Host Response and Dysfunction in the CNS during Chronic Simian Immunodeficiency Virus Infection

Eleanor S. Roberts, Salvador Huitron-Resendiz, Michael A. Taffe, Maria Cecilia G. Marcondes, Claudia T. Flynn, Caroline M. Lanigan, Jennifer A. Hammond, Steven R. Head, Steven J. Henriksen, and Howard S. Fox

Molecular and Integrative Neurosciences Department and DNA Array Core Facility, The Scripps Research Institute, La Jolla, California, 92037

CNS abnormalities can be detected during chronic human immunodeficiency virus (HIV) infection, before the development of opportunistic infections or other sequelae of immunodeficiency. However, although end-stage dementia caused by HIV has been linked to the presence of infected and activated macrophages and microglia in the brain, the nature of the changes resulting in the motor and cognitive disorders in the chronic stage is unknown. Using simian immunodeficiency virus-infected rhesus monkeys, we sought the molecular basis for CNS dysfunction. In the chronic stable stage, nearly 2 years after infection, all animals had verified CNS functional abnormalities. Both virus and infiltrating lymphocytes (CD8+ T-cells) were found in the brain. Molecular analysis revealed that the expression of several immune response genes was increased, including CCL5, which has pleiotropic effects on neurons as well as immune cells. CCL5 was significantly upregulated throughout the course of infection, and in the chronic phase was present in the infiltrating lymphocytes. We have identified an altered state of the CNS at an important stage of the viral–host interaction, likely arising to protect against the virus but in the long term leading to damaging processes.

Key words: HIV; SIV; CCL5; RANTES; CD8; CTL; brain; neuroAIDS; cognitive; gene array

Introduction

Abnormalities of the CNS resulting from human immunodeficiency virus (HIV) infection are usually studied during end-stage disease, when individuals with acquired immunodeficiency syndrome (AIDS) suffer from a range of disorders including HIV-associated dementia (HAD). However, most people living with HIV have a long-term, reasonably asymptomatic, condition before the development of immunodeficiency and the full range of HIV-related complications. Still, throughout this chronic infection, CNS function is often aberrant.

A number of analyses have documented such alterations. Asymptomatic HIV+ patients have been found to be significantly impaired in tests of verbal memory and psychomotor speed (Villa et al., 1993). Other studies have revealed changes or impairment in measures of choice reaction, visuomotor coordination, global attentional performance (Kokkevi et al., 1991), spatial working memory (Sahakian et al., 1995), and fine motor control (Hestad et al., 1993; Stern et al., 2001; Chang et al., 2002).

Even a mild degree of neurocognitive disorder is associated with neuroimaging abnormalities. Magnetic resonance imaging (MRI) studies have found decreased volumes in cortical, limbic, and striatal structures (Jernigan et al., 2005), as well as selective thinning of the cerebral cortex (Thompson et al., 2005). Additionally, functional MRI has revealed increased brain activation in HIV-infected individuals, interpreted as an increased usage of brain reserve capacity to maintain normal cognitive functions (Ernst et al., 2003). In addition, CNS neurophysiological abnormalities have been found in HIV-infected individuals, including abnormalities in the spontaneous encephalogram (EEG) (Gabuzda et al., 1988; Parisi et al., 1989) and changes in brainstem auditory sensory evoked potential latencies (Pagano et al., 1992; Pierrelli et al., 1993; Reyes-Contreras et al., 2002).

The analysis of the etiology and pathogenesis of CNS abnormalities in the chronic stage of HIV is quite limited, hampering their analysis. One study had found elevations in major histocompatibility complex class II (MHC-II) and cytokines in the brains of those with chronic HIV infection (An et al., 1996). However, a more accessible window for sampling the CNS in chronic HIV infection is examination of the CSF. In the CSF, increases in IgG, neopterin, and β2-microglobulin (β2M) have been found (McArthur et al., 1988; Bogner et al., 1992), with elevated β2M found to be predictive of development of CNS disorders in asymptomatic HIV-infected individuals (Brew et al., 1996).

Taken as a whole, these studies indicate that, although the period between acute HIV infection and overt AIDS may be reasonably asymptomatic, the CNS undergoes changes that could be progressive and cause additional damage as the disease worsens. Access to human brain tissue during this stage is rarely possible, making determination of the basis for CNS involvement problematic. The simian immunodeficiency virus (SIV)-infected rhesus macaque provides an excellent model for mimicking the CNS abnormalities induced by HIV in humans. Here, we report a longitudinal study of CNS function in SIV-infected rhesus ma-
caques, after which the animals were killed in the stable phase of infection, while still asymptomatic but with demonstrable CNS dysfunction. Molecular analyses of brain tissue was performed to uncover the effect of this chronic infection on the brain.

Materials and Methods
Rhésus macaques. For these experiments, we used rhesus macaques free from SIV, type D simian retrovirus and Herpes B virus; these animals were obtained from Charles River Laboratories (Key Lois, FL), Labs of Virginia (Yemassee, SC) and Covance (Alice, TX). Experiments were performed with the approval and guidelines of the Animal Care and Use Committee of The Scripps Research Institute.

In this study, four monkeys were first trained in the behavioral tests (detailed below), followed by electrophysiological analysis (details to follow) for baseline values. These monkeys were then intravenously inoculated with a cell-free stock of SIVmac182 (Lane et al., 1995; Gaskill et al., 2005). Additional behavioral and electrophysiological analyses were performed during the chronic period of infection, and the subjects were subsequently killed at 92–114 weeks post-inoculation (p.i.) (denoted the chronic group). Three of the four monkeys were treated with a CD8 depleting antibody, by a slight modification of the originally described protocol (Schmitz et al., 1999), at either 30 or 66 weeks p.i. (25–61 weeks pretermination), which led to a transient depletion of CD8+ T-cells. This experiment was performed to examine the effect of increasing viral load on behavioral parameters and did not alter disease course compared with untreated infected animals. Viral load rose briefly, increasing viral load on behavioral parameters and did not alter disease course.

Another group of six uninfected animals (control group) did not receive SIV injections but underwent mock inoculation. One monkey from the control group also received a CD8 T-cell-depleting regime at 16 weeks pretermination. Four additional groups were also investigated in comparisons with untreated infected animals. Viral load rose briefly, increasing viral load on behavioral parameters and did not alter disease course.

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Histopathological analysis. Sections (6 μm) of formalin-fixed, paraffin-embedded tissue were mounted on glass slides, deparaffinized with xylene, hydrated in graded alcohols, and either subjected to hematoxylin and eosin staining (Sigma-Aldrich, St. Louis, MO), immunohistochemical staining, or in situ hybridization (ISH) analysis. Immunohistochemical staining followed a basic indirect protocol, using an antigen retrieval (AR) method (heated to 95°C in the indicated buffer for 45 min and then left for 20 min to steep). Primary antibodies were anti-CD163 (Novocastra, Newcastle-upon-Tyne, UK) and anti-human leukocyte antigen (HLA)-DR (Zymed, San Francisco, CA) mouse monoclonal antibodies (both using citrate buffer for AR), anti-CD8 (Panomics, Fremont, CA), rabbit polyclonal antibody, and anti-RANTES/CCL5 (R&D Systems, Minneapolis, MN) goat polyclonal antibody (both using Tris/Urea buffer for AR). Staining reactions were developed with the NovaRed chromogen (Vector Laboratories, Burlingame, CA), followed by a hematoxylin counterstain. Controls included omission of the primary antibody and use of irrelevant primary antibodies. ISH was performed as described previously using 35S-labeled single-strand RNA probes consisting of a fragment of the SIVmac nef (negative factor) gene (Fox et al., 2000). In addition to the antisense probe hybridization, controls included sense probe hybridization and omission of the probe. Sections were counterstained with methyl green (Vector Laboratories).

Flow cytometry. Cells were isolated from the brain and stained with a mixture of antibodies as described previously (Marcondes et al., 2001).

For each task, -scores were calculated using the mean and SD of the

<table>
<thead>
<tr>
<th>Group</th>
<th>Monkey</th>
<th>Day p.i.</th>
<th>Frontal lobe viral load</th>
<th>Signs/symptoms</th>
<th>AIDS pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>298</td>
<td>NA</td>
<td>NA</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>331</td>
<td>NA</td>
<td>NA</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>332</td>
<td>NA</td>
<td>NA</td>
<td>None</td>
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<td>None</td>
</tr>
<tr>
<td>337</td>
<td>NA</td>
<td>NA</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>382</td>
<td>NA</td>
<td>NA</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>393</td>
<td>NA</td>
<td>NA</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Acute</td>
<td>335</td>
<td>13</td>
<td>2711</td>
<td>Anorexia, rash</td>
<td>None</td>
</tr>
<tr>
<td>346</td>
<td>13</td>
<td>746</td>
<td>None</td>
<td>None</td>
<td>None</td>
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<tr>
<td>347</td>
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<td>1239</td>
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<td>None</td>
<td>None</td>
</tr>
<tr>
<td>430</td>
<td>13</td>
<td>38,721</td>
<td>Fever</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Postacute</td>
<td>350</td>
<td>73</td>
<td>178</td>
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<td>None</td>
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<td>352</td>
<td>75</td>
<td>264</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>353</td>
<td>77</td>
<td>2442</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>354</td>
<td>80</td>
<td>161</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Early chronic</td>
<td>324</td>
<td>270</td>
<td>20</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>360</td>
<td>183</td>
<td>15</td>
<td>None</td>
<td>None</td>
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<tr>
<td>387</td>
<td>191</td>
<td>633</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>407</td>
<td>226</td>
<td>64</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Chronic</td>
<td>227</td>
<td>799</td>
<td>nd (&lt;5)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>290</td>
<td>723</td>
<td>207</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>291</td>
<td>647</td>
<td>147</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>304</td>
<td>646</td>
<td>709</td>
<td>None</td>
<td>None</td>
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</tr>
<tr>
<td>383</td>
<td>133</td>
<td>1,142,004</td>
<td>Anorexia, diarrhea, edema</td>
<td>SIVE</td>
<td>SIVE</td>
</tr>
<tr>
<td>417</td>
<td>56</td>
<td>775,060</td>
<td>Anorexia, lethargy</td>
<td>SIVE</td>
<td>SIVE</td>
</tr>
<tr>
<td>418</td>
<td>82</td>
<td>5,777,621</td>
<td>Anorexia, fever</td>
<td>SIVE</td>
<td>SIVE</td>
</tr>
<tr>
<td>494</td>
<td>105</td>
<td>916,066</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

Monkeys are grouped by stage of infection. Time of sacrifice, viral load in frontal lobe RNA (in viral equivalents per microgram total RNA), prominent clinical signs and symptoms at time of sacrifice, and simian AIDS histopathology are given. NA, Not applicable; nd, not detectable. The asterisk indicates that the incidental B-cell lymphoma found in animal 290 did not involve the CNS.

For each task, -scores were calculated using the mean and SD of the
Table 2. Sequences of the oligonucleotide primers and probes used in qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>j2M1</td>
<td>gccgctcagctggctctc</td>
<td>cggatgtgatagtaaccaaga</td>
<td>cggctcagctggctctc</td>
</tr>
<tr>
<td>CCL5c</td>
<td>aacgttgcaagtgtc</td>
<td>tggcagctacggaggtt</td>
<td>tggcagctacggaggtt</td>
</tr>
<tr>
<td>GPP1a</td>
<td>cggagcggggtttgctttt</td>
<td>gagcttcgagcagttccttc</td>
<td>gagcttcgagcagttccttc</td>
</tr>
<tr>
<td>HLA-DRAa</td>
<td>tctcaagcctggagttt</td>
<td>caccctgtgatgtgaagga</td>
<td>caccctgtgatgtgaagga</td>
</tr>
<tr>
<td>HIF1Ma</td>
<td>attgctctggctctcggtc</td>
<td>agatctggctctggctctcggtc</td>
<td>agatctggctctggctctcggtc</td>
</tr>
<tr>
<td>lgG6c</td>
<td>gggaaggtctcagctggtc</td>
<td>ggttgctctggctctgagtg</td>
<td>ggttgctctggctctgagtg</td>
</tr>
<tr>
<td>18S</td>
<td>cggagactcactcaagaaaa</td>
<td>cggagactcactcaagaaaa</td>
<td>cggagactcactcaagaaaa</td>
</tr>
</tbody>
</table>

Primers and probes were designed based on rhesus monkey DNA sequences and analyzed by a “web-based program from Genecreator,” PrimerExpress software, or from published studies of Hofmann-Lehmann et al (2000) or Gerard et al (2000).

*For detection of lgp6c, a mixture of two forward primers was used, differing at the bases in parentheses, in order to quantify all three lgp heavy chains.

Table 3. Behavioral testing results

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Acc</th>
<th>Lat</th>
<th>Acc</th>
<th>Lat</th>
<th>Rel</th>
<th>Move</th>
<th>PR</th>
<th>Lat</th>
<th>No. of tasks abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>227</td>
<td>x</td>
<td>✓</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>2 of 5</td>
</tr>
<tr>
<td>290</td>
<td>✓</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td>✓</td>
<td>2 of 5</td>
</tr>
<tr>
<td>291</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>nt</td>
<td>nt</td>
<td>✓</td>
<td>x</td>
<td>1 of 4</td>
</tr>
<tr>
<td>304</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>3 of 5</td>
</tr>
</tbody>
</table>

Animals were trained on a series of behavioral tasks, and their performance was compared for the 6 months before SIV infection and the 6 months before termination. *Statistically significant difference in task n, no disruption; mt, not tested; SSS, self-ordered spatial search; D/NMS, delayed nonmatching-to-sample; PR, progressive ratio; BMS, bimanual motor skills; Acc, accuracy; Lat, latency; Rel, release; Move, movement.

Baseline performance to compare measurements obtained during the final 6 months of infection, using Excel software (Microsoft, Redmond, WA). Performance on any task was considered impaired when the Z-score was less than −1.96 for two consecutive months or less than −2.58 for the last month preceding the killing of the animals. These criteria correspond to a 95 and 99% confidence interval around the baseline. The behavioral data on one of the animals reported here (#227) has been published previously (Weed et al., 2004).

Neurophysiology. Electrophysiology of brainstem auditory evoked potentials (BASEPs) was performed monthly on ketamine (20 mg/kg)-anesthetized animals as described previously (Prospero-Garcia et al., 1996). Positive waves in BASEPs were identified and denoted P1, P2a, P2b, P3, P4, and P5. In visual evoked potentials (VEPs) and auditory evoked potentials (AEPs), three positive and three (VEP) or two (AEP) negative waves; respectively, were identified. Average peak latencies were calculated for the six measurements before infection, and the six measurements before termination were then compared between preinfection and posttermination measurements using Student’s t-test with Excel software.

RNA extraction. Total RNA was purified from samples using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the protocol of the manufacturer, with an additional centrifugation step to remove cellular debris. RNA was further purified (RNeasy mini kit; Qiagen, Valencia, CA), and the quantity of total RNA was assessed by 600 nm UV absorption, with quality verified by gel electrophoresis and analysis of the ribosomal RNA bands.

Viral quantitation. Viral load in brain tissue was determined on RNA, purified as above, using the quantitative branched DNA (bDNA) signal amplification assay performed by Bayer Reference Testing Laboratory (Emeryville, CA), using 25 μg of RNA. In animals killed in the acute stage of infection, three of the four viral load values (excluding monkey #430) have been published previously (Marcondes et al., 2001). Values for the postacute group were also investigated previously in that study; however, the results presented here are a repeated measurement of the tissue RNA levels, using a more sensitive assay than that applied to the previous samples (now allowing the quantification of virus in one sample, which was previously below the limits of detection). Values are also presented for the results presented here are a repeated measurement of the tissue RNA levels, using a more sensitive assay than that applied to the previous samples (now allowing the quantification of virus in one sample, which was previously below the limits of detection). Values are also presented for the results presented here are a repeated measurement of the tissue RNA levels, using a more sensitive assay than that applied to the previous samples (now allowing the quantification of virus in one sample, which was previously below the limits of detection). Values are also presented for the results presented here are a repeated measurement of the tissue RNA levels, using a more sensitive assay than that applied to the previous samples (now allowing the quantification of virus in one sample, which was previously below the limits of detection).

Quantitative reverse transcription (qRT)-PCR. Specific RNA transcripts were quantified through the use of real-time PCR using dual-labeled hydrolysis probes. The primers and probe sequences selected for use here were designed for rhesus macaque sequences either by reference to previous studies, by the PrimerExpress software (Applied Biosystems, Foster City, CA), or through the Genescript online tool (https://www. genescript.com/ssl-bin/app/primer), and are listed in Table 2. Our protocol for qRT-PCR was described in detail previously (Burudi et al., 2002). To compute the relative amounts of each cytokine mRNA in the samples, the average cycle threshold (Ct) of the primary signal for 18S was subtracted from that for each cytokine to give changes in Ct (ΔCt). In this manner, the degree of changes in expression (the differences in ΔCt or ΔΔCt) are determined, which are log, relative units. Statistical analysis was done using Student’s t-test (Excel) or ANOVA plus Tukey’s honestly significant difference multiple-range test using Statgraphics software (StatPoint, Herndon, VA).

Results

Chronic SIV infection results in CNS dysfunction

Four rhesus monkeys were injected intravenously with SIVmac182 and studied for CNS functional abnormalities over the course of the resulting chronic infection. Before infection, the animals were trained to perform a variety of tasks to enable investigators to score cognitive and motor functions and then compare behaviors during the 6 months before the animals were killed with those obtained at the preinfection baseline. Once trained, performance on such tasks is stable over time, and performance is marked abnormal in animals progressing to simian AIDS with SIV encephalitis (Weed et al., 1999, 2003, 2004). In the chronically infected animals studied here, animals were clinically asymptomatic and exhibited stable plasma viral loads. However, all of these infected animals developed significant deficits in bimanual motor skills (Table 3). In addition, three of the four monkeys had other abnormalities; two showed deficits in the self-ordered spatial search task (testing spatial working memory), and the third was slow in reaction release times and in progressive-ratio schedule of reinforcement.
As a second measure of CNS function, neurophysiological testing was performed to assess sensory evoked potentials. Such measurements are stable over time in uninfected animals but become abnormal relatively early after SIV infection (Horn et al., 1998; Fox et al., 2000). Analysis of BSAEPs revealed significant delays in the P3 (Fig. 1A), P4, and P5 waves of all animals. Because the P1 wave is generated via the auditory nerve, the examination of interpeak latencies (Fig. 1B, P3–P1) reveals that this delay is localized within the CNS. Because delays in the earlier waves, i.e., P3, can slow the later waves, the P4 and P5 changes may not be independent abnormalities, and indeed, examination of the P4-P3 and P5-P3 interpeak latencies did not reveal significant delays, except for the P4-P3 latency in animal #290. Although delays in some of the cortical evoked potentials were found in these animals, there were no consistent changes in VEP or AEP latencies (data not shown). Alterations in the evoked potentials occurred before behavioral changes were evident. Thus, these two independent measures of CNS function were abnormal in these chronically SIV-infected animals.

SIV and infiltrating immune cells are present in the brain

After necropsy, we evaluated the viral load in the frontal lobe (as presented in Table 1), caudate, midbrain, and cerebellum of the infected monkeys (Fig. 2A) and compared these with plasma and CSF viral loads measured within 2 weeks before the animals were killed. Tissues were extensively perfused to eliminate any contribution from blood. Viral RNA was present in the brains of all four animals. Two monkeys had virus in all five of the areas examined, and the others were positive for virus in four areas of the brain. However, although three of the animals had $10^{4.7}$ to $10^{6.1}$ log$_{10}$ viral equivalents per milliliter of virus in the plasma and $10^{2.9}$ to $10^{3.2}$ log$_{10}$ viral equivalents per milliliter of virus in the CSF, no virus was detectable in these fluids in the other animal (#304; limit of detection $10^{3.1}$ log$_{10}$ viral equivalents per milliliter). Thus, SIV is present in the brain during the chronic infection period, regardless of levels of peripheral or CSF viremia.

We had reported previously that, during the postacute stage of infection, a significant number of CD8+ T-cells [cytotoxic T lymphocytes (CTLs)] infiltrate the brains of SIV-infected monkeys. To examine whether the presence of such an infiltrate persists in longer-term infection, lymphoid cells were isolated from the brains of the four chronically infected and six control animals and subjected to flow cytometric analysis (Fig. 2B). Both natural killer (NK) cells and CD4+ T lymphocytes were present but did not differ between the infected and uninfected animals. However, a three-fold increase in the number of CD8+ T lymphocytes could be found in the brains of the chronically infected group relative to the uninfected controls.

SIV infection induces CNS molecular changes

Our previous success in using human oligonucleotide-based gene arrays to identify upregulated genes in the brains of SIV-infected animals, both in the acute and terminal encephalitic stages (Roberts et al., 2003, 2004), prompted us to apply the same methodology to seek transcripts upregulated at least two-fold in this chronic phase over values found in uninfected controls. Table 4 details the mean expression values, SDs, fold-change values and p values for the seven genes found to be increased in the brains of chronically SIV-infected monkeys (p < 0.01). Error bars represent SEM.
change, we performed qRT-PCR using primers and probes derived from the rhesus monkey sequences for these genes. Transcripts for CCL5, HLA-DRα, IgG heavy chain (IgGhc) (using a mix of primers and a probe recognizing all three heavy chains), G1P3, IFITM1, and, for the HLA class I genes (HLA-A and HLA-C), the expression of β2M, their common light chain, was measured. Relative to the levels found in the frontal lobes of uninfected controls, all six of the tested transcripts were confirmed to be increased during chronic SIV infection (Table 4). Expression of these upregulated genes was also assessed in three noncortical areas of the brain: the caudate, cerebellum, and midbrain. Except for β2M and IFITM1 in the caudate, expression of all of the genes was significantly upregulated in these other regions of the CNS (Fig. 3).

SIV infection progresses through several distinct phases, including (1) the acute phase, which incorporates disease symptoms as well as brain transcriptional changes (Madden et al., 2004; Roberts et al., 2004); (2) the postacute phase, during which the acute phase resolves and a relatively asymptomatic period ensues; (3) an early chronic period, when a steady-state viremia has developed; (4) the chronic phase investigated here, with stable clinical, viral, and immune parameters over one and one-half years after infection; and (5) simian AIDS, in which some of the animals exhibit frank neurological symptoms and SIV encephalitis. Using RNA samples from the frontal lobes of animals killed at these three antecedent stages, end-stage SIV, and uninfected control animals, we examined the temporal pattern of changes for six of the genes found to be upregulated in the chronic infection phase.

All of the gene transcripts differed significantly over the stages of infection (Fig. 4). Post hoc testing of the ANOVA revealed distinct groupings. With the exception of G1P3, which peaked during the acute stage, expression of the upregulated genes was highest at end-stage SIV encephalitis (SIVE). Examination of individual genes reveals that CCL5 expression rose sharply in the acute phase, but dropped, although not to control levels, in the postacute stage.

The level of CCL5 transcripts then rose at the early chronic and chronic stages and reached its highest level in SIVE. For IgGhc, in contrast, only levels at the chronic and SIVE stages differed significantly from the uninfected control group. β2M and HLA-DRα levels differed significantly from uninfected controls at the acute, chronic, and SIVE stages. The remaining gene transcripts, G1P3 and IFITM1, although differing across the infection stages,
Discussion

In this study, we performed a molecular analysis of the brains of four chronically SIV-infected monkeys whose behavioral and electrophysiological abnormalities of the CNS were evident. At this chronic phase of infection, averaging 23 months p.i., all animals had viral RNA in the brain, and transcriptional profiling of the frontal cortex revealed the upregulation of a discrete set of genes. These results signify an altered state of the CNS at a distinct stage of the viral–host interaction and may reflect protective as well as damaging processes.

Previously, we and others have demonstrated functional CNS deficiencies in asymptomatic SIV-infected rhesus monkeys, including abnormalities in sensory evoked potentials, behavioral/cognitive abnormalities, and motor problems (Murray et al., 1992; Prospero-Garcia et al., 1996; Gold et al., 1998; Raymond et al., 1998; Weed et al., 2004). Here, during the chronic period, the functional impairments found with cognitive/motor testing (bimanual motor task in all monkeys, and spatial working memory in two of the four monkeys), as well as abnormalities in the BSAEPs (in all of these SIV-infected monkeys) simulate those found deficient in HIV-infected patients. By using samples from these SIV-infected animals, we believe we can effectively model effects on the brain during chronic HIV infection and examine the basis for the resulting dysfunctions.

The alterations in the latencies of BSAEPs confirm our previous findings in SIV-infected monkeys and extend them to relate these abnormalities to those in the cognitive and motor spheres. Abnormalities in BSAEPs can be early indicators of neurological disruptions and have been reported in HIV infection (Goodin et al., 1990; Pagano et al., 1992; Pierelli et al., 1993; Reyes-Contreras et al., 2002), as well as in other causes of neurodegeneration and dementia including Creutzfeldt-Jakob, Alzheimer’s, and a number of childhood neurodegenerative diseases (Chen et al., 1986). Although the exact mechanisms resulting in the electrophysiological alterations induced by SIV, HIV, and the other disorders are unknown, the ability to identify preclinical signs of CNS dysfunction in these conditions can enable early detection of susceptible individuals and allow the testing of measures to prevent the development of clinical sequelae.

Postmortem we found SIV RNA in the brain of all four infected monkeys; however, no correlation was discernable between viral RNA levels in the brain and viral loads in the plasma or CSF. For instance, in animal #304, the virus, if present in the plasma, CSF, or occipital lobe, was below the limit of detection, whereas the frontal lobe of this monkey contained more virus than any brain region of the other monkeys. ISH confirmed productive virus infection in the brain, localized in perivascular cells, consistent with findings in acute SIV infection and end-stage SIVE (Chakrabarti et al., 1991; Williams et al., 2001).

To obtain a global assessment of the changes in the chronically infected brain, the extent of transcription in the frontal lobe was analyzed by using gene array technology. Thereby, we noted significant upregulation of seven genes in the frontal lobes: CCL5, HLA-DRα, IgG1c, GIP3, IFITM1, HLA-A, and HLA-C. Quantitative RT-PCR was used to confirm their upregulation in the frontal lobe, as well as in other regions of the brain.

We found CCL5, also known as RANTES, to be expressed at a significantly increased level at each stage of infection compared with controls. CCL5 expression can be induced in astrocytes, and potentially other CNS cells, by proinflammatory cytokines (Oh et al., 1999), as well as by HIV infection of microglia (Si et al., 2002). However, although some degree of staining of other cell types or their...
processes can be observed, here in chronic infection the predominant cell type in which CCL5 is found is infiltrating lymphoid cells, likely CD8+ T-cells. Indeed, CCL5 is present in HIV-specific CD8+ CTLs (Wagner et al., 1998). Interestingly, in contrast to other chemokines/cytokines and cytotoxic granule proteins, CCL5 is stored in a unique secretory compartment, with rapid release after T-cell activation (Catalfamo et al., 2004), thus with the capacity for a brisk effect on immune as well as neural cells.

Because CCL5 has been shown to inhibit HIV-1 infection (Cocchi et al., 1995) and can protect from gp120-mediated neurotoxicity (Kaul and Lipton, 1999); the increases here may be part of the protective measures of the CNS against infection. Additionally, because another study showed that CCL5 treatment of a differentiated neuronal cell line led to induction of genes involved in neuronal survival, synaptogenesis, and neurite outgrowth (Valerio et al., 2004), this also may imply a protective function for CCL5 on neurons. However, there are also detrimental aspects associated with the presence of CCL5. For instance, CCL5 application resulted in apoptosis in a neuroblastoma cell line (Cartier et al., 2003). It is also a chemoattractant, and memory and effector CTLs efficiently migrate in response to CCL5 (Fukada et al., 2002), leading to immune cell infiltration as found in the increased numbers of CD8 T-cells found here. Such a response is likely aimed at protecting against the virus but, over a chronic course, can potentially damage neurons and other CNS cells. Although a low level of CCL5 may be protective, the rising amounts we found as infection progressed may eventually lead to various mechanisms of damage to the CNS.

An essential component of the immune system, the MHC system, was represented in our monkeys by upregulation of both class I (HLA-A and HLA-C) and class II (HLA-DRα) transcripts. Because the MHC class I genomic region encoding the Mamu (Maca ca mulatta, rhesus monkeys) genes is quite different in rhesus than that found for HLA (Otting et al., 2005), the common class I light chain β2M, which is regulated in a similar manner to MHC class I genes (van den Elsen et al., 2004), was examined as a surrogate. Indeed, β2M was significantly upregulated in the chronic phase, likely reflecting immune activation. Although quite prominent in end-stage HIV/SIV encephalitis, immunohistochemistry failed to identify a difference in HLA-DR staining of the brain between chronically infected and uninfected animals. However, more sensitive analysis using flow cytometry indeed has reported that HLA-DR is increased on microglia in the chronic stage of SIV infection (Scheller et al., 2005), likely reflecting the increase in mRNA detected by the gene array and qRT-PCR.

The humoral arm of the adaptive immune system also undergoes alterations in this situation (i.e., we find increases in the level of IgGhc transcription). As expected for chronic exposure to an antigen, the highest levels of expression occur as exposure time increases. Humoral immunity in the brain has not been extensively studied in HIV infection, although intrathecal anti-HIV antibody production was identified early in the AIDS pandemic (Resnick et al., 1985) and can be associated with neurological disorders (Fainardi et al., 2001). A study of brain-resident, antibody-secreting cells (plasma cells) performed in SIV-infected rhesus macaques (Sopper et al., 1998) revealed that the frequency of anti-gp120 plasma cells increased with time of infection and amounted to, on average, 15% of all plasma cells, far exceeding the 2–4% in blood, spleen, and lymph nodes. Although we have not been able to detect B-cells by flow cytometry or immunohistochemistry in the brains of SIV-infected monkeys (our unpublished observations), the antigens identified by the antibodies used in those studies are not expressed by plasma cells. Viruses from the brains of infected monkeys can show increased sensitivity to neutralization by antibodies (Puffer et al., 2002; Song et al., 2004). Therefore, humoral immune responses within the brain may be an important protective arm against the viral attack within the CNS.

The final two genes identified by the gene array in the chronically SIV-infected brain, G1P3 and IFTTM1, are known as interferon-inducible genes and have poorly characterized functions, although they are implicated in antiviral activities. No increases in any of the interferons were seen by qRT-PCR at this stage (our unpublished observations); however, as we hypothesized previously, other cytokines or infection itself may be responsible for the upregulation of G1P3 and IFTTM1 (Roberts et al., 2004).

The following picture emerges of the brain in this chronic stage of SIV infection. A low level of productive viral infection coexists with an immune cell response, maintaining a stable steady-state balance of virus and the immune response against it. Some of this response is relatively nonspecific, such as the increase in the interferon-inducible genes as well as HLA molecules, whereas the adaptive immune response, manifested by CD8+ CTLs and antibody-producing cells, are likely enriched for specific reactivity against the virus. We have identified a molecule made by CTLs, CCL5, which, after a peak in the acute phase of infection, is elevated, and gradually increases over time to this chronic phase. CCL5 has direct antiviral action and the ability to attract other immune cells into the brain, including CTLs, thus playing an important role in the equilibrium existing in the infected brain. CCL5 also has a number of varied effects on neurons themselves. Although a chronic host-pathogen interaction can be tolerated by some physiological systems, the brain is a unique organ. Through CCL5 and perhaps other products of the protective immune response, neurons themselves can be affected, enabling us to detect CNS functional abnormalities before the onset of frank neurological disorders later in the disease course. Viral infection of the brain, which occurs early after HIV/SIV inoculation, may upregulate factors we see here such as CCL5, which may be short-term protective measures but are, in the long term, harmful.

Highly active antiretroviral therapy (HAART) can be quite efficacious in lowering peripheral viral load, prolonging life span, and lowering the incidence (annual number of new cases) of CNS disorders caused by HIV. However, with the advent of HAART, the total prevalence (total number of people currently afflicted) of HIV-related cognitive impairment has either not changed or is possibly increasing (Sacktor et al., 2002; Dore et al., 2003; Cysique et al., 2004; Tozzi et al., 2005). Furthermore, a recent neuropathological autopsy study of HAART-treated HIV-infected individuals revealed a surprising level of neuroinflammation, with high levels of microglial activation and macrophages in the brain (Anthony et al., 2005). Possibly, this therapy does not reach sufficient concentrations within the brain to inhibit viral replication, thus requiring agents with better blood–brain barrier penetration (Letendre et al., 2004).

In conclusion, the many instances of injurious changes during the chronic period of SIV infection documented here indicate that labeling this phase “asymptomatic” is a dangerous misnomer. Although some of the factors we identified may protect the CNS, others may lead to the behavioral, physiological, and neuronal malfunctions we have observed in these cases. Alterations within the brain defined here can provide a basis for calculating the effect of therapy on the CNS.
References


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