Selective Neuronal Vulnerability in HIV Encephalitis

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Abstract. Recent studies of human immunodeficiency virus type 1 (HIV-1) encephalitis have shown that in addition to well-established white matter damage, the neocortex shows thinning, loss of large neurons and dendritic damage. In order to identify neuronal populations affected in HIV encephalitis and to determine how neuronal damage relates to the severity of HIV infection within the nervous system, we quantified parvalbumin (PV) and neurofilament (NF) immunoreactive neurons in the frontal cortex and hippocampus. We found that in the neocortex, the density of NF+ and PV+ neurons was independent of severity of HIV encephalitis, and therefore changes in these neuronal subsets did not account for previously reported neuronal loss. However, neuritic processes of PV+ neurons were fragmented, atrophic and in some cases distended. In contrast to the frontal cortex, there was a trend toward decreased density of PV+ neurons in the hippocampus which only reached significance in the CA3 layer where there was a 50-90% decrease in PV+ neurons. This decrease was closely correlated with the severity of HIV encephalitis. Double-label immunocytochemical analysis confirmed neuritic damage to interneurons. These results suggest that HIV encephalitis differentially involves specific subpopulations of neurons. Since direct HIV infection of neuronal cells was not detected, damage to PV+ cells and fibers may be indirectly mediated by cytokines released by HIV-infected microglia.

Key Words: HIV encephalitis; Interneurons; Laser confocal imaging; Neurofilament; Parvalbumin.

INTRODUCTION

Studies of the nervous system in acquired immunodeficiency syndrome (AIDS) have shown that human immunodeficiency virus type 1 (HIV-1)-mediated brain damage is not limited to white matter and basal ganglia (1-5), but also affects cortical gray matter (6-9). Cortical pathology in HIV encephalitis is characterized by loss of large neurons in orbito-frontal (8), temporal and parietal cortices with a 20% reduction in cortical width (7). Moreover, laser confocal microscopy and quantitative Golgi analysis of frontal cortex in severe HIV encephalitis shows a 40% loss of dendritic area, and a 40-60% loss of spine density along the apical dendrites of large pyramidal neurons (10). The affected dendrites were dilated, tortuous, and vaculated with decreased length and branching. Whether all neurons or a select subpopulation (e.g. large pyramidal cells) are affected during HIV encephalitis is not known (11, 12). To characterize the neuronal subpopulations affected, we used confocal laser microscopy and morphometric techniques to study the frontal cortex and hippocampus of AIDS cases with and without HIV encephalitis. Antibodies against parvalbumin (PV), a calcium binding protein present in interneurons (13), and neurofilament (NF), a family of intermediate and high molecular weight proteins present in all neurons but immunocytochemically detectable in large pyramidal cells (14), were used to characterize the involved neurons in HIV encephalitis.

MATERIALS AND METHODS

Samples and Immunohistopathological Staging

A total of 26 AIDS cases from UCSD Medical Center with postmortem times between 4 and 12 hours (h) and without pathological evidence of anoxic damage to Sommer's sector were available for study. A summary of the major systemic and neuropathological findings in each case are presented in Table 1. Tissue blocks from the frontal cortex and hippocampus at the level of the geniculate body were fixed overnight in 2% paraformaldehyde and 40 μm thick sections were cut with a Dosaka microtome (Tokyo, Japan). Formalin-fixed tissue blocks from the frontal cortex, basal ganglia, thalamus, hippocampus, midbrain and cerebellum were stained with hematoxylin and eosin for routine histopathological examination (3). Additional sections of frontal cortex and basal ganglia were immunolabeled with a monoclonal antibody against the HIV envelope protein gp41 (Genetics Systems, Seattle, WA) to semiquantitatively assess viral burden, and with a polyclonal antisem to glial fibrillary acidic protein (GFAP, Dako, Carpinteria, CA) to assess nonspecific central nervous system (CNS) damage, as previously described (11). All immunocytochemical markers were assessed on individual scales, and scores were interpreted separately for each region. Gliosis was studied by immunocytochemistry for GFAP and each region scored from 0 to 3; 0 = no difference in immunocytochemical staining from previously reported seronegative controls (11), 1 = thin glial processes dispersed among a histologically unremarkable neuropil, 2 = glial cells with well defined processes stained for GFAP, some of which outlined small vessels, and 3 = glial cells with abundant somal GFAP staining and thick and abundant processes within a histologically perturbed neuropil. Only areas without evidence of opportunistic infections were assessed for gliosis. Levels of HIV antigen expression were assessed by immunocytochemistry with
TABLE 1
Summary of Systemic and Neuropathologic Alterations in 26 Seropositive AIDS Cases

<table>
<thead>
<tr>
<th>Group</th>
<th>Case no.</th>
<th>Neuropathologic findings</th>
<th>Systemic disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>No to mild HIV</td>
<td>1</td>
<td>Pituitary microadenoma</td>
<td>Pneumocystis pneumonia; CMV</td>
</tr>
<tr>
<td>encephalitis</td>
<td>2</td>
<td>Mild chronic meningitis</td>
<td>KS; hemorrhage</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Mild chronic meningitis</td>
<td>Pneumonia</td>
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<tr>
<td></td>
<td>4</td>
<td>Mild chronic meningitis</td>
<td>Sepsis</td>
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<tr>
<td></td>
<td>5</td>
<td>Subacute encephalitis</td>
<td>CMV; Candidiasis</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>White matter gliosis</td>
<td>KS</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Mild chronic meningitis</td>
<td>Sepsis</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Mild chronic meningitis</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>Moderate HIV</td>
<td>9</td>
<td>Chronic meningitis</td>
<td>CMV; Cryptococcosis; KS</td>
</tr>
<tr>
<td>encephalitis</td>
<td>10</td>
<td>Microglial meningitis</td>
<td>Pneumocystis pneumonia</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>White matter gliosis</td>
<td>CMV</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Mild chronic meningitis</td>
<td>KS</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>CMV; Cryptococcosis</td>
<td>KS</td>
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<tr>
<td></td>
<td>14</td>
<td>Chronic meningitis</td>
<td>Pneumocystis pneumonia</td>
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<td>15</td>
<td>Cryptococcosis meningitis</td>
<td>Pneumonia</td>
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<tr>
<td></td>
<td>16</td>
<td>Microglial encephalitis</td>
<td>CMV pneumonia</td>
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<tr>
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<td>17</td>
<td>Microglial encephalitis</td>
<td>Pneumonia</td>
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<tr>
<td></td>
<td>18</td>
<td>Bacterial and CMV meningitis</td>
<td>Sepsis</td>
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<tr>
<td></td>
<td>19</td>
<td>Microglial encephalitis</td>
<td>Sepsis</td>
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<tr>
<td></td>
<td>20</td>
<td>Lymphoma (meninges)</td>
<td>Disseminated lymphoma</td>
</tr>
<tr>
<td>Severe HIV</td>
<td>21</td>
<td>Microglial encephalitis</td>
<td>CMV; Cryptococcosis</td>
</tr>
<tr>
<td>encephalitis</td>
<td>22</td>
<td>Microglial encephalitis</td>
<td>Pneumocystis; Sepsis</td>
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<tr>
<td></td>
<td>23</td>
<td>White matter gliosis</td>
<td>Pneumocystis; CMV pneumonia</td>
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<td>24</td>
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<td>White matter gliosis</td>
<td>KS</td>
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<tr>
<td></td>
<td>26</td>
<td>Microglial encephalitis</td>
<td>Pneumonia</td>
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CMV = cytomegalovirus, KS = Kaposi sarcoma.

anti-gp41 monoclonal antibodies and each region was scored on a scale from 0 to 2; 0 = no cells stained for gp41, 1 = less than two cells stained for gp41 in an average of five 20× fields, and 2 = more than two cells stained for gp41 in an average of five 20× (0.25 mm²) fields. This compressed scale was chosen to assure maximum inter-observer reliability. All assessments were done blindly by two authors (CAW and CLA) followed by simultaneous viewing and resolution of any discrepancies. A composite gp41 or GFAP score for each case was derived by summing the individual scores of the three regions (neocortical gray matter, subcortical white matter, deep gray matter) (range for composite gp41 was 0-6 and range for composite GFAP was 0-9). Therefore, a composite gp41 score, denominated gp41 SUM, was obtained for each case. Consistent with our previous study (11), cases with gp41 SUM of 0-1 were classified as having absent or minimal HIV-1 encephalitis, cases with composite gp41 SUM of 2-3 were classified as moderate HIV encephalitis, and cases with composite gp41 SUM of 4-6 were classified as severe HIV encephalitis.

Immunocytochemistry and Morphometry

Separate 40 μm thick sections from the frontal cortex (26 cases) and hippocampus (23 out of the 26 cases were available for immunolabeling) were single immunolabeled with antibody recognizing PV (mouse monoclonal, 1:1,000, Sigma Chemical Co., St. Louis, MO) or NF (mouse monoclonal SMI 31, 1:1,000, Sternberger Immunocchemicals, Baltimore, MD). The antibody specificities are described elsewhere (14). Briefly, nonspecific staining was blocked with 10% normal horse serum before overnight incubation at 4°C with specific primary antibody at 4°C (15). The free-floating sections were then washed and incubated with biotinylated goat anti-mouse IgG followed by avidin D-HRP (ABC Elite, Vector Labs, Burlingame, CA) and reacted with diaminobenzidine (DAB) containing 0.001% hydrogen peroxide. Anti-PV and anti-NF immunostained cells were counted in ten consecutive fields (0.1 mm² each) along the side of the gyrus using a 40× objective and a gridded 10× ovarian lens. For the frontal cortex results were expressed as immunostained neurons per sq mm per layer and in the hippocampus as immunostained neurons per sq mm per subdivision of the Cornus Ammonis (CA) (16). Additional vibratome sections from the hippocampus of all cases were immunostained with gp41 with the technique described above. Results were expressed as numbers of gp41 positive cells per sq mm.

Double Immunolabeling and Laser Confocal Imaging

Free-floating sections were double immunolabeled with a mixture of the mouse monoclonal antibodies recognizing PV and the rabbit polyclonal antiserum recognizing NF as previously described (7). Sections were reacted with biotinylated goat anti-rabbit IgG followed by a mixture of horse anti-mouse IgG fluorescein isothiocyanate (FITC) and avidin Texas red (Vector Labs; 1:75 and 1:100, respectively). The double immunolabeled sections were transferred to gelatin coated slides and mounted under glass coverslips with anti-fading media (4% n-propyl gal- late, Sigma Chemical Co.). All sections were processed simul-
neuronally under identical conditions. The immunofluorescent labeling protocol was repeated in order to assess reproducibility of immunostaining.

The double immunolabeled sections were viewed with a Zeiss 63× (N.A. 1.4) objective on a Zeiss Axiosvert 35 microscope (Germany) with attached laser confocal scanning system MRC 600 (Bio-Rad, Watford, UK) (7). The Texas red channel collected the NF immunoreactive (NF+) neurons and the FITC channel collected corresponding images of the PV immunoreactive (PV+) neurons. Each vibratome tissue section was scanned through a depth of 10 μm. Each tissue was analyzed as a series of 25 0.5 μm thick optical sections. The digitized video images were stored and processed with MRC software.

Statistical Methods

Differences among the groups were tested using non-paired, two-tailed Student's t-test. Pearson product-moment correlation, the r value, was calculated with simple linear regression analysis running in the StatView II program. All results were expressed as mean ±SEM.

RESULTS

Immunocytochemical Analysis and Morphometry

Frontal Cortex: Of the 26 cases analyzed 8 were HIV seropositive cases with no or minimal HIV immunoreactivity in the CNS (gp41 SUM = 0–1), 12 displayed a moderate degree of HIV encephalitis (gp41 SUM = 2–3) and 6 had severe HIV encephalitis (gp41 SUM = 4–6). In the control cases with no or minimal HIV in the CNS, antibodies to NF immunolabeled an average of 145 pyramidal neurons per sq mm in layers 2 and 3, and 155 pyramidal neurons in layer 5 (Fig. 1A). In AIDS cases with moderate and severe HIV encephalitis, there was a slight decrease in NF immunoreactive neurons in layers 2, 3 and 5, but these differences were not statistically significant (p = 0.57) (Fig. 1A). Consistent with previous reports, immunocytochemical staining for PV labeled a subpopulation of non-pyramidal neurons mostly distributed in layers 2–4, and to a lesser extent in layers 5–6 (Fig. 2). In cases with no to minimal HIV in the CNS, immunocytochemical staining for PV immunolabeled an average of 65 neurons per sq mm in layers 2 and 3, and 36 neurons per sq mm in layer 4 (Fig. 1B). No statistically significant differences were observed in cases with moderate and severe CNS infection by HIV. Despite the absence of significant differences in the density of PV+ neurons in the neocortex, the PV+ neurons in the moderate and severe HIV encephalitis cases presented striking morphological abnormalities when compared to controls with no or minimal HIV in the CNS (Fig. 2). In moderate and severe HIV encephalitis cases PV+ neurons had shrunken cell bodies and fragmented and distended neurites (Fig. 2).

Hippocampus: In cases with no or minimal HIV infection within the CNS, antibodies to NF immunolabeled an average of 200 pyramidal neurons per sq mm in CA3 to CA1, and 165 in CA4. AIDS cases with moderate or severe HIV encephalitis did not show a significant decrease in NF+ neurons in regions CA4, CA2 and CA1. Only CA3 showed a slight (10–15%) decrease in NF+ neurons, but this difference was not statistically significant (p = 0.09) (Fig. 3A). Antibodies to PV immunostained small groups of non-pyramidal neurons in all regions of the hippocampus (Fig. 4). The cell bodies of these neurons were most commonly distributed immediately below the pyramidal cell layer with several neuritic projections that went through the pyramidal cell layer and the stratum radiatum toward the molecular layer (Fig. 4). The pyramidal cells were not labeled with antibodies to PV, but they were surrounded by abundant PV+ fibers and terminals. An average of 32 PV+ neurons were found in all four areas per section of the control cases with minimal to no HIV in the CNS (Fig. 3B). In these cases,
50% of the PV+ neurons were in CA1 and the other 50% were distributed throughout CA2, CA3, and CA4. There were no significant differences in the amounts of PV+ neurons per region in CA1 and CA2 in cases with moderate to severe HIV encephalitis (Fig. 3B). However, the CA3 region displayed a significant (50%) decrease ($p < 0.02$) in cases with moderate HIV encephalitis, and a 90% decrease ($p < 0.02$) in the cases with severe HIV encephalitis. The CA4 region showed a trend toward decrease of PV+ neurons but the differences in density of these cells did not correlate with severity of HIV encephalitis ($p = 0.13$). Those PV+ neurons present in moderate to severe HIV encephalitis were shriveled and displayed few neuritic processes, some of them with severe dystrophic changes.

**Statistical Analysis:** Linear regression analysis correlating cellular density in the frontal cortex and hippocampus versus the degree of involvement of the CNS by HIV showed a significant negative correlation between CA3 PV+ neurons and severity of HIV encephalitis (gp 41 SUM) ($r = -0.614$, $p < 0.002$, $n = 23$) (Fig. 5). Linear regression analysis between gp41 SUM and counts of gp41 positive cells in the hippocampus showed a significant correlation between these two variables ($0.61$, $p < 0.001$) further supporting the notion that gp41 SUM is a good general indicator for the degree of involvement in the brain. No significant correlations were observed when the density of NF+ or PV+ neurons in the frontal cortex or in CA1, CA2 and CA4 areas of the hippocampus were plotted against the degree of HIV-mediated damage in the CNS.

**Laser Confocal Imaging of Double-Labeled Sections**

Analysis of sections double immunolabeled for PV and NF showed that a subpopulation of the PV+ non-pyramidal neurons contained NF immunoreactivity. PV immunolabeled terminals and fibers were also observed around the cell bodies of NF+ pyramidal neurons. In HIV encephalitis cases with moderate to severe damage of the CNS, PV+ neurites were usually fragmented and distended and they lacked NF immunoreactivity (Fig 6).

**DISCUSSION**

The present study examined subpopulations of hippocampal and frontal cortex neurons in autopsy cases of HIV encephalitis. There was a slight trend toward decreased numbers of NF+ pyramidal neurons in either region. In contrast, there was selective damage to PV+ neurons in region CA3 of the hippocampus and, to a lesser extent, in layers 2 and 4 of the frontal cortex in cases with moderate to severe HIV encephalitis. Parvalbumin is a calcium binding protein of approximately 12 kilodalton.

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**Fig. 2.** Differential patterns of anti-PV immunoreactivity in the frontal cortex of AIDS cases. (A, B, C) Cases with no or mild HIV encephalitis displayed strong immunolabeling of interneurons in layers 2-6. (D, E, F) Cases with moderate HIV encephalitis showed a slight decrease in cell body size as well as some dilatation (arrowhead) and fragmentation of neuritic processes. (G, H, I) Cases with severe HIV encephalitis presented PV+ neurons that were shrunken and depleted of neuritic processes. A, D, G x 40, B, C, E, F, H, I x 250.
Fig. 4. Differential patterns of anti-PV immunoreactivity in the hippocampus of AIDS cases. (A, B, C) Cases with no or mild HIV encephalitis presented strong immunolabeling of interneurons in CA2 and CA3. (D, E, F) Cases with moderate HIV encephalitis showed a slight decrease in cell body size and branching of neuritic processes. (G, H, I) Cases with severe HIV encephalitis displayed PV+ neurons that were shrunken and had several dystrophic neurites (arrowheads). sr = stratum radiatum, sp = stratum pyramidale, so = stratum oriens. A, D, G ×40, B, C, E, F, H, I ×250.
molecular weight (17) that is enriched in inhibitory interneurons that contain gamma amino butyric acid (13). Some PV+ neurons could also contain other neurotransmitters (18). In HIV encephalitis, the PV+ hippocampal neurons were more severely affected than the PV+ neurons in the neocortex. In analogy with the known selective vulnerability of CA1 to anoxic damage, the heterogeneous damage of interneurons in HIV encephalitis could be the result of differential topographic vulnerability of interneurons. The present study showed that both by unit area as well as relative to the amounts of NF+ pyramidal neurons, the hippocampus has fewer PV+ cells as compared to the neocortex. Furthermore, the immunocytochemical analysis showed damage to the neurites of the interneurons. In fact, the overall density of interneurons in CA3 was lower than in all other hippocampal regions. This characteristic might explain why this region displayed a greater amount of damage than other regions of the hippocampus. Therefore, since this region has a lower interneuronal density to begin with, milder injury to inhibitory circuitry could be translated into greater damage. In contrast to this particular vulnerability of PV+ cells in HIV encephalitis, previous studies in the cortex of patients with epilepsy (19, 20) and in cases with Alzheimer’s disease (21) have shown that these subsets of interneurons are relatively resistant to injury. Pneumonia and terminal anoxia are commonly observed in AIDS. Because of the known effects of anoxia on hippocampal CA1 neurons, we excluded such cases from our analysis (sensitively confirmed by no evidence of decreased NF+ neurons in the cases studied). The relative resistance of PV+ neurons to anoxic damage (22–25) strengthens the conclusion that the changes we observed were not due to simple terminal anoxic events.

The degree of damage to PV+ in the hippocampus was correlated with the severity of the HIV encephalitis. Because HIV-1 does not directly infect significant numbers of neurons (if any), damage to interneurons must result from indirect effects (2, 26–30). From this study the close correlation of interneuronal damage and degree of CNS HIV burden (severity of HIV encephalitis) suggests that the damage to PV+ neurons may be mediated by factors or virus released by infected microglia. Studies have shown that under in vitro conditions, HIV can infect neural cell lines (31). Among the many potential toxic secretory products of microglia, interleukin 1 (IL-1) has been hypothesized to mediate neuritic damage in HIV encephalitis. Presumably this effect would be mediated through interleukin receptors on neurons. Autoradiographic studies in the mouse brain in combination with studies of quinolinic acid lesions have shown that IL-1 binding sites in the hippocampus were localized to intrinsic neurons (32). Therefore, it is possible that damage to PV+ cells and fibers could be mediated by cytokines released by infected microglia, for which interneurons have specific receptors.

Classical studies in Golgi-impregnated hippocampus have described two basic types of intrinsic non-pyramidal neurons: a) basket cells with thick dendrites and few neurites, and b) non-pyramidal non-basket cells (33, 34). A recent study of PV+ neurons in the human hippocampus (35) showed that these cells are distributed throughout the three layers of the hippocampus. The CA3 region contains three subtypes of PV immunolabeled interneurons in the stratum oriens, three subtypes in the stratum pyramidale, and a single type in the stratum radiatum. The CA3 region has a strong recurrent excitation system, formed by axon collaterals with adjacent pyramidal neurons and strong synaptic inhibition from interneurons (36). Clinical studies have reported epileptic activity in a small group of children (37) and adult patients with HIV encephalitis (38, 39). Initiation of this activity might be controlled by interneurons as experimental work in the rat hippocampus has shown (40). Therefore, damage to the inhibitory neurons in the hippocampus similar to that with PV immunocytochemistry could trigge synchronized neuronal discharges. Further studies will be necessary to determine if the interneuron damage in the hippocampus in our cases is associated with abnormal CNS electrical activity as well as with other clinical aspects of the AIDS dementia complex.
Fig. 6. Laser confocal imaging of frontal cortex sections double immunolabeled for NF and PV. For each panel, the image on the left corresponds to NF immunolabeling and the image on the right corresponds to PV immunolabeling. (A) In cases with no HIV encephalitis, PV+ terminals were abundant around perikarya (arrows) of NF+ pyramidal neurons. (B) In cases with moderate or severe HIV encephalitis, NF+ neurons were distorted but PV+ terminals and neurites were preserved around apical dendrites. (C) NF+ pyramidal neurons (nf) did not immunolabel for PV. Some PV+ interneurons (pv) did contain neurofilaments. (D) In cases with severe HIV encephalitis, PV+ neurites were abnormally dilated (arrow). ×750.

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