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Effects of extracellular human immunodeficiency virus type 1 Vpr protein in primary rat cortical cell cultures

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Recent evidence suggests that HIV-1 Vpr exists in soluble form in the serum and cerebrospinal fluid (CSF). Further, its abundance in the bloodstream, and the CSF, and its activity on other cell types suggest that it could have an effect on brain activity. Using mixed embryonic rat brain cultures as a model to examine the effects of physiological concentrations of extracellular Vpr protein, Vpr-induced cell death was observed. We also observed similar Vpr-induced effects in enriched primary cortical rat astrocytes, as well as in the C6 glioma cell line. Vpr-induced cell death observed in the astrocytic cells appeared to be caused primarily by a necrotic mechanism, although a few apoptotic nuclei were also present. We did not observe Vpr-induced effects on any primary cortical neurons, although we did observe Vpr-induced cell death in hippocampal neurons and astrocytes. Finally, we observed no cell cycle effects due to extracellular Vpr protein. This data points out that different cell types are affected by the toxic effects of extracellular Vpr protein, and that differential toxic effects of extracellular Vpr protein are observed in similar cell types. *Journal of NeuroVirology* (2000) **6,** 202–220.

Keywords: neurons; astrocytes; cortex; apoptosis; C6

Introduction

We were interested in examining the cytotoxic potential of HIV-1 proteins on cells of the central nervous system (CNS). There is abundant evidence suggesting the HIV-1 envelope protein, gp120, activates a cascade of events that cause neurotoxicity. Macrophage/microglia activated by HIV gp120 cause neurodegenerative processes through several mechanisms (Diop *et al*, 1994; Dreyer and Lipton, 1995; Genis *et al*, 1992; Heyes *et al*, 1989; Lipton, 1993; Lipton and Rosenberg, 1994). Through an indirect astrocytic mediated mechanism, gp120 causes neuronal membrane depolarization, increased levels of intracellular Ca^{2+}, and ultimately neuronal cell death. HIV gp120 directly alters ion transport in astrocytes, ultimately triggering a pathway leading to neuronal depolarization. Heat-inactivated virions or soluble gp120 have been shown to stimulate the amiloride-sensitive Na^+/H^+ antiport, glutamate efflux, and increased extracellular K^+ (Benos *et al*, 1994). These elevated extracellular glutamate and K+ levels possibly trigger neuronal depolarization, and the opening of NMDA receptor ion and voltage-dependent Ca^{2+} channels.

We focused on the cytotoxic/neurotoxic potential of the HIV-1 regulatory protein, Vpr. Vpr is one regulatory protein shown to be a significant constituent of the virion, and has been shown to exist in the serum of HIV+ individuals in significant concentrations (Cohen *et al*, 1990; Lavallee *et al*, 1994; Levy *et al*, 1994; Yuan *et al*, 1990). Further, it has been suggested that Vpr is active in a soluble, cell- and virus-free form (Levy *et al*, 1994). Additionally, detectable concentrations of Vpr have been observed in the cerebrospinal fluid (CSF) of HIV+ individuals, and Vpr appears to exist in higher concentrations in the CSF of HIV+ individuals displaying neuropathogenic symptoms (Levy *et al*, 1994).

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Vpr is packaged in virions through its interaction with the Gag p6 protein at equimolar concentrations to the Gag proteins (Cohen *et al*, 1990; Kondo *et al*, 1995; Lavallee *et al*, 1994; Lu *et al*, 1993; Paxton *et al*, 1993). It is essential for viral replication in macrophage (Balliet *et al*, 1994; Connor *et al*, 1995), where it is believed to function, in part, to allow the nuclear localization of the preintegration complex in these nondividing cells (Heinzinger *et al*, 1994). Recent data suggest a role for vpr in prevention of chronic infections in these cells (He *et al*, 1995; Jowett *et al*, 1995; Rogel *et al*, 1995). Vpr was shown to prevent replication, and induce differentiation of muscle cell lines, suggesting a role for this protein in the control of cell division (Levy *et al*, 1993). Subsequently, cells expressing *vpr* were shown to be disrupted in their progression through the cell cycle, arresting at the G2 phase, possibly regulating this and other effects through the glucocorticoid pathway (Ayyavoo *et al*, 1997a; Jowett *et al*, 1995; Mahalingam *et al*, 1997; Withers-Ward *et al*, 1997; Zhao *et al*, 1994, 1996). However, there is still debate as to whether vpr-induced cell cycle arrest is cytostatic or leads to apoptosis (Ayyavoo *et al*, 1997a, Bartz *et al*, 1996; Poon *et al*, 1997; Stewart *et al*, 1997).

Vpr has been observed to form cation-selective channels in membranes (Piller *et al*, 1996). It was further suggested that the channel structure is stabilized by the hydrophobic interactions of the Vpr-terminal alpha helical domain, and the lipid membrane. In a more recent study, that same group has shown that extracellular vpr protein can cause cell death in hippocampal neurons (Piller *et al*, 1998). This effect appears to be due to the ability of Vpr to form channels in the hippocampal neuron membrane. Consequently, at least one published study examining the role played by extracellular Vpr protein in the CNS suggests that extracellular Vpr protein has a direct effect on neuronal activity. To further examine what role the HIV-1 Vpr protein plays in neurological disorders associated with HIV-1 infection, we screened for the effect(s) of Vpr on mixed primary cortical brain cell cultures. The results of this study show that extracellular Vpr protein causes cytotoxic effects on primary cortical astrocytes, but has no effect on primary cortical neurons. Additionally, the primary cortical astrocytes appear to be dying through necrotic rather than apoptotic induced mechanisms.

Results

Vpr-induced cytotoxicity in mixed neuronal cell cultures

Tissue from the neocortex of 16 day rat embryos was harvested and dispersed onto coated plates (Figure 1A). These cultures were grown for 14 days, and

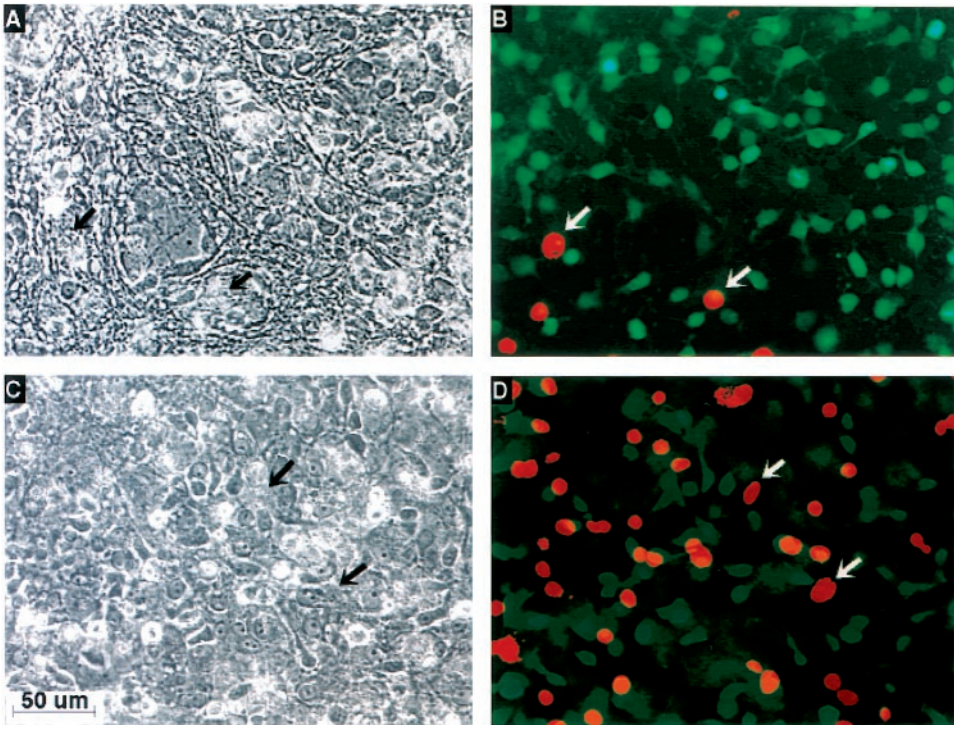

Figure 1 Vpr-induced cytotoxicity in mixed cortical cell cultures. The neocortex of 16 day rat embryos were harvested, dispersed onto coated plates, and grown for 14 days. These cultures were either untreated (**A**, **B**), or treated (**C**, **D**) with 200 pg/ml of HIV-1 Vpr for 36 h. Subsequently, the cultures were assayed for cells displaying permeable membranes, indicative of dead or dying cells, by staining with fluorescein diacetate (**B**, **D**; green) and propidium iodide (**B**, **D**; red). Matched phase images of the fluorescent fields are also displayed (**A** matches **B**; **C** matches **D**). Arrows mark several propidium iodide staining cells identified in the matching images.

then used in the initial studies to examine potential effect(s) of Vpr. In these experiments, the mixed cultures were either untreated, or exposed to various concentrations of Vpr for 36 h. As a positive control, the cultures were treated with the viral protein, gp120, which had previously been shown to cause cytotoxicity in several different cell types in mixed brain cultures (Lannuzel *et al*, 1997; Meucci and Miller, 1996; Muller *et al*, 1992; Scorziello *et al*, 1997). At each time point, duplicate cultures for each Vpr, or gp120 concentration were assayed for cells displaying permeable membranes, usually indicative of dead or dying cells, using a fluorescein diacetate/propidium iodide (FD/PI) assay (Figure 1B,D). A graphic compilation of the quantitative data from a number of these experiments is shown in Figure 2. As can be seen, chronic exposure to picomolar concentrations of Vpr caused a statistically significant increase in the number of PI-labeled cells in the mixed cortical cell cultures (compare Figure 1B to D) beginning at 1 pg/ml of Vpr ($P<0.001$; Figure 2), and increasing over the entire range of protein concentrations analyzed (Figure 2). Additionally, chronic exposure to picomolar concentrations of gp120 caused statistically significant increases in the number of PI-labeled cells in these cultures beginning at the lowest concentration of the gp120 protein analyzed, 1 pg/ml ($P<0.001$; Figure 2). As in the case of Vpr, gp120 caused significant increases in the number of labeled cells over the entire range of concentrations analyzed (Figure 2). The data suggest that exposure to the Vpr protein caused an increase in the number of dead or dying cells in these cultures.

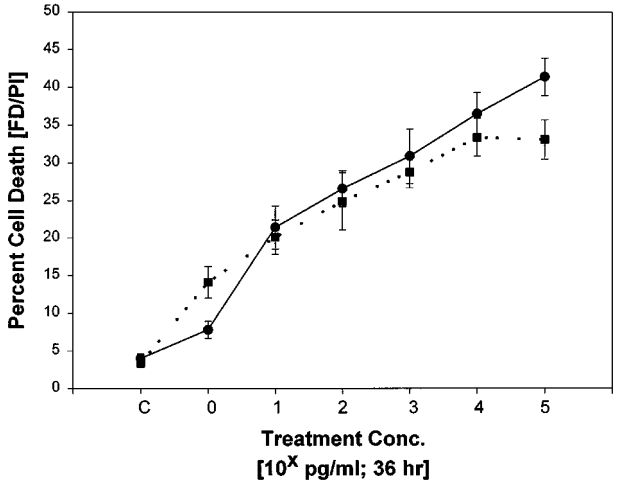

Figure 2 Percentage of cell death in mixed neuronal cell cultures as a function of protein concentration. The neocortex of 16 day rat embryos was harvested, dispersed onto coated plates, and grown for 14 days. These cultures were either untreated, or treated with various concentrations of Vpr or gp120 for 36 h. Subsequently, the cultures were assayed for the number of propidium iodide (PI)-stained cells (dead or dying cells), and the fluorescein diacetate (FD)-stained cells (living cells) were counted for each field scanned (six fields were scanned per plate) per plate in each condition to get the total cell count. The percentage dead/dying cells was then calculated as the number of propidium iodide stained cells divided by the total number of cells. This data from several identical experiments was collated, standard errors of the averages were calculated, and this collated data is shown plotted against the treatment concentration ($10\times$). On the y-axis, C denotes the percentage cell death in the control or untreated condition. Circles denote Vpr-treated cultures, and squares denote gp120-treated cultures.

This effect could be due to the Vpr protein, or to some contaminant associated with Vpr in the buffer. To show that the observed effect(s) was due to the Vpr protein, the Vpr protein solution was preincubated with an antibody specific for the Vpr protein, or with preimmune serum. Subsequently, duplicate cultures were exposed to either no protein, the Vpr-antibody-treated Vpr protein solution, the preimmune serum-treated Vpr protein solution, or the untreated Vpr protein solution, for 24 h at 200 pg/ml. We selected this concentration of Vpr after determining from previous studies that this Vpr protein concentration roughly approximates the concentration found in the CSF of AIDS patients with central nervous system involvement (Levy *et al*, 1994). The cultures were again assayed using the FD/PI assay, and the results are displayed in Table 1. Cultures exposed to preimmune serum treated Vpr protein looked similar to those cultures exposed to the untreated Vpr protein, having significant increases in the number of PI-labeled cells in those cultures (Table 1, compare row 2 to row 3). Alternatively, exposure of the cultures to the Vpr-specific antibody pretreated protein solution reduced the number of labeled cells to background, or untreated levels (Table 1, compare row 4 to row 1). This clearly suggests that the putative cytotoxic effect(s) are due specifically to the Vpr protein.

Vpr-induced cytotoxicity analyzed for apoptosis or necrosis

The Vpr-induced increases in the numbers of PI-labeled cells observed in the FD/PI assayed mixed cortical cell cultures could be due to necrosis or apoptosis. The next step was to attempt to distinguish between those two possibilities. In a series of experiments, the mixed cultures were either untreated, or treated with 200 pg/ml of Vpr for various time periods ranging from 1 to 144 h. Gp120-induced effects on these cultures were also examined simultaneously as a control. At each time point duplicate cultures were assayed for apoptosis using (1) the Annexin V assay (Table 2), which highlights (fluorescently labels the cytoplasmic membrane) cells displaying a specific cytoplasmic membrane perturbation of phosphatidylserine unique to apoptotic cells early in the programmed death cycle; or (2) the TUNEL assay (Figure 3A–E; Table 2), which highlights (fluorescently labels) cells with chromatin strand breaks which is also a hallmark feature of apoptosis. The quantitative data

Table 1 Specificity of vpr-induced cell death in mixed neuronal cell cultures

Exposure (hours)	*Conditions*[a] *Agent*	*Conc.* (pg/ml)	*Per cent cell death*[b]	*Relative ratio of cell death*[c]
24	UT		6.6±0.5 [1770]	1.0
	Vpr	200	35.5±3.8 [871]	5.2
	Vpr	200+preimmune	40.9±1.3 [902]	5.6
	Vpr	200+Vpr Ab	8.9±1.7 [946]	1.3

[a]Treatment conditions on each culture. Exp.=Time of exposure to the appropriate treatment; Agent=treatment the cultures were exposed to; Conc.=concentration of the agent in pg/ml. [b]This is a collation of the data gathered from two experiments for each condition. The number of propidium iodide stained cells (dead or dying cells), and fluorescein diacetate stained cells were counted for each field scanned (six fields were scanned per plate) per plate in each condition to get the total cell count. The fraction of dead cells was then calculated as the number of propidium iodide stained cells divided by the total number of cells, and is shown with the standard error. The total cells counted for each condition ranged from 871–946. [c]The relative fold cell death is the average fraction of dead cells for each treatment relative to the average basal (untreated=UT) amount of cell death. To calculate this we divided the average percentage of cell death caused by a specific treatment condition by that of the appropriate average basal amount of cell death. preimm.=preimmune serum; Ab=antibody. Using the Sigma Plot computer program, the level of significance (*P*) of the difference between the appropriate untreated control and each treatment condition was measured using the Student's unpaired *t*-test. Using this analysis, both the vpr, and vpr+preimmune serum treatment conditions had levels of cell death that were significantly different from the untreated control levels ($P<0.001$). Alternatively, the vpr+Vpr antibody treatment condition had levels of cell death that was not significantly different from the untreated control levels ($P>0.05$).

Table 2 Vpr-induced apoptosis in mixed neuronal cell cultures

Row	*Exposure* (hours)	*Conditions*[a] *Agent*	*Conc.* (pg/ml)	*Per cent apoptosis*[b]	*Relative ratio of apoptosis*[c]
Annexin V assay					
1	1–24	UT		4.22±0.26	1.0
2	24–144	Vpr	200	15.87±1.35	3.8
3	1–24	Vpr	200	33.03±1.96	7.8
4	24–144	gp 120	26 000	17.34±1.28	4.1
5		gp 120	26 000	36.66±1.79	8.7
TUNEL assay					
6		UT		2.99±0.35	1.0
7	2	Vpr	200	39.40±3.29	13.2

[a]Treatment conditions used on each culture. Exposure=Time of exposure (hours) to the appropriate treatment; Agent=the treatment that those cultures were exposed to; Conc.=the concentration of the agent in pg/ml. [b]The total number of cells were determined for each field (Hoechst staining) scanned (six fields per plate) for each treatment condition. The percentage of the total cells with labeled DNA (TUNEL), or labeled cell membrane (Annexin V) was then calculated, and is displayed for each treatment condition with the calculated standard error. [c]The relative ratio of apoptosis is the average percentage of labeled cells for each treatment relative to the average basal (untreated=UT) percentage of labeled cells. To calculate this we divided the average percentage apoptosis for that specific treatment condition by that of the appropriate average basal percentage of apoptosis. Using the Sigma Plot computer program, the level of significance (*P*) of the difference between the appropriate untreated control and each treatment condition was measured using the Student's unpaired *t*-test. Using this analysis, all treatment conditions had levels of apoptosis that were significantly different from the untreated control levels ($P<0.001$).

from a number of these experiments is displayed in Table 2. For both the Vpr treatment, and gp120 treatment, the quantitative data from the Annexin V analyses performed on monolayers treated for time periods between 1 and 24 h (considered a short exposure) were pooled and considered as one data set. Similarly, the quantitative Annexin V data for exposure periods between 24 and 144 h (considered a long exposure) was also pooled and considered as one data set. A 2 h exposure time was used during experiments assayed by the TUNEL assay.

Short, chronic exposure (1–24 h) of the mixed cell cultures to 200 pg/ml concentrations of Vpr caused a statistically significant increase in the levels of labeled cells observed in these cultures, as assayed by Annexin V. Under these conditions, a statistically significant 3.8-fold increase in the numbers of labeled cells was observed ($P<0.001$; Table 2, row 2). This can be compared to a 4.1-fold increase in labeled cells observed in cultures treated with 26 000 pg/ml of gp120 ($P<0.001$; Table 2, row 4). Similar results were observed when mixed cell cultures were assayed by the TUNEL assay (Table 2, compare rows 6 and 7 to rows 1 and 2; Figure 3, compare B to D).

Long, chronic exposure (24–144 h) to 200 pg/ml of Vpr caused even higher levels of labeled cells in these cultures (Table 2, compare rows 1 and 2 to 3). Again, we compared this to increases caused by long chronic exposure to 26 ng/ml of gp120 (Table 2, row 5). Increases in the number of Annexin V FITC-labeled cells in the presence of Vpr were

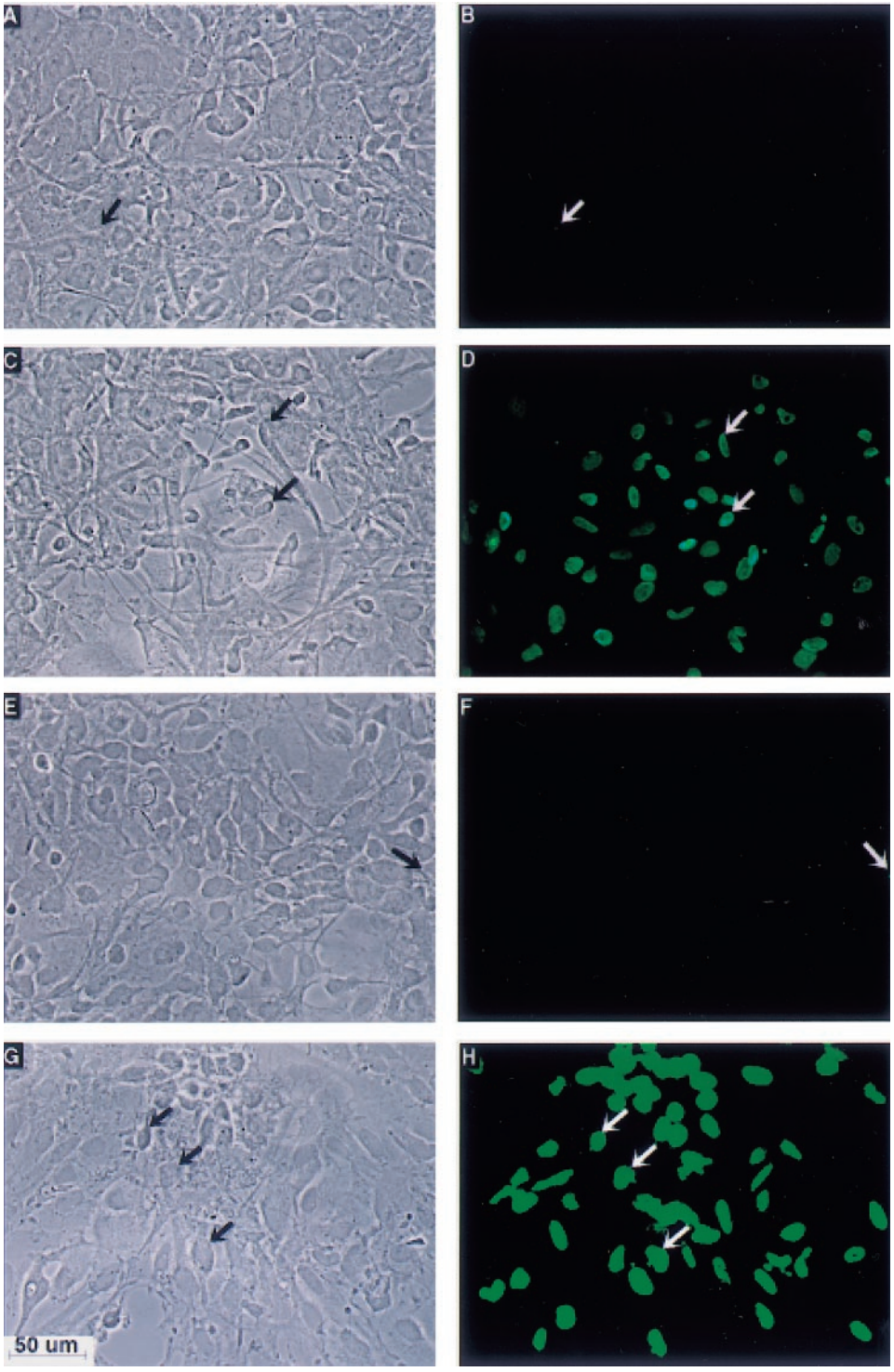

Figure 3 Vpr-induced apoptosis in cell cultures. (**A–D**) Mixed cultures were either untreated (**A**, **B**), or treated with 200 pg/ml of Vpr (**C**, **D**) for 4 h. (**E–H**) Cultures of enriched E16 rat primary GFAP staining (astrocytic) cells were either untreated (**E**, **F**), or treated with 200 pg/ml of Vpr (**G**, **H**) for 4 h. Both sets of cultures were then assayed for apoptosis using the TUNEL assay, highlighting (fluorescently labeling) cells with chromatin strand breaks. Matched phase images (**A**, **C**, **E**, **G**) of the fluorescent fields (**B**, **D**, **F**, **H**) are displayed. Within the images, arrows mark several FITC/TUNEL staining cells identified in the matching phase images.

observed to be dose-dependent over a similar concentration range to that examined in Table 1 (data not shown). These data suggest that Vpr-induced effects are due to apoptotic mechanisms in these cells. However, necrosis causes random DNA strand breakage which could be labeled by the

TUNEL assay, as well as causing membrane disruption which would allow membrane labeling by the Annexin V assay. Thus, these assays alone are not definitive.

Vpr-induced effects appear to act on astrocytic cells
We observed increases in the number of Annexin V and TUNEL-labeled cells in mixed cortical cell cultures, suggesting Vpr induction of apoptosis in these cultures. However, it was unclear what specific cell type(s) contained within these mixed cortical cultures were being affected. To address this issue, we began looking for potential effect(s) of the Vpr protein on enriched cell populations starting with: (1) enriched rat primary GFAP staining (astrocytic) cells; and (2) an astrocytic cell line (C6 cells). Because astrocytic cells in the adult brain would probably be nonproliferating, any effects of circulating viral proteins would have to be exerted on nonproliferating astrocytic cells. Consequently, we attempted to reproduce this condition by doing these experiments on confluent cultures, as well as on actively growing primary cultures. In the initial experiment, cultures of the enriched primary cells were treated with 200 pg/ml of Vpr for 12 h. Duplicate cultures were assayed using the TUNEL assay (Figure 3B,D,F,H; Table 3A) for labeled cells, which should indicate apoptotic cells, and also using Annexin V (data not shown). Significant increases in the number of TUNEL-labeled cells were observed (Figure 3, compare F to H; Table 3A compare UT to Vpr). Additionally, we acquired double-labeling images from the enriched primary astrocytic cultures for (1) DNA strand breaks either untreated or treated (as monitored by TUNEL assay; Figure 4A or E, respectively) and (2) the labeling for astrocytic cells either untreated or treated (as monitored by GFAP staining; Figure 4B or F, respectively) generating a simultaneous TUNEL/GFAP-labeled image (Figure 4C or G, respectively). In the Vpr treated cultures (Figure 4G), the TUNEL/GFAP image clearly displayed GFAP-labeled cells (astrocytes) that also were FITC/TUNEL-labeled for DNA strand breaks. On close examination, some of the TUNEL-labeled nuclei displayed condensed nuclei morphologically characteristic of apoptotic nuclei (Figure 4G, upper lefthand corner). However, many of the TUNEL-labeled nuclei do not have the condensed nuclear appearance. A comparative examination of the GFAP stained images (Figure 4, compare B to G) also points to an overall larger nuclear and cytoplasmic area of the cells, as well as more diffuse (and less) GFAP labeling of the cytoplasm of the cells. The detailed imaging data hint at both Vpr-induced apoptotic as well as necrotic effects in the astrocytic cell cultures. These effects were also observed both in confluent cultures, as well as in actively growing cultures. We observed similar apoptotic effects in pure cultures of primary human astrocytes (data not shown).

As observed in the primary mixed cell cultures, chronic exposure to Vpr caused increases in the number of PI-labeled cells in the C6 cell cultures (Table 3B, compare UT to Vpr). A 12 h exposure to picomolar concentrations of Vpr induced a statistically significant increase in the number of PI-labeled cells in C6 glioma cell cultures. This increase began at 10 pg/ml of Vpr ($P > 0.05$; Figure

Table 3 Vpr-induced cytotoxic effects in astrocytic cell cultures

A. Primary astrocytic cultures

	Conditions[a]		*Per cent*	
Exp. (hours)	*Agent*	*Conc.* (pg/ml)	*Dead cells*[b,d]	*Apoptotic cells*[c,d]
12	UT		3.73 ± 0.38	3.29 ± 0.36
12	Vpr	200	31.87 ± 3.15	43.10 ± 3.20

B. C6 cell cultures

	Conditions		*Per cent*	
Exp. (hours)	*Agent*	*Conc.* (pg/ml)	*Dead cells*	*Apoptotic cells*
12	UT		6.53 ± 1.86	3.27 ± 0.82
12	Vpr	200	39.81 ± 3.53	23.63 ± 2.64
12	gp120	26 000	58.14 ± 5.38	24.25 ± 3.01

[a]Treatment conditions used on each culture. Exp.=Time of exposure to the appropriate treatment; Agent=the treatment the cultures were exposed to; Conc.=concentration of the treatment agent in pg/ml. [b]This is a collation of all the data gathered from multiple experiments for each condition. The number of propidium iodide stained cells (dead or dying cells), and the fluorescein diacetate stained cells were counted for each field scanned (six fields were scanned per plate) in each condition to get the total cell count. The fraction of dead cells was then calculated as the number of propidium iodide stained cells divided by the total number of cells, and is shown with the standard error. [c]The total number of cells were determined for each field (Hoechst staining) scanned (six fields per plate) for each treatment condition. The percentage of the total cells with labeled DNA (TUNEL) was then calculated, and is displayed for each treatment condition with the calculated standard error. [d]Using the Sigma Plot computer program, the level of significant (P) of the difference between the appropriate untreated control and each treatment condition was measured using the Student's unpaired t-test. Using this analysis, all treatment conditions had levels of apoptosis that were significantly different from the untreated control levels ($P > 0.001$).

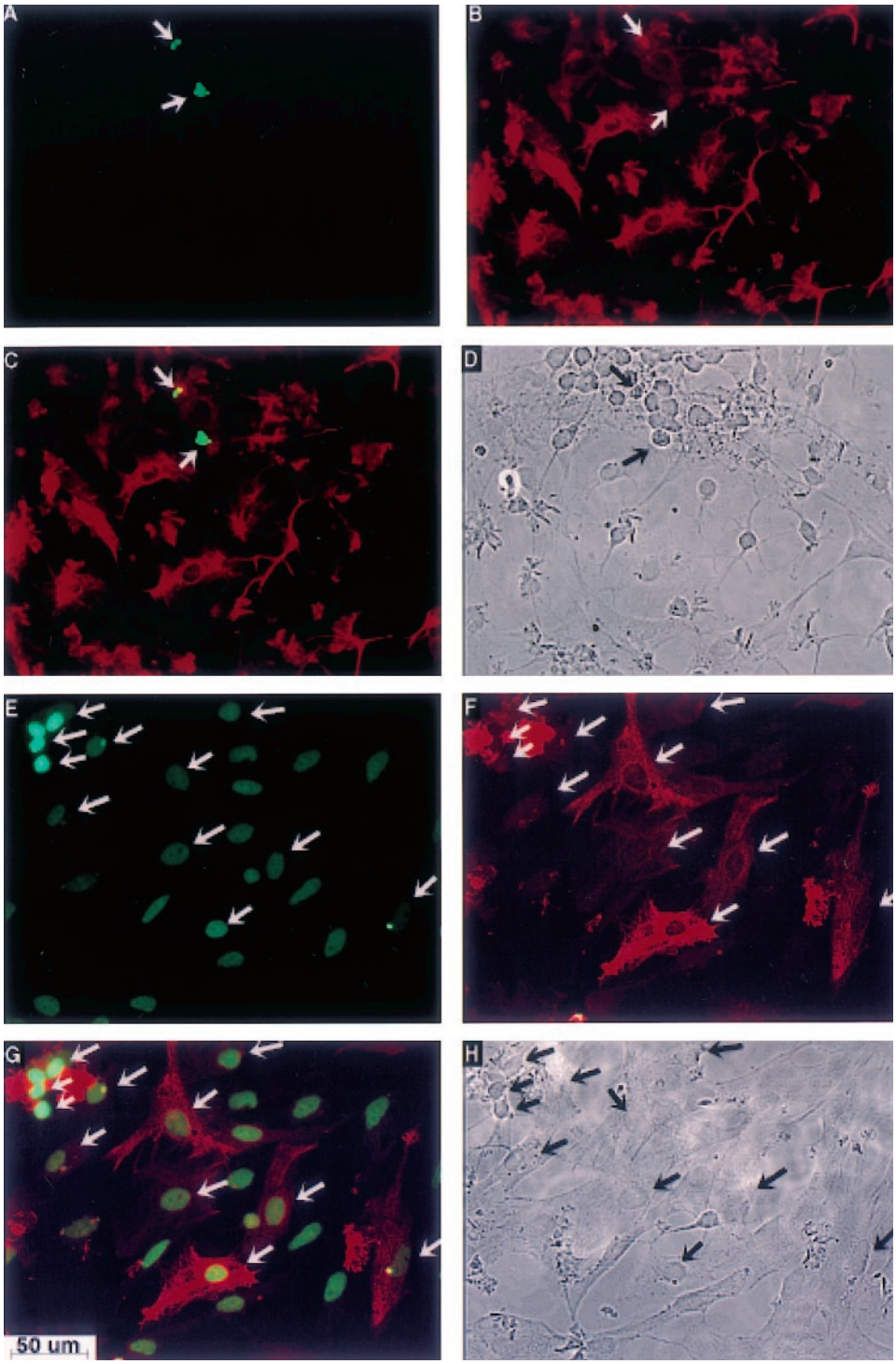

Figure 4 Vpr-induced effects in astrocytic cells. Cultures of enriched E16 rat primary GFAP staining (astrocytic) cells were either untreated (**A–D**), or treated with 200 pg/ml of Vpr (**E–H**) for 4 h. Both sets of cultures were then assayed for apoptosis (**A**, **E**) using the TUNEL assay, highlighting (FITC) cells with chromatin strand breaks. Simultaneously, the cultures were stained for GFAP (**B**, **F**), identifying astrocytic cells, using the fluorescent marker Cy3. Matched phase images (**D**, **H**) of the fluorescent fields are also displayed. Several FITC/Cy3 double-stained cells are identified with arrows between the matching images (**A–D**, and **E–H**).

5), became statistically significant between 10 pg/ml and 100 pg/ml ($P<0.001$; Figure 5), and increased over the entire range of concentrations analyzed. Finally, the Vpr-induced effect(s) on the C6 cultures were unclear as they appeared to be both apoptotic and necrotic in nature. Again, a

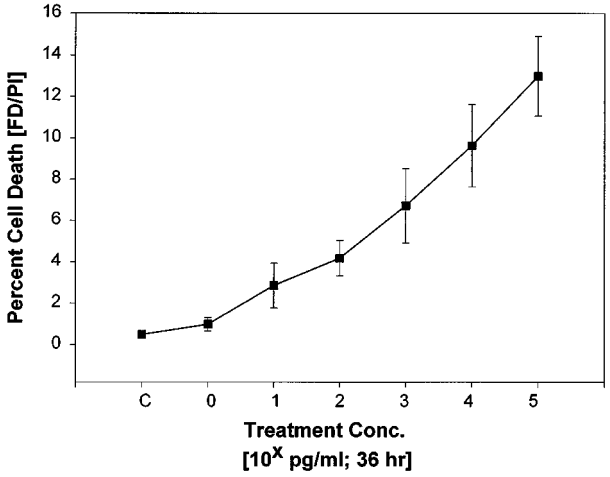

Figure 5 Percentage of cell death in C6 glioma cell cultures as a function of the Vpr concentration. C6 glioma cultures were untreated, or treated with various concentrations of Vpr for 12 h. The cultures were then assayed for PI stained cells (dead or dying cells), and FD stained cells (living cells). These were counted for each field scanned (six fields were scanned per plate) in each condition, and then added to get the total cell count. The percentage dead/dying cells was then calculated as the number of PI-stained cells divided by the total cells. Data from multiple identical experiments was collated, standard errors of the averages calculated, and the resulting data plotted against the treatment concentration ($10\times$). On the y-axis, C denotes the percentage cell death in the control or untreated condition.

significant increase in the number of TUNEL-labeled cells was observed in the Vpr-treated cultures (Figure 6D; Table 3B) compared to the untreated cultures (Figure 6B; Table 3B). However, the morphological appearance of the cells, with no condensed nuclei suggested necrotic effects.

DNA fragmentation

To resolve the issue of whether Vpr is inducing apoptosis or necrosis, we used neutral gel electrophoresis of extracted DNA to look for the laddering banding pattern suggestive of internucleosomal cleavage indicative of apoptosis. C6 cells or primary human astrocytes were either untreated, or treated for 24 h with 200 pg/ml of Vpr, or with 10 mM ceramide, which has been shown to cause apoptosis and the DNA laddering banding pattern in this particular cell line (Yasugi *et al*, 1995). Cultures were then harvested for DNA, and the resultant DNAs electrophoresed and analyzed for DNA fragmentation (Figure 7). Neutral gel electrophoresis of the extracted DNAs revealed the laddering characteristic of apoptotic cells in the ceramide treated cultures of both C6 cells and primary human astrocytes (Figure 7A,B, lane 3). In the Vpr-treated cultures, laddering is observed in both cell types (Figure 7A,B, lane 2). Thus, the evidence suggests that apoptosis is evident in the Vpr-treated cultures.

Flow cytometric analysis of the Vpr-induced effects on C6 cells

Several studies have shown that cells expressing *vpr* appear to be disrupted in their progression through the cell cycle, arresting at the G2 phase (Ayyavoo *et al*, 1997a; Jowett *et al*, 1995; Mahalingam *et al*, 1997; Withers-Ward *et al*, 1997; Zhao *et al*, 1996). Vpr has been observed to form cation-selective channels in membranes (Piller *et al*, 1996), and has been shown to cause cell death in hippocampal neurons, apparently due to this same ability to form channels in the hippocampal neuron membrane (Piller *et al*, 1998). Several studies examined the effects of extracellular Vpr on rhabdomyosarcoma cells (Ayyavoo *et al*, 1997b; Levy *et al*, 1995; Refaeli *et al*, 1995) as well as on both primary and transformed monocytes/macrophage (Levy *et al*, 1995). However, physical evidence that the Vpr protein is being internalized is not given in any of these studies. The only solid physical evidence on the localization of extracellular Vpr protein was obtained by Piller *et al* (1998) who suggested that extracellular Vpr protein interacts at the plasma membrane of cells.

We addressed this issue by treating C6 cultures with extracellular Vpr, examining those cultures for potential effects on the cell cycle using FACS analysis, and comparing this data to that from *vpr* transfected C6 cells. These cultures were stained with PI and sorted for DNA content, which is indicative of the phase of the cell cycle. Simultaneously, the *vpr* expression vector, pVpr, was cotransfected into C6 cultures with a vector coding for an expression marker, IL2r. These cultures were analyzed in a two color sort using flow cytometry, first for cells expressing IL2r, which will be transfected cells, and second those transfected cells, which were also stained with PI, were sorted for DNA content, indicative of the cell cycle phase. The results shown in Figure 8 are a compilation of at least three independent transfections and FACS analyses. In Figure 8A, the cell cycle effects of extracellular Vpr on C6 cultures are shown. The graph displayed in dotted lines are untreated C6 cells, with the plotted results displaying the normal cell cycle pattern with most cells in the G1 phase, and a smaller shoulder covering S phase, and G2/M phase. Treatment of similar cultures with extracellular Vpr protein appears to have no effect on the cell cycle pattern (Figure 8A, solid line) which looks almost identical to that of the untreated cells (broken line). In Figure 8B, the cell cycle effects of Vpr protein expressed endogenously from a transfected Vpr expression vector are shown, and clearly display the G2/M cell cycle arrest pattern observed by other researchers. The graph displayed in dotted lines are C6 cells transfected only with IL2r, the transfection marker. Again, as in Figure 8A, the plotted results display the normal cell cycle pattern with most cells in the G1 phase, and a smaller

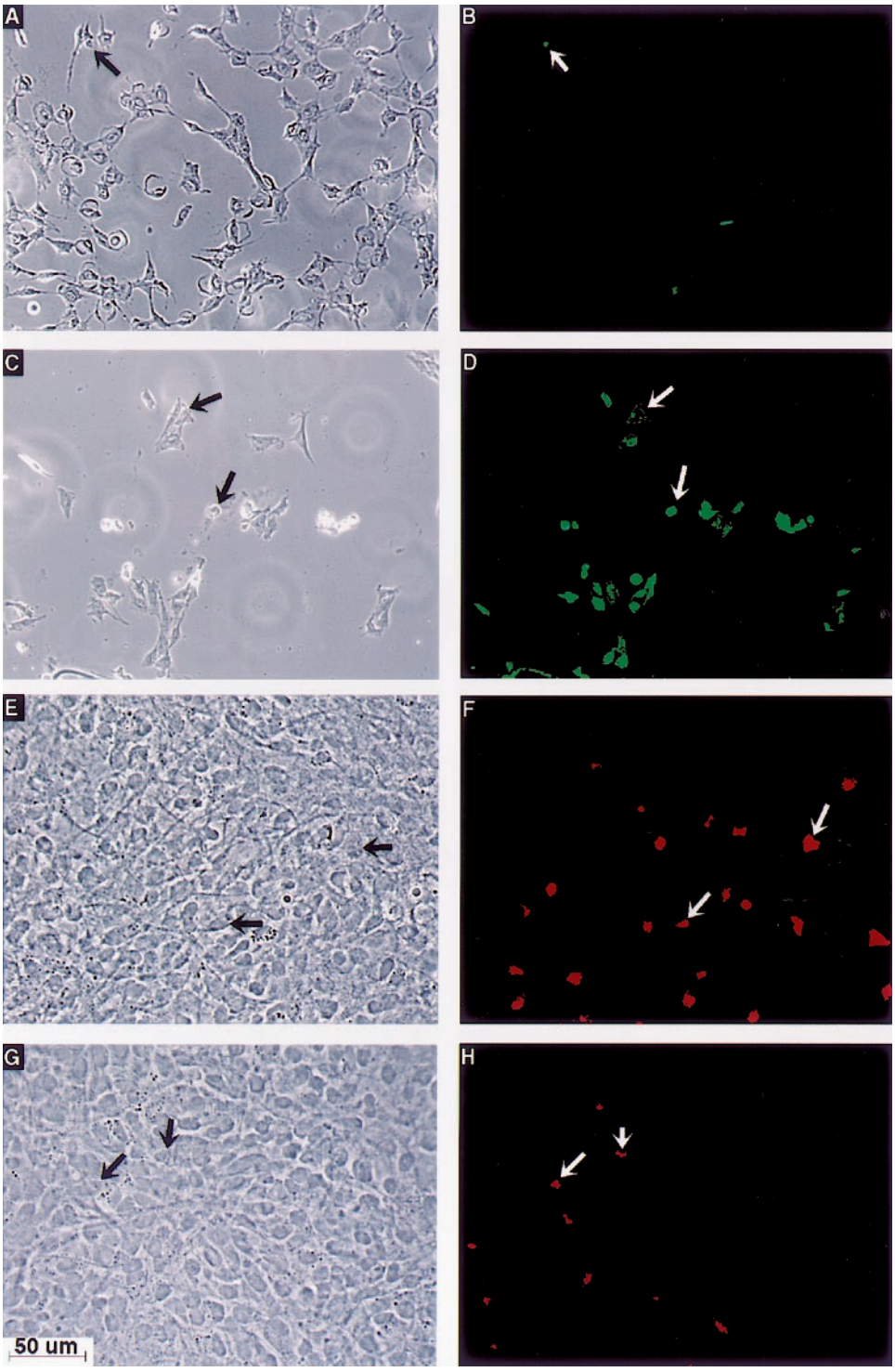

Figure 6 Potential Vpr-induced genomic DNA effects in C6 glioma cell cultures. (**A–D**) C6 glioma cells kept in log phase growth in culture were either untreated (**A**, **B**), or treated with 200 pg/ml Vpr (**C**, **D**) for 12 h. Subsequently, the cultures were assayed for apoptosis using the TUNEL assay, highlighting (FITC) cells with chromatin strand breaks. Matched phase images (**A**, **C**) of the fluorescent fields (**B**, **D**) are displayed, and within the images arrows mark several FITC/TUNEL staining cells identified in both the images. Examination of mixed cell cultures for Vpr-induced effects on NSE staining cells. (**E–H**) Mixed cortical cultures were either untreated (**E**, **F**) or treated with 200 pg/ml of Vpr (**G**, **H**) for 4 h. Cultures were then assayed for NSE (**F**, **H**), identifying neurons, using the fluorescent marker Cy3. Matched phase images (**E**, **G**) of the fluorescent fields are also displayed. Several NSE expressing cells (Cy3) are identified with arrows between the matching images (**A–D**, and **E–H**).

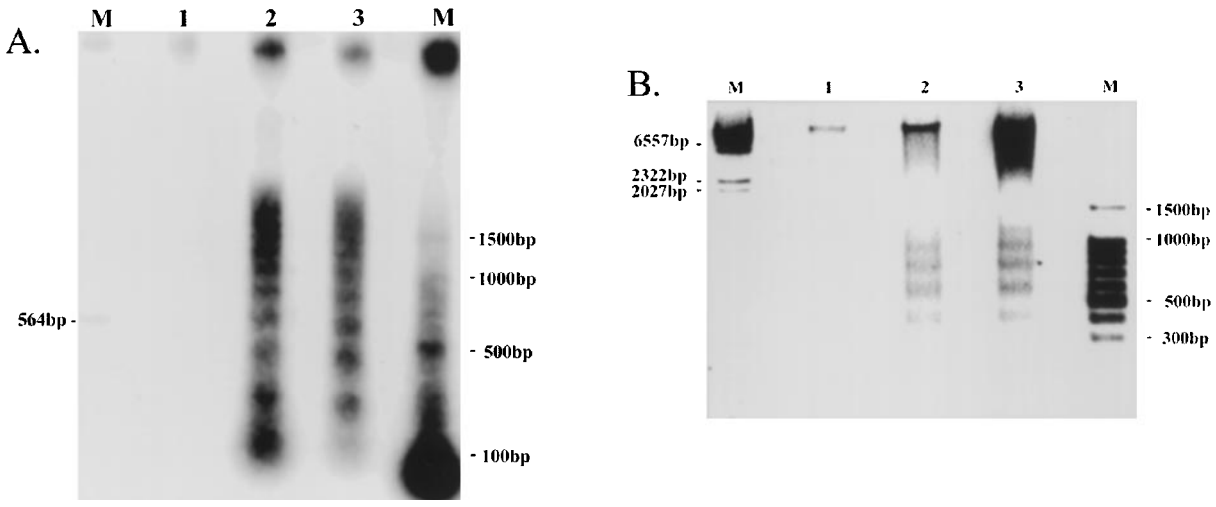

Figure 7 Neutral gel electrophoresis of extracted DNA from untreated, Vpr-treated, and ceramide-treated C6 glioma cells (**A**), and primary human astrocytic cell cultures (**B**). Cells were either untreated (lane 1), or treated for 24 h with 200 pg/ml of Vpr (lane 2), or with 10 mM ceramide (lane 3). Cultures were then harvested for DNA, and the resultant DNA's electrophoresed and analyzed for DNA fragmentation. (M) markers: left-most lane are lambda *Hind*III digested markers, and right-most lane are the DNA ladder markers; (*) bands representative of internucleosomal cleavage products suggesting apoptosis.

shoulder covering S phase, and G2/M phase (Figure 8B). However, the solid line graph depicting the C6 cells transfected with both IL2r and pVpr displayed a significant reduction in the G1 phase peak with a concomitant increase in the G2/M phase peak. The combined results clearly suggest that, although as previously observed, endogenously expressed Vpr protein causes the G2/M cell cycle arrest phenomenon, extracellular Vpr protein does not appear to cause the G2/M cell cycle arrest in similar cells exposed to that protein.

Vpr-induced effects on primary cortical and hippocampal neurons

It appears from the collected evidence that the Vpr-induced effects were acting on astrocytic cells. However, it was still unclear whether Vpr had any effect on the primary cortical neurons contained in the mixed cell cultures. To address this issue, mixed cultures were either untreated, treated with 200 pg/ml of Vpr protein, or treated with 26 ng/ml of gp120, which has already been shown to cause cytotoxicity in several studies. For each condition, duplicate cultures for Vpr, or gp120 were assayed for the percentage of neurons in the cultures using immunocytochemical staining with neuron-specific-enolase (NSE; Figure 6; Table 4). The fraction of total cells in the culture that were NSE staining (neurons) was significantly reduced in the Vpr treated cultures (Figure 6, compare F and H; Table 4, compare row 1 to rows 2–4) from an average of about 26% for untreated cultures to an average of about 10% for Vpr treated cultures. A similar effect was observed for gp120-treated cultures, which displayed an analogous change (Table 4, compare rows 1 to rows 5 and 6). However, we were never able to identify apoptotic neurons in the Vpr-treated cultures as assayed by screening for NSE/TUNEL double-labeled cells in the cultures, although we could identify NSE/TUNEL-labeled neurons in cultures treated with gp120 (data not shown). We used other neuron specific antibodies (e.g., anti-synaptophysin) and observed similar results (data not shown). The observation of no effect on cortical neurons due to extracellular Vpr protein was consistent over a number of separate experiments.

A recent study suggested that free, extracellular Vpr protein can be inserted into the cell membrane of hippocampal neurons forming cation-selective ion channels which subsequently disrupt the cellular ionic gradient and cause cell death (Piller *et al*, 1998). The effects of Vpr described by Piller *et al* could similarly explain the effects of Vpr on astrocytes we describe. However, as stated above, we did not observe any effects on primary cortical neurons. We next looked at the effect(s) of Vpr on mixed hippocampal cultures to determine whether we would also observe Vpr-induced hippocampal neuron toxicity. Mixed hippocampal cultures were either untreated, or treated with 200 pg/ml of Vpr for 24 h. Duplicate cultures were assayed for effects on either astrocytes, or neurons using cell-specific staining (GFAP or NSE), and for apoptosis using the TUNEL assay. Significant increases in the number of TUNEL/FITC-labeled cells were observed in the Vpr treated cultures (Figure 9B,F) when compared to the untreated cultures (Figure 9D,H). Additionally, in the Vpr treated cultures (Figure 9B), the

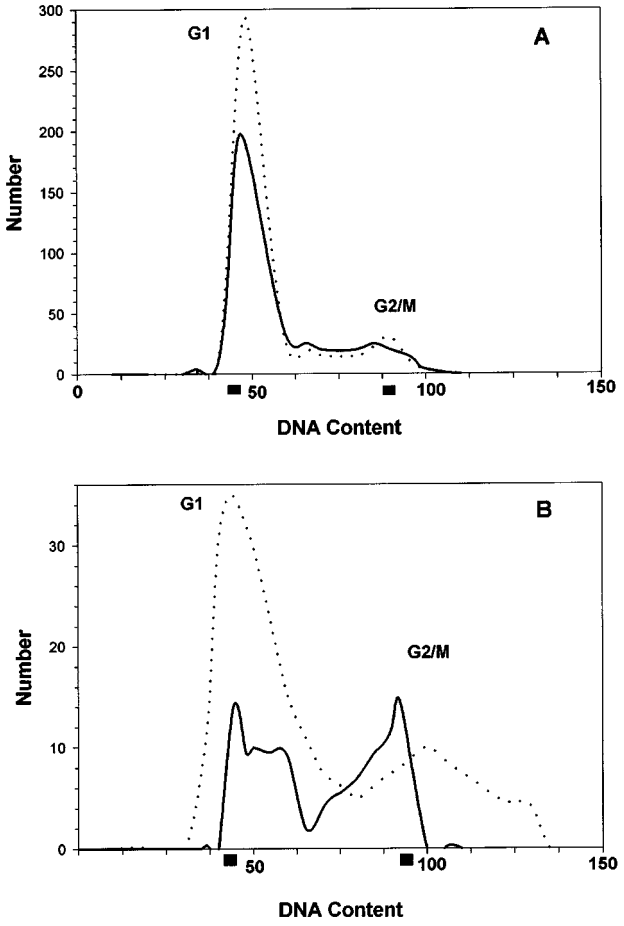

Figure 8 Flow cytometric analysis of C6 glioma cell cultures either exposed to extracellular Vpr protein or transfected with a Vpr expression vector. (**A**) C6 glioma cell cultures were exposed to 200 pg/ml of Vpr protein for 24 h, harvested, fixed, stained with PI, and FACS analyzed. The cell data gathered from the FACS analysis was graphed by plotting the proportion of the cells sorted (y-axis) against the relative concentration of DNA in the cell (x-axis). This generates a graph that predicts the proportion of cells in G1, S and G2/M phases of the cell cycle. Graph of the untreated cultures is illustrated by the dotted line, and the data from cultures exposed to extracellular Vpr protein is illustrated by the solid line. (**B**) C6 cultures were cotransfected with pVpr and pMLSV N1/N4-S, incubated for 48 h, harvested, and fixed. Subsequently, they were stained with a CD25 (anti-interleukin-2 receptor, human) mono-clonal, FITC conjugated antibody and PI, and FACS analyzed first for IL-2r/transfected cells, and second for PI/DNA content. The cell data gathered from the FACS analysis was graphed by plotting the proportion of the cells sorted (y-axis) against the relative concentration of DNA in the cell (x-axis) generating a graph predicting the proportion of cells in G1, S and G2/M phases of the cell cycle. The graph of cultures transfected with IL-2r only is illustrated by the dotted line, with the data from cultures transfected with both IL-2r and pVpr being illustrated by the solid line.

double TUNEL/GFAP image clearly displayed GFAP-labeled cells (astrocytes) that also were FITC/TUNEL-labeled for DNA strand breaks. Finally, in the Vpr treated cultures, the double TUNEL/NSE image (Figure 9F) clearly displayed NSE-labeled cells (neurons) that also were FITC/TUNEL-labeled for DNA strand breaks. We used other neuron specific antibodies (e.g., anti-synaptophysin) and observed similar results (data not shown). This data clearly shows Vpr-induced cytotoxicity in hippocampal neurons, and hints at apoptotic effects in hippocampal neurons.

Discussion

A consistent, and significant increase in the fraction of PI-stained cells was observed when E16 rat primary mixed cortical cell cultures were chronically exposed to the Vpr protein. A number of studies have shown that gp120 causes cell death in rat and human brain cell cultures, which was observed as an increase in the fraction of PI-stained cells in a FD/PI assay (Lannuzel *et al*, 1997; Meucci and Miller, 1996; Muller *et al*, 1992). The observed Vpr-induced effect on cortical cell cultures was compared to the cytotoxic effect caused by gp120 in similar cultures. The increases in PI staining caused by Vpr at any specific protein concentration were similar to increases caused by gp120. Both proteins caused increasing amounts of PI staining over the entire range of concentrations analyzed. A significant reduction, essentially to background levels, in the fraction of PI-stained cells was observed when the Vpr protein solution was pretreated with an anti-Vpr antibody, and subsequently used to treat the cultures. This was true whether the antigen-antibody complexes were removed with beads before using the protein solution to treat the cultures, or whether the antigen-antibody complexes were left in the protein solution. Alternatively, a preimmune serum had no effect on Vpr-induced increases in PI staining in the cultures. This evidence suggests that the extracellular Vpr protein, like gp120, is cytotoxic on exposure to primary rat cortical cultures. We also have screened an untagged recombinant Vpr protein in these assays and have observed identical results to those described above using the tagged recombinant protein (unpublished data).

Data from both the TUNEL assay, and the Annexin V assay suggest that this chronic, Vpr-induced cytotoxicity causes DNA strand breakage and membrane disruption characteristic of apoptosis. Increases in the amount of Annexin V assayed cell membrane labeling were observed over a range of Vpr exposure times, as well as over a range of Vpr concentrations (data not shown). For example, in the Annexin V assay, at 200 pg/ml Vpr, a 3.8-fold increase in the number of membrane-labeled cells was observed. This is compared to a 4.1-fold increase observed in cultures treated with 26 000 pg/ml of gp120, which has already been shown to induce apoptosis in mixed brain cell cultures. Data from Vpr and gp120 treated cells assayed using the TUNEL assay displayed a similar

Table 4 Effect of Vpr exposure on per cent neurons in mixed neuronal cell cultures

Exposure (hours)	*Conditions*[a] *Agent*	*Conc.* (pg/ml)	*Per cent neurons*[b]	*Relative ratio of neurons*[c]
	UT		26.19±1.24	1.00
24	Vpr	200	10.72±0.86	0.42
48	Vpr	200	8.37±2.57	0.31
72	Vpr	200	11.67±1.65	0.43
24	gp120	26 000	11.10±1.92	0.41
72	gp120	26 000	9.48±3.65	0.35

[a]Treatment conditions used on each culture. Exp.=Time of exposure to the appropriate treatment; Agent=treatment the cultures were exposed to; Conc.=concentration of the agent in pg/ml. UT=untreated. [b]This is a collation of all data gathered from at least two experiments for each condition. The total number of cells were determined (Hoechst staining) for each field scanned (six fields per plate) for each treatment condition. The percentage of the total neuron-specific enolase (NSE) antibody/FITC-labeled cells was then calculated, and is displayed for each treatment condition with the calculated standard error. [c]The relative ratio of neurons is the average percentage of NSE/FITC-labeled cells observed following each treatment relative to the average basal (UT) percentage of NSE/FITC-labeled cells. [d]Using the Sigma Plot computer program, the level of significance (*P*) of the difference between the appropriate untreated control and each treatment condition was measured using the Student's unpaired *t*=test. Using this analysis, all treatment conditions had levels of apoptosis that were significantly different from the untreated control levels ($P>0.001$).

pattern to the Annexin V data. Consequently, this statistically significant increase in the fraction of TUNEL- or Annexin-V labeled cells suggested that exposure of the cultures to the Vpr protein caused these effects in one or more of the cell types in these cultures. Finally, the observed levels of Vpr-induced strand breakage (as measured by TUNEL), or membrane perturbation (FD/PI) in these cultures was similar to levels induced by gp120. However, necrosis causes random DNA strand breakage which could be labeled by the TUNEL assay, as well as membrane disruption which would allow membrane labeling by the Annexin V assay. Thus, these assays alone are not definitive.

We next determined which of the several cell types contained within these cultures were being affected by Vpr. We looked at the potential effect(s) of the Vpr protein on: (1) enriched rat primary GFAP staining (astrocytic) cells; (2) an astrocytic cell line (C6 glioma cells); and primary human astrocytes. The data from several different assays clearly demonstrates that Vpr protein caused a statistically significant increase in the fraction of TUNEL-labeled cells in chronically exposed cultures. Further, we were able to definitively identify specific TUNEL-labeled astrocytes, distinguished as also being GFAP-labeled cells. However, morphological analysis of the cells in these cultures suggested that both apoptosis and necrosis were present, which is still consistent with the TUNEL and Annexin V data. Necrosis also causes membrane disruption which would allow cytoplasmic membrane labeling by the Annexin V assay. Thus, the data at this point could be consistent with either necrosis or apoptosis in the astrocytic cells fraction of the cortical cultures. However, clearly some fraction of the cells in the Vpr-treated cultures appear to be going through apoptosis, as evidenced by the DNA fragmentation assay. A clear apoptotic ladder can be observed for both C6 cells and primary human astrocytes exposed to Vpr protein, but not in unexposed cultures. Thus, the combined data suggest evidence of both necrosis and apoptosis in these cultures. Further, the effects of Vpr protein appear to occur in both human and rat astrocytes.

A recent study suggests that extracellular Vpr protein can be inserted into the cell membrane of hippocampal neurons causing cation-selective ion channels which subsequently disrupt the cellular ionic gradient and cause cell death (Piller *et al*, 1998). It is possible that disruption of the cellular ionic gradient could lead to either necrosis or apoptosis, depending on the severity of the disruption and the specificity of the resultant ion channels. The effects observed by Piller *et al* could similarly be causing the effects we observed in cortical astrocytes, C6 cells, and human astrocytes. It is interesting that Piller *et al* mention they observed no cytotoxic effects of Vpr protein on C6 glioma cells in unpublished preliminary experiments (Piller *et al*, 1998). However, they show no data, and draw no definitive conclusions from it. Alternatively, we have performed multiple experiments with both cultured C6 glioma cells, primary rat cortical as well as hippocampal astrocytes, and primary human astrocytes. All of these experiments clearly indicate that Vpr protein is cytotoxic in astrocytes.

We observed no Vpr-induced effect(s) on primary cortical neurons although we did observe a definite Vpr-induced cytotoxicity (which is possibly apoptotic) on primary hippocampal neurons, as well as hippocampal astrocytes. Additionally, it appears from the Vpr-treated culture images that the treated hippocampal neurons stained for NSE, which in our hands stains primarily the cell body, do not stain nearly as intensely for the NSE protein as those neurons in the untreated cultures. This observation suggests that Vpr induced a reduction in expression of the NSE protein. Most importantly, the data clearly suggests that the Vpr protein has a differ-

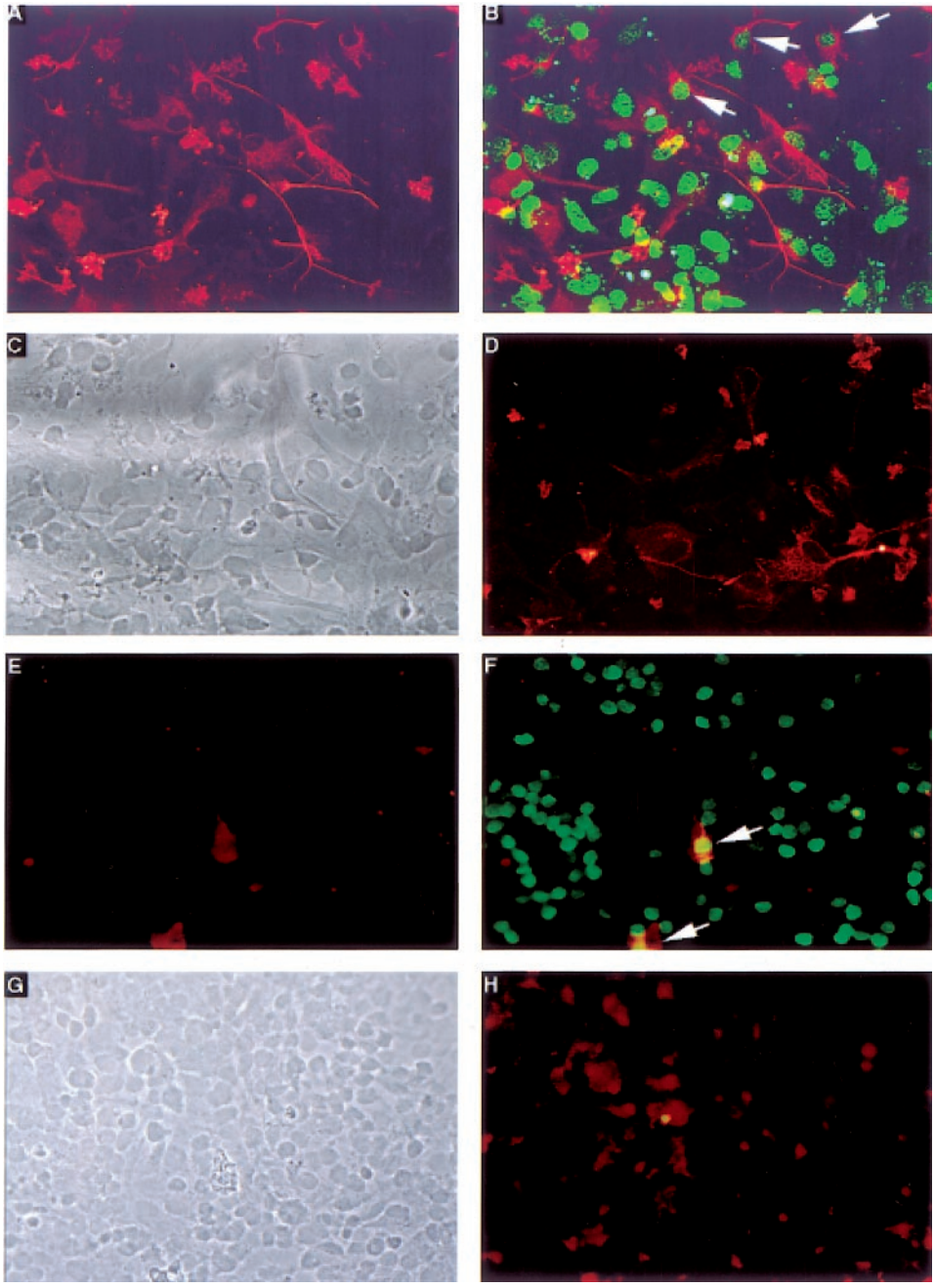

Figure 9 Vpr-induced effects in primary rat hippocampal cell cultures. The hippocampal region of 16 day rat embryo brains was harvested, dispersed onto coated plates, and grown for 7 days. These cultures were either untreated (**D**, **H**), or treated (**A – C**, **E – G**) with 200 pg/ml of Vpr for 24 h. The resultant slides were assayed for apoptosis using the TUNEL assay, highlighting (FITC) cells with chromatin strand breaks. (**A – D**) Display mixed hippocampal cell cultures stained for GFAP (astrocytes; Cy3). (**E – H**) Display mixed hippocampal cultures stained for NSE (neurons; Cy3). Matched phase images (**C**, **G**) of the fluorescent fields (**A**, **B**, and **E**, **F**) are displayed. Within the double TUNEL/GFAP image (**B**) arrows mark several double staining cells (apoptotic hippocampal astrocytes). Similarly, within the double TUNEL/NSE image (**F**) arrows mark several double staining cells (apoptotic hippocampal neurons).

ential ability to affect neurons. However, the differential Vpr-induced effects we observed in neurons do not seem to extend to similar effects on astrocytes, as Vpr clearly seems to affect all astrocytes thus far examined. Interestingly, we have also screened for Vpr-induced effects on peripheral neurons (superior cervical ganglion neurons), and observed no effect on these peripheral neurons also (unpublished data). Piller *et al* (1998) suggest that Vpr, once associated with the cell membrane, may only be induced into active channel activity as a function of that cell's resting membrane potential. They infer that unpublished preliminary data on the lack of effect of Vpr protein on C6 glioma cells

support this idea. However, our definitive studies on the strong effect of Vpr protein on C6 glioma cells and primary human astrocytes appears not to support their model.

Data gathered in this study suggest that the cellular effects of extracellular Vpr protein are not due to the G2 arrest phenomenon observed by other researchers when the Vpr protein is expressed endogenously in the affected cell (Ayyavoo *et al*, 1997a; Jowett *et al*, 1995; Mahalingam *et al,* 1997; Withers-Ward *et al*, 1997; Zhao *et al*, 1996). In one study, researchers have observed HIV-1 regulatory protein-induced cytotoxic/apoptotic effects on primary astrocytes (He *et al*, 1997). They concluded that on infection of mixed brain cultures with HIV-1, expression of the Nef protein induced the receptor tyrosine kinase, c-kit, in astrocytes through transactivation, which subsequently induced apoptotic death in those cells. However, they were observing intracellularly expressed Nef protein, which was subsequently affecting those cells through intracellular mechanisms. In a second series of studies which examined the effects of extracellular Vpr on rhabdomyosarcoma cells (Ayyavoo *et al*, 1997b; Levy *et al*, 1995; Refaeli *et al*, 1995) as well as both primary and transformed monocytes/macrophage (Levy *et al*, 1995), the data suggested that, in these cell types, extracellular Vpr does appear to cause cell cycle effects. However, physical evidence that the Vpr protein is being internalized was not given in any of these studies, suggesting that all the observed effects, including the cell cycle effects, could be receptor-mediated at the cell membrane. In fact, if one extrapolates from the one solid piece of physical evidence on the location of extra cellularly applied Vpr protein obtained by Piller *et al* one would believe that extracellular Vpr protein interacts at the plasma membrane of the astrocytes (Pillar *et al*, 1998).

However, our results in combination with those of Piller *et al* (1998) suggest several other possible scenarios specifically concerning neurons. One, the lipid bilayer makeup of hippocampal neurons could be different from those of cortical and SCG neurons. Effects on Vpr-induced effects due to differences in lipid content is suggested by the first paper by Piller *et al* (1996) in which they show channel formation by the Vpr protein in *in vitro* formed planar lipid bilayers containing no other proteins/peptides. Two, hippocampal neuron membranes contain a protein(s) that enhances the ability of Vpr to form channels. Three, cortical and SCG neuron membranes contain a protein(s) that disrupts that ability of Vpr to form channels. We are currently examining these and other possibilities for why Vpr would have differential effects in similar and different cell types.

The Vpr-treated cortical cell cultures were examined using NSE staining for changes in the fraction of primary neurons in the cultures over time. The data clearly indicated that cortical neurons, as identified by NSE labeling, were disappearing from those cultures as a function of time. However, as discussed above, we found no clear cases of double-labeled cortical neurons on examination of these cultures for either PI/NSE-labeled cells, or TUNEL/NSE-labeled cells. Alternatively, double-labeled cells were identified in the gp120 treated cultures. In these cortical mixed cultures, the astrocytes form a basement monolayer on top of which the neurons then attach themselves. One likely scenario is that Vpr-induced killing of the basement astrocytic cell monolayer caused the neurons attached to these astrocytes to detach and float away, although it did not kill the attached neurons. Consequently, no change would be observed in the number of dying or apoptotic neurons, although changes in the fraction of neurons per culture would be observed.

Our observations on the cytotoxic and necrotic/apoptotic effects of Vpr protein on astrocytic cells, and the lack of a Vpr-induced effect on cortical neurons are quite clear. Additionally, the observation on (1) lack of extracellular Vpr protein-induced effects on the cell cycle of C6 cells, and (2) the lack of extracellular Vpr protein induced cytotoxic effect on cortical neurons with a strong cytotoxic effect on hippocampal neurons make two points. First, Vpr may act at different physical sites on different cell types. By this we suggest that Vpr acts at the cell membrane in astrocytes or neurons, but in other cells (e.g., rhabdomyosarcoma cells) it can be internalized acting endogenously in the cytoplasm. Second, Vpr appears to have differential effects on similar cell types. We observed no cytotoxic effects of extracellular Vpr protein on cortical neurons, but both Piller as well as ourselves have observed clear cytotoxic effects of extracellular Vpr protein on hippocampal neurons.

As was mentioned above, detectable concentrations of Vpr have been observed in the cerebrospinal fluid (CSF) of HIV+ individuals, and Vpr appears to exist in higher concentrations in the CSF of HIV+ individuals displaying neuropathogenic symptoms (Levy *et al*, 1994). However, in this study, the issue of which cell/compartment extracellular CSF Vpr protein is expressed in is not addressed, and we start with the fact that significant concentrations of Vpr protein exist in the CSF in HIV-infected patients, and ask what potential effect(s) this could have. The concentration of Vpr protein used in our experiments (200 pg/ml) was roughly calculated to be close to the concentrations that would be observed *in vivo* in the CSF during an HIV-1 infection, and thus would be physiologically relevant. Also, the central nervous system of infected patients would be exposed to this concentration of circulating Vpr for much longer than our primary cultures were exposed to Vpr. There is no direct clinical evidence to suggest this, and an

analysis of this phenomenon *in vivo* during the early and mid-stages of an HIV infection would be very difficult, if not impossible. However, it is conceivable that Vpr could produce similar, or perhaps even more robust effects. Subsequent changes, such as a large reduction in the number of astrocytic cells in the brain could lead to significant changes in the central nervous system physiology.

Materials and methods

Proteins, antibodies, constructs and other reagents The Vpr protein was expressed and purified from *E. coli*, and contains a tag sequence located at the C-terminal (SPAWRRASVLEDYKDDDKGHHHHHH), which is recognizable by a monoclonal antibody (Zhao *et al*, 1994). The gp120 protein was obtained from Intracel (Issaquah, WA, USA; Cat.#12001), is the IIIB variant, and was measured to be $>90\%$ pure as estimated by Coomassie blue gel staining. The following antibodies were used: a Vpr rabbit polyclonal antibody (AIDS Research and Reference Reagent Program, Catalog #3252); a Glial Fibrillary Acidic Protein (GFAP) antibody (Boehringer Mannheim, Indianapolis, IN, USA); a neuron-specific enolase (NSE) mouse monoclonal antibody, NCL-NSE2 (Novacastra Lab, Ltd., UK); a goat anti-mouse IgG(H+L), $F(ab')_2$-FITC antibody (Boehringer Mannheim, Indianapolis, IN, USA); sheep anti-mouse IgG $F(ab)_2$-Cy3 antibody (Sigma, St. Louis, MO, USA); a CD25 (anti-interleukin-2 receptor human) monoclonal, FITC-conjugated antibody (Becton Dickinson, San Jose, CA, USA). Ceramide was obtained from RBI (Natick, MA, USA). The pVpr expression vector was constructed in the laboratory. It was designed by cloning a full length Vpr gene PCR fragment from pNL4-3 into the pCR3.1-uni vector (Invitrogen) constructed with a HA tag. Expression from this vector was checked both by ICA and Western analysis using either the Vpr antibody, or an HA antibody (data not shown). pMLSV N1/N4-S, is an SV40 promoted expression vector containing the interleukin 2 receptor, alpha gene (ATCC #39890). It was obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA.

Cell cultures

16 day embryonic rat cortical mixed cell cultures Primary cell cultures were prepared from the isolated neocortex of the fetus of 16 day timed pregnant female Sprague-Dawley rats using a procedure similar to that described by Furschpan and Potter (1989). Briefly, the excised neocortex regions from E16 rats were put in chilled media in a 15 ml tube. The tissue was then triturated with a 5 ml plastic pipette 50 times. Large tissue chunks left were allowed to settle, and the dispersed cell/media was removed to another 15 ml tube. The dispersed cells were counted, and assayed for viability by trypan blue exclusion. Plates were prepared with a hole drilled out, and a well formed by mounting a glass coverslip (Assistent Deckglaser/Carolina Biological Supply, Burlington, NC, USA) over the hole using sylgard (Dow Corning, Midland, MI, USA). Plates were dried at 37°C for 2 h, and sterilized by exposure to UV light using a 15 W G15TB/GL-15 germicidal lamp for 30 min at 30 cm. Subsequently, the glass well bottoms were exposed to a 100 μl mixture of 33 μg/ml laminin, and 0.1 mg/ml poly-D-lysine in ddH_2O (Boehringer Mannheim, Indianapolis, IN, USA) overnight at 37°C, followed by three rinses with 3 ml of ddH_2O. The plates were then filled with Hanks Balanced Salt Solution, without phenol red (Gibco), and stored at 37°C. The dispersed mixed rat cortical cells were plated at 2×10^5 cells/well in 100 μl plating media, and incubated at 37°C in a CO_2 incubator for several hours to allow the cells to adhere. Then, 1.0 ml of additional growth media was added to the plates, and the plates were reincubated at 37°C in the CO_2 incubator, feeding every 3–4 days. The cultured cells were used for experiments at about 12–14 days postplating.

16 day embryonic rat hippocampal mixed cell cultures These cultures were prepared from the isolated hippocampal region of the fetus of 16 day timed pregnant female Sprague-Dawley rats essentially as described above for cortical cultures. These cultures were used for experiments at about 5–7 days postplating.

Culture medium Premedium contained DMEM with high glucose (Gibco, Gaithersburg, MD, USA), 10 ml of 100 mM sodium pyruvate, and 4.5 ml of modified stable vitamin mix [0.6 g L-proline, 0.6 g L-cystine, 0.2 g p-aminobenzoic acid, 80 mg Vitamin B12, 0.4 g I-inositol, 0.4 g choline chloride, 1.0 g fumaric acid, 16 mg Coenzyme A, 20 mg DL-6,8-Thiootic acid, and 1.0 mg d-Biotin (Sigma) per 200 ml solution]. 500 u/ml penicillin; 0.5 mg/ml streptomycin; 8.4 mg/ml α-ketoglutaric acid, monosodium salt (Sigma, St. Louis, MO, USA); 5 ml of putrescine, 1.6 mg/ml (Sigma); 5 ml of transferrin, 5 mg/ml (Sigma); and 5 ml of I..T.S., 0.5 mg/ml insulin, 0.5 mg/ml sodium selenite, 0.5 mg/ml human transferrin (Sigma) were then added to 100 ml of premedium. This was filter sterilized using 0.22 μm Millipore millex-GS filters (Millipore, Bedford, MA, USA). After filtration, 4% rat serum was additionally added to make plating media, and 2% rat serum was added to make growth media.

Enriched GFAP-positive cell cultures Astrocytes were prepared by harvesting the mixed embryonic rat cultures 14 days postplating using 1× Trypsin-EDTA (Gibco). The harvested cells were diluted,

and replated on 35 mm tissue culture plates (NUNC, Napierville, IL, USA) coated with 0.5 ml of a 25 μg/ml collagen preparation (Sigma). Cells were plated at 1.2×10^5 cells/plate, and incubated at 37°C for 7–14 days. Cultures were obtained that contained between 70 and 90% GFAP-positive cells.

C6 cell cultures C6 rat glioma cells (American Type Culture Collection, #CCL-107, NJ, USA) were maintained in DMEM with high glucose, without sodium pyruvate, and supplemented with 10% FBS (Gibco), 1% glutamine (Gibco), 1% antibiotic/antimycotic (Gibco), and 1% nonessential amino acids (Gibco).

Primary human astrocyte cultures Primary human astrocytes were obtained from Biowhittaker Technologies (Wikersville, MD, USA). These were cultured as per the instruction from Biowhittaker.

Immunoprecipitation
An aliquot of Vpr at 200 ng/μl was mixed *in vitro* with 2 μl of rabbit anti-Vpr antibody, or rabbit IgG, and incubated at 37°C overnight. Part of the antibody treated solution was used directly in cytotoxicity assays, and part was mixed with 100 μl of anti-rabbit IgG agarose beads (Sigma, St. Louis, MO, USA) and incubated overnight at 4°C. This suspension was centrifuged, and the supernatant then used in cytotoxicity assays.

Cell viability assay (FD/PI)
In initial experiments, the cultures were treated with Vpr and gp120 protein at varying concentrations in growth media, and for various lengths of time, or with ceramide at 10 mM in growth media (see Results). In most experiments, the cultures were treated with 200 pg/ml of Vpr. This concentration was selected as being roughly equivalent to the concentration of Vpr in the CSF of AIDS patients with neurological pathologies. We did this calculation using data from two sources: (1) data gathered by Levy *et al* (1994) which they used to determine the optical densities (OD) of p24 and Vpr in the serum and CSF of AIDS patients without neurological pathologies ($\sim$1100 for p24, and $\sim$400 for Vpr in serum) and AIDS patients with neurological pathologies ($\sim$1100 for p24, and $\sim$1600 for Vpr in serum, and $\sim$1800 for Vpr in CSF); and (2) data gathered over the course of many studies (Saltarelli *et al*, 1996) suggesting that the average concentration of p24 in AIDS patient serum is roughly 100 pg/ml. OD of Vpr in CSF/OD of p24 in serum=1.45. (1.45)$\times$(100 pg/ml)=145 pg/ml. Thus, the rough concentration of Vpr in the CSF of AIDS patients with neurological pathologies is $\sim$145 pg/ml. We rounded it to 200 pg/ml for easy dilution.

The culturing media was then aspirated off the cultures, and the cells washed three times with 1$\times$PBS. 10 μl of stock Fluorescein Diacetate (FD; Sigma), made by diluting 50 mg FD in 10 ml acetone, was diluted into 2.5 ml fresh DPBS to make FD working solution. Then, 1 ml of FD working solution was added to 0.3 ml of a propodium iodide (PI; Sigma) stock, made by diluting 1 mg PI into 50 ml DPBS, to make the FD/PI cocktail. Enough FD/PI cocktail was added to cover the cell monolayer on the coverslip, followed by incubation at 22–24°C for 3 min. The coverslips were inverted onto a microscope slide, and the cultures were then viewed immediately by epifluorescence at 450 excitation, 520 barrier, using a computer controlled microscope system based on a Zeiss microscope (Carl Zeiss, Thornwood, NY, USA). Microscopic images were obtained using a charged coupled device (CCD) camera, MC 100 SPOT, 60910 (Photonic Science, East Sussex, UK). Images were examined using Image-Pro Plus 2.0 (Media Cybernetics, Silver Springs, MD, USA) software.

Apoptosis assays
Annexin V apoptosis assay Apoptosis was monitored through the rapid redistribution of phosphatidylserine (PS) from the interior face of the plasma membrane to the outer plasma membrane surface. This event is an active process, but its function is unclear. Annexin V is a member of a family of proteins with high Ca^{2+}-dependent affinities for aminophospholipids, and has been shown to have affinity for PS. Following the manufacturer's procedure for the ApoAlert™ Annexin V Apoptosis Kit (Clontech, Palo Alto, CA, USA), briefly, the coverslips were washed with PBS, and covered with 51.4 μl of Annexin V-FITC/PI working solution. Annexin V-FITC/PI working solution is 150 μl of 1$\times$ binding buffer, 3.75 μl of Enhanced Annexin V-FITC (stock: 20 μg/ml in Tris-NaCl buffer), 0.3 μl of PI (50 μg/ml in 1$\times$ binding buffer), mixed well. The coverslip was inverted onto a drop of Annexin V-FITC/PI working solution on the glass slide, and incubated at room temperature for 15 min in the dark.

TUNEL assay Apoptotic generated DNA free ends were labeled *in situ* using terminal deoxynucleotidyl-transferase (TdT) to incorporate exogenously added labeled nucleotides. This label was then visualized directly by fluorescence. Cells on coverslips were washed with PBS, and fixed for 30 min at room temperature (RT) with 4% paraformaldehyde, in PBS, pH 7.4. They were then washed with PBS, and permeabilized with 0.1% Triton X-100 for 10 min at RT. The slides were rinsed twice with PBS, and air dried for 2 min. Then, 50 μl of TUNEL reaction mixture was added per coverslip, and incubated for 1 h at 37°C, followed by three rinses

with PBS (these are manufacturer's procedures for the In Situ Cell Death Detection Kit, AP; Boehringer Mannheim). In both apoptotic assays, total cell counts were determined by counterstaining fixed cultures with 20 μg/ml of Hoechst 33258. Finally, all specimens were observed by epifluorescence on a computer controlled microscope system based on a Zeiss microscope (Carl Zeiss), and the images were obtained, and examined using a CCD camera (Photonic Science), and Image Pro software (Media Cybernetics).

Agarose gel electrophoresis/DNA fragmentation
Untreated or treated cells were collected, and DNA was harvested from each treatment condition (Herrmann *et al*, 1994). Briefly, the cells were washed with PBS, pelleted at 1600 × g for 20 min at 4°C, and resuspended in 50 mM Tris-HCl, pH 7.5, 20 mM EDTA buffer at about 10^7 cells/ml. The cells were then treated twice with lysis buffer: 1.0% NP-40 (Sigma) in the same Tris/EDTA buffer at RT for 2 min followed by centrifugation at 1600 × g for 5 min. After the last spin, SDS was added to the supernatant to a final concentration of 1%, and RNase A (Sigma) was added to final concentration of 5 mg/ml. The solution was subsequently incubated at 56°C for 2 h. Then, protease K (Promega) was added to the solution to 2.5 μg/ml, and incubated at 37°C for 2.5 h. Following this, ammonium acetate was added to a final concentration of 4 M, 0.7 volumes of isopropanol was added, and the DNA precipitate was put at −20°C overnight. This chilled solution was centrifuged at 14 000 r.p.m. for 30 min, and the pellets were washed twice with 70% ethanol, dried, and resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Eighteen μg of DNA sample per condition was prepared in neutral loading buffer (0.02% bromophenol blue, 5% glycerol, 0.1% SDS, and 50 μg of ethidium bromide) and loaded onto a neutral agarose gel (1.7%), and run at 50 V for 2.5 h. DNA size standards were *Hind*III digested lambda DNA, and DNA ladder markers (Promega). Separated DNA fragments were visualized by fluorescent staining using ethidium bromide.

Cell typing
Cells on coverslips were washed with 1 × PBS, and fixed with 4% paraformaldehyde in PBS. Cells treated with Neuron-Specific Enolase Ab (NSE), which specifically identifies neurons, were fixed for 20 min at RT, rinsed twice with PBS, and blocked with 10% goat serum (Gibco) in PBS at RT for 1 h. Cells treated with Glial Fibrillary Acidic Protein (GFAP) Ab, which specifically identifies astrocytes, were fixed similarly, followed by blocking and permeabilization with 10% goat serum, 0.5% Triton X-100 in PBS at RT for 1 h. Then, 50 μl of GFAP Ab (1 : 1000), or NSE Ab (1 : 200) was applied in the blocking solution, and incubated at RT for 30 min. Following this, the coverslips were washed once in PBS for 10 min followed by incubation with a 1 : 100 dilution of a secondary FITC-tagged Ab (Boehringer Mannheim), or a 1 : 300 dilution of a secondary Cy3 tagged Ab (Sigma) in blocking solution for 30 min at RT. After four washes in 1 × PBS, the coverslips were mounted on slides with one drop of Vectashield (Vector Laboratories, Burlingame, CA, USA). Finally, all specimens were observed by epifluorescence on a computer controlled microscope system as described above.

ICA/TUNEL The coverslips were washed, and initially stained using the TUNEL procedure described above, followed by secondarily being stained for either NSE or GFAP as described above. After rinsing, the coverslips were mounted on slides with Vectashield. Slides were observed by epifluorescence on a computer controlled microscope system based on a Zeiss microscope as described above. Identical images were taken of each field using phase, and epifluroescence with FITC filters, and rhodamine filters for Cy3. Images were subsequently examined for cells simultaneously staining for FITC and Cy3 using Image Pro software.

Flow cytometry
The double staining method used for analyzing Vpr-transfected cells was adapted from the technique of Schmid *et al* (1991). Briefly, 1×10^6 C6 glioma cells were transfected, using Lipofectamine (GibCo/BRL, Gaithersburg, MD, USA), with 20 μg total DNA (17 ug of the pVpr expression construct made in the laboratory, 3 μg of pMLSV N1/N4-S, an IL-2Ra expression vector), and the cells incubated for 24 h. After the cells were washed with PBS, they were stained with a monoclonal antibody to IL-2 R, human, FITC-conjugated in 200 μl PBSAz (PBS, 0.1% NaAz) for 30 min at 4°C in the dark. Subsequently, the cells were washed twice in PBSAz, and resuspended in 0.3% paraformaldehyde in PBS for 1 h on ice, and subsequently permeabilized within 0.2% Tween 20 in PBS for 15 min on ice (Jowett *et al*, 1995). They were stained with propidium iodide (10 μg/ml) and RNase-A (25 000 U) in FACS buffer (PBS, 2% FCS, 0.1% NaAz) for 30 min at 4°C in the dark. Approximately 10 000 − 20 000 cells were subsequently analyzed using an ABI FACScan cell sorter (Becton Dickinson). The first sort was for FITC-positive (transfected cells) single cells, with gating performed to exclude debris and cell clumps. Subsequently, the FITC-positive single cell subpopulation was sorted for PI fluorescence (stage of the cell cycle). The sum of broadened rectangles (SOBR) mathematical model was used to predict the proportion of cells in G1, S and G2+M phases of the cell cycle using Cellfit (Becton Dickinson). This data was shown to be in agreement with that acquired using Lysis II software (Becton Dickinson).

Analysis of data
SigmaPlot 4.0 (Chicago, IL, USA), a statistical analysis program, was used to compile the image analysis data. This data was subsequently analyzed by the Student's *t*-test, using a two-factor, unpaired test. In this analysis, data gathered using the various treatment conditions were compared to those gathered in the untreated control conditions. For this study, significance, or lack of significance of the data was set at $P > 0.001$, or $P < 0.05$.

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