic cascade could provide important insights into the evolution of CNS disease. In addition, information regarding the identity and expression of potential toxins at various disease stages could help to clarify the nature of long-term interactions of virus within the brain. The current studies addressed two main issues: (1) Can toxic activity be detected in HIV(+) CSF prior to the development of neurological disease?; and (2) How do *in vivo* toxic factors affect neurons? The ultimate goal of these studies, using longitudinal analyses, will be to identify the relationship between toxic factors in CSF and clinical disease progression.

A subset of HIV(+) CSF samples is neurotoxic in vitro

The data show that CSF from HIV-infected humans can contain factors that are toxic to neurons *in vitro*. The toxic factors are active in rat cortical cultures providing a convenient and reliable detection system. Based on this assay, 32.5% of the HIV(+) CSF samples was found to have toxic activity significantly greater than HIV(−) CSF controls. The toxic activity was present in CSF from asymptomatic, symptomatic and AIDS patients reinforcing the view that such factors appear throughout the course of CNS disease. The level of toxicity is relatively low, as should be expected, representing a loss of approximately 2.5% of the cultured neurons. This mimics the level of neuronal loss seen *in vivo* and suggests similar potencies *in vivo* and *in vitro*. The factor(s) present in the CSF of HIV(+) patients have a relatively low molecular weight (<30 kDa), are relatively stable and induce a gradual accumulation of intracellular Ca^{2+} with little acute excitotoxic actions. Thus a number of putative large or unstable toxic factors including viral envelope proteins (Lipton *et al*, 1991; Brenneman *et al*, 1988; Nath and Geiger, 1998) and nitric oxide (Dawson *et al*, 1993; Adamson *et al*, 1996) are unlikely to be the active factors in the CSF. In addition, the actions on neurons do not support the presence of direct excitotoxins such as glutamate and quinolinate (Heyes *et al*, 1991a,b). The absence of these factors in the CSF however, does not negate their potential contribution to toxicity in brain parenchyma. Indeed, it is likely that a variety of factors may contribute to the development of disease. Interactions of virus or viral proteins with cellular targets and generation of nitric oxide may precede the release of more stable toxins or may represent independent steps in the chain of events leading to neurotoxicity. Combined interactions of virus exposure with toxin accumulation are supported by the substantial improvement in the correlation with early clinical disease status when the additive effects of CSF toxins and viral burden were evaluated.

Previous assays for specific substances in brain and CSF have provided a list of potential neurotoxins that could contribute to our current results. The most prominent candidates include TNF-α (Grimaldi *et al*, 1991; Nuovo and Alfieri, 1996; Wesselingh *et al*, 1997), IL-6 (Sopper *et al*, 1996), PAF (Gelbard *et al*, 1994a) and NTox (Giulian *et al*, 1996). Each of these cytokines/toxins have been shown to promote neurotoxicity *in vitro* (Westmoreland *et al*, 1996; Yeung *et al*, 1995; Holliday *et al*, 1995; Giulian *et al*, 1996). In addition, a number of viral proteins other than gp120 could contribute to toxicity. The small regulatory protein, Tat, has been shown to be toxic to neurons (Magnuson *et al*, 1995; New *et al*, 1997) and may be secreted from infected cells (Ensoli *et al*, 1993). In addition, the viral proteins Nef and Rev are abundantly synthesized in astrocytes, particularly within the brains of patients with AIDS dementia (Ranki *et al*, 1995). However, the role of these latter proteins in the pathogenesis of AIDS dementia has been questioned (McPhee *et al*, 1998). Future longitudinal analyses of toxic CSF and pharmacological characterization studies should help to clarify the potential role of these substances *in vivo* during disease progression.

HIV(+) CSF induces a gradual increase in intracellular Ca^{2+} in selected neurons

Neurons that are sensitive to the toxic effects of HIV(+) CSF exhibit a unique pattern of Ca^{2+} accumulation. After exposure to the CSF, a slow increase in intracellular Ca^{2+} develops over a period of approximately 1 h and in the absence of any significant acute Ca^{2+} influx. This response is similar to the late phase of Ca^{2+} accumulation described by Tymianski *et al* (1993) following NMDA receptor stimulation and the delayed Ca^{2+} increase observed in ciliary ganglion neurons exposed to feline leukemia virus envelope protein (Mitchell *et al*, 1997). In the studies of Tymianski *et al* (1993), the magnitude of the late Ca^{2+} accumulation predicted which cells would die. The slow accumulation followed the acute NMDA response but was independent of NMDA receptor activity and extracellular Ca^{2+}. The late increase was attributed to a breakdown in intracellular Ca^{2+} homeostasis, although the precise mechanism was not determined. Although several putative toxins appear to require NMDA receptor activation (Gelbard *et al*, 1994b; Lipton, 1992b; Dawson *et al*, 1993;

Giulian *et al*, 1996; New *et al*, 1997), the mechanisms underlying the long-term destabilization of intracellular Ca^{2+} are not clear. A role for intracellular release of Ca^{2+} has been suggested in some studies showing that dantrolene, an antagonist of ryanodine receptor mediated Ca^{2+} release, can partially block the toxic effects of nitric oxide (Dawson *et al*, 1993) or HIV gp120 envelope protein (Nath *et al*, 1995). We have seen similar Ca^{2+} responses when neural tissue is exposed to infectious virions of feline immunodeficiency virus (unpublished results) suggesting the release of similar toxic factors. Thus, the response of neural cells to virus *in vitro* may give rise to the release of toxins similar to those that appear in CSF *in vivo*.

It is important to note that only a fraction of the cultured cells die or respond with robust Ca^{2+} accumulation (e.g. Figure 8) after exposure to the CSF toxins. This selective cellular vulnerability is similar to that described for exposure of neural cultures to HIV envelope protein (Nath and Geiger, 1998) or to free virus in the feline immunodeficiency virus model (Meeker *et al*, 1996) and may reflect the processes that lead to selective neuronal loss *in vivo* (Wiley *et al*, 1991; Everall *et al*, 1991; Gray *et al*, 1991; Meeker *et al*, 1997). Thus, this model may provide the opportunity to more precisely define the interactions that determine which neurons will die.

Clinical correlates of HIV(+) CSF toxicity
Establishing a relationship between toxic activity in CSF and disease is the most difficult aspect of these experiments and can be only partially addressed in the context of these studies due to high variability, the current lack of longitudinal analyses and a limited number of Tox(+) samples. Future longitudinal studies are particularly important because the temporal relationship between viremia, toxin accumulation and neurological disease is currently unknown. In the current studies only a single time point has been examined for each patient and therefore trends must be viewed cautiously. Over time, many factors may contribute to CNS disease progression. For this reason it is particularly important to follow the Tox(+) asymptomatic individuals to determine if they will develop neurological disease.

Previous studies of CSF viral RNA burden have shown significant correlations with neurological disease (Robertson *et al*, 1998; McArthur *et al*, 1997; Brew *et al*, 1997). However, these correlations are restricted to AIDS patients with neurological disease or encephalitis and show little predictive value early in the course of disease. Our results on this small set of patients indicate that additive models that take into account both the presence of virus and the accumulation of putative toxins in the CSF may have useful predictive validity. Thus, assays for toxicity in the CSF of HIV-infected patients could provide indices of neurological disease progression and lead to valuable insights into CNS pathogenesis.

Materials and methods

Preparation of CSF samples
CSF was centrifuged at 2500 r.p.m. for 15 min aliquoted and frozen at $-80^{\circ}C$. Prior to use in the experiments, the CSF was thawed, and half of the aliquot ($\sim 500\ \mu l$) centrifuged through an ultrafiltration membrane with a 30 000 dalton cutoff. The ultrafiltrate and the remaining raw CSF was then frozen on dry ice in 100 μl aliquots and stored at $-80^{\circ}C$.

Primary cultures of rat cortex
Fetuses at E17-18 were removed from pregnant female Long-Evans rats terminally anesthetized with isoflurane. The fetuses were removed from the uterus and were placed in ice cold HEPES-buffered Hank's balanced salt solution (HBSS). The brain was removed from the cranium within a laminar flow hood and rinsed three times in fresh sterile HBSS. The dura-arachnoid membrane was removed and the brains processed for punch cultures or dissociated cultures.

Cortical punch cultures These, used for calcium imaging, were taken from frontal-parietal cortex using a 23 ga blunt-tip needle connected to a 1 ml syringe filled with sterile Dulbecco's Modified Eagle Medium (DMEM) +10% fetal bovine serum (FBS) +20 μg/ml gentamycin (complete medium). The punched pieces of tissue extracted with mild suction were then gently ejected onto poly-L-lysine (0.1 mg/ml) treated glass coverslips and transferred directly to a 5% CO_2 incubator at $35-36^{\circ}C$ with just enough medium to keep them wet. The tissue punches generally attached within 2 h and were supplemented with additional medium approximately 2–4 h after plating. After 6–8 days *in vitro*, cortical neurons have migrated into the region surrounding the punch. These neurons have morphological features typical of mature cortical neurons and were easily identified for imaging studies. Cells were fed with complete medium every 2 days.

Dissociated cultures These were prepared from the remaining cortical tissue. Pieces of cortex were transferred to calcium-magnesium-free HBSS (CMF-HBSS), minced and incubated in 5–10 ml of CMF-HBSS containing 2 units/ml Dispase and 2.5 units/ml DNase for 10–15 min at $37^{\circ}C$. The

tissue was then gently triturated using 5–6 passes through a sterile 10 ml pipette. The pieces were allowed to settle for 2 min and the suspended cells were transferred to a 50 ml culture tube containing 20 ml of complete medium. The remaining tissue was re-suspended in 5 ml of CMF-HBSS and the trituration procedure repeated. After approximately 6–8 cycles of trituration the final tube of cells was centrifuged at 400 r.p.m. for 10 min to collect the cells as a loose 'pellet'. The supernatant was checked for residual cells and then aspirated. The cells were resuspended in complete medium at a concentration of approximately 500 000 cells/ml. The cells were then seeded into 48-well plates at a density of approximately 100 000 cells/cm^2. The resulting cultures contained a mixed population of neurons, glia and microglia.

Evaluation of CSF cytotoxicity

Dissociated cultures seeded at identical densities in multiwell plates were used for cytotoxicity analysis to provide optimal uniformity between cultures. Cultures were screened prior to use to remove those with poor growth, non-uniformity or other problems. Evaluation of CSF effects on the cultured neurons was accomplished by washing the cultures twice in HBSS followed by addition of 250 μl/well of serum-free DMEM containing CSF at concentrations ranging from 1:5 to 1:1000 with each condition run in triplicate. Controls included a minimum of 9 wells/plate receiving an equal volume of artificial CSF (aCSF in mM; NaCl 130, KCl 5.0, $CaCl_2$ 2.3, $MgCl_2$ 1.3, $NaHCO_3$ 24, glucose 10) as well as CSF samples obtained from HIV-negative [HIV(−)] individuals. HIV(−) CSF was collected from patients receiving CT-myelograms with no known underlying neurological disease. The cells were incubated for 18–28 h under standard culture conditions (37°C, 5% CO_2). At the end of the incubation, 100 μl of medium was collected from each well for the subsequent assay of lactate dehydrogenase (LDH) activity. The cells were then washed twice in HBSS and incubated in a 2 μM solution of ethidium homodimer in DMEM under standard culture conditions for a period of 30 min (37°C in a 5% CO_2 humidified incubator). After three washes in HBSS to remove excess stain, the fluorescent red nuclei of dead cells were systematically imaged and counted in triplicate fields from each culture at a final magnification of 106×. The red fluorescent nuclei provide a very high signal to noise with virtually no background. The digital image of the fluorescent nuclei was highlighted and the size of each nucleus measured automatically using a Bioquant Image Analysis System (R & M Biometrics). Each highlighted object was then filtered based on size (μm^2) and objects counted which fell in the range of cell nuclei (calibrated from stained cells cultured under the same conditions). The mean number of dead cells was calculated for each well and corrected for basal cell death measured in wells treated with aCSF. The per cent increase (or decrease) in cell death relative to basal was then calculated and a 95% confidence limit calculated from the cell death observed in cultures treated with HIV(−) CSF. This limit was then used to determine which HIV(+) CSF samples had significant cytotoxic activity.

Assay for lactate dehydrogenase activity in the tissue culture medium was accomplished using the Optimized LDH Assay kit (Sigma Chemical Co.). Each CSF sample was analyzed in triplicate according to the following modified protocol. Reagent A (NADH and phosphate buffer, pH 7.5) was added in a volume of 1.25 mls to a tube containing 100 μl of culture medium, the tube was gently mixed and allowed to react for 1 min. Reagent B (pyruvate solution) was added in a volume of 50 μl, the solutions mixed and incubated for 30 s. The conversion of pyruvate to lactate by LDH with the corresponding oxidation of NADH to NAD^+ was measured by the decrease in absorbance at 340 nm at 1 min intervals over a total period of 3 min. The rate of decrease (absorbance units/min) is proportional to the LDH activity (U/ml) in the sample. The assay was found to be linear for at least 4 min.

Correlation of the results obtained from the ethidium homodimer *versus* LDH for assays run at the same time generally yielded good concordance but correlations were modest. The largest direct comparison between cell death and LDH for an assay in which 42 samples were run in parallel gave a correlation of 0.658.

Assessment of apoptotic cell death

After quantification of cell death based on the ethidium homodimer stain, selected cultures were fixed for 10 min in 4% paraformaldehyde, 0.1 M phosphate buffer, pH 7.4. Deoxynucleotidyl terminal transferase end labeling of DNA fragments was accomplished using the TdT FragEL kit (Amersham Corp.). The cells were rinsed in 0.05 M Tris-buffered Saline (TBS), 3×5 min and treated for 5 min at room temperature with 2 μg/ml proteinase K in 10 mM Tris buffer, pH 8.0. The cells were again rinsed 3×5 min in TBS and then incubated in 0.6% H_2O_2 for 15 min. After rinsing 3×5 min in TBS, TdT buffer was added to the cells and allowed to stand for 30 min. TdT labeling reaction mix and TdT enzyme, premixed on ice, were then added to the cells and incubated at 37°C for 1.5 h. The reaction was stopped, the cells washed and avidin-HRP added in blocking solution. Cells were reacted with diaminobenzidine, washed and counterstained with methyl green. The density of stained

nuclei was evaluated and compared with results from the ethidium homodimer stain. Positive controls in which the cultures were treated with DNase yielded robust, widespread staining.

Digital morphometry and calcium imaging

After a minimum of 8 days *in vitro*, coverslips containing cortical punches were washed twice in HEPES-buffered artificial CSF (HEPES-aCSF) composed of the following salts (mM concentration): NaCl 137, KCl 5.0, $CaCl_2$ 2.3, $MgCl_2$ 1.3, HEPES 10, glucose 20; pH=7.36–7.40, osmolality=290 mOsm/kg. After the second wash, a 4 μM solution of the fluorescent Ca^{2+} indicator, Fluo-3 AM (Molecular Probes, Inc.), dissolved in HEPES-aCSF was added and the cells were allowed to incubate for 40 min in a humidified chamber at room temperature. The working Fluo-3 AM solution was made by diluting from a 2 mM stock in DMSO. After incubation in the Fluo-3 AM, cells were washed three times with HEPES-aCSF and the coverslip was transferred to Biophysica chamber for the imaging studies. A volume of 0.5 ml of HEPES-aCSF was placed on the coverslip. After triplicate measurements of basal Ca^{2+} in the cells, an additional 0.5 ml aliquot of HEPES-aCSF containing a 1 : 10 dilution of human CSF was applied to the culture (final dilution of 1 : 20). Images were collected over a period of 2 h after addition of the CSF.

A Bioquant Meg M Image Analysis System coupled to an Olympus IMT-2 microscope was used for the Ca^{2+} measurements. For most analyses, cells were imaged at a final magnification of 1634×. Regions were selected to contain large cortical neurons. If possible, smaller neurons and microglia, were included for comparison. Images of the cell and basal fluorescence were collected using an MTI camera coupled to a Genesis II image intensifier. The mean of three pre-stimulation measurements $\pm$ s.d. was used to define the range of values considered to be basal Ca^{2+} fluorescence. The proportional increase in fluorescence intensity within each cell was then measured relative to the average of the baseline measurements to correct for cell to cell differences in dye loading and intrinsic fluorescence ($\Delta F/F_{baseline}$).

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