



Effect of Combined Antiretroviral Therapy on Brain and Lymphoid Tissue Virus Burden in An Accelerated Model of SIV Encephalitis in Rhesus Macaques

Lakshmanan Annamalai*¹, Susan Westmoreland¹, Douglas Pauley¹, Kenneth Williams², Eva Ratai³, Margaret Lentz³, Raymond Schinazi⁴, Norbert Bischofberger⁵, R. Gilberto González³ and Shawn P. O'Neil¹

¹New England Primate Research Center, Harvard Medical School, Southborough, MA, ²Boston College, Chestnut Hill, MA, ³A. A. Martinos Center for Biomedical Imaging, Massachusetts General Hospital, Charlestown, MA, ⁴Emory University School of Medicine/Veterans Affairs Medical Center, Atlanta, GA and ⁵Gilead Sciences, Foster City, CA.

Abstract

Background: Like lymphoid tissue, brain serves as an important anatomical reservoir for HIV. Highly active antiretroviral therapy (HAART) has been shown to the prevalence of HIV-associated dementia; however, the direct impact of HAART on brain virus burden in relation to peripheral lymphoid tissue virus burden remains unknown.

Methods: We used CD8-depletion model of SIV encephalitis (SIVE) to investigate the effect of short-term combined antiretroviral therapy (CART) on virus burden in brain and peripheral lymphoid tissues in rhesus monkeys. Eight monkeys were inoculated with SIVmac251 and depleted of CD8⁺ T cells. Four monkeys received CART (consisting of daily subcutaneous injections of Raltegravir and Tenofovir) for 28 days, beginning 28 days post-inoculation, while the remaining four animals served as untreated controls. Brain specimens (frontal cortex, putamen, hippocampus, and brainstem) and lymphoid tissues (spleen, bone marrow, and mesenteric, axillary, and inguinal lymph nodes) were collected during necropsy and the tissue virus burdens were measured by real time RT-PCR. In addition, the extent of viral replication in tissues was evaluated by computer image analysis on tissue sections subjected to *in situ* hybridization (ISH) for SIV RNA.

Results: Tissue virus burdens were lower in all compartments of brain and lymphoid tissues from animals that received CART; however, the differences were statistically significant only in frontal cortex, inguinal lymph node, mesenteric lymph node, spleen, and bone marrow ($p < 0.03$). Productively infected cells were localized by ISH in the brain parenchyma of both treated and untreated macaques; however, significantly fewer SIV+ cells were observed in frontal cortex and brainstem from animals that received CART as compared to untreated controls. Surprisingly, we were unable to localize viral RNA by ISH in many of the brain lesions that were present in treated animals.

Conclusions: Although virus burdens were lower in both brain and lymphoid tissues from monkeys that received short-term CART as compared to untreated controls, statistically significant differences were measured in only 1 of 4 regions of brain parenchyma in contrast to 4 of 5 lymphoid organs. This suggests that longer periods of therapy may be required to reduce brain virus burden than lymphoid virus burden, which may reflect the longer half-life of the principal target cells in the brain (macrophages) versus lymphoid tissues (T lymphocytes).

Supported by NIH grants NS050041, NS048831, MH61232, and RR01618.

Methods

In situ hybridization (ISH) for SIV

Productively infected cells in formalin-fixed, paraffin-embedded sections of brain were localized by ISH for SIV RNA, as described elsewhere. Tissue sections were deparaffinized in xylene and rehydrated in graded ethanol to diethyl pyrocarbonate (Sigma Chemical Co., St. Louis, MO) treated water. Endogenous alkaline phosphatase activity was blocked with levamisole (Sigma). Tissue sections were hybridized in HCl (Sigma), digested with proteinase K (Roche Diagnostics Corp., Indianapolis, IN), oxidized in acetic anhydride (Sigma), and hybridized overnight at 50°C with a digoxigenin-labeled antisense riboprobe which spans the entire genome of the SIVmac239 molecular clone of SIVmac251 (Lofstrand Labs, Gaithersburg, MD). The following day, tissue sections were washed extensively and bound probe was detected by immunohistochemistry (IHC), using alkaline phosphatase-conjugated sheep anti-digoxigenin F(ab) fragments (Roche) and the chromogen, nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, Roche). Sections were counterstained with nuclear fast red (Vector Labs, Burlingame, CA). Sections of brain from a rhesus macaque with SIVmac251 encephalitis served as both positive control (when incubated with SIV sense probe) and negative control (when reacted with SIV sense probe). Additional negative controls included sections of brain from uninfected macaques reacted with SIV antisense probe.

Computer Image analysis

SIV infected cells were enumerated in sections of brain tissue from each of the SIV infected macaques by computer image analysis as described elsewhere. Images of tissue sections were captured without manipulation using a Olympus 3-CCD T60C color video camera mounted on a Olympus Vanox-SI microscope and analyzed using NIH Image J software.

Quantitation of tissue virus burden. The relative cell-associated virus load was measured in four regions of brain parenchyma (frontal cortex, putamen, hippocampus, and brainstem) from each infected macaque. For each anatomic compartment, virus burden was measured by computer image analysis, counting the number of chromogen positive cells per unit area (mm²) on tissue sections that had been subjected to a carefully controlled ISH assay for SIV RNA. Particle size and threshold parameters were established on control sections of brain from infected and uninfected macaques that had been incubated with SIVmac239 antisense probe and processed in parallel with experimental sections. The mean virus burden for each anatomic compartment of brain (regional mean) was determined for each SIV-infected macaque by counting the number of chromogen positive cells in 20 random fields at 200x magnification, and dividing by the total area analyzed (reported as SIV infected cells per mm²).

Immunohistochemistry for CD68

Cells expressing CD68 were localized in sections of brain by IHC, using KP1 (anti CD68, DAKO Corp.) and a commercial kit (ABC Elite, Vector Laboratories). Formalin-fixed, paraffin-embedded sections of brain were deparaffinized in xylene and rehydrated through graded ethanol to distilled water. Endogenous peroxidase activity was blocked by incubation in 3% H₂O₂, and antigen retrieval was accomplished by microwaving sections for 20 minutes in citrate buffer (DAKO). Sections were then incubated for 30 minutes at room temperature with antibodies, and reacted sequentially with biotinylated secondary antibody and horseradish peroxidase-conjugated avidin DH. Antigen-antibody complex formation was localized by development in the chromogenic substrate 3, 3'-diaminobenzidine (DAKO). Tissue sections were counterstained in Mayer's hematoxylin (Sigma-Aldrich), cleared, and cover-slipped with permanent mounting medium.

RNA isolation and Real Time RT-PCR

Brain and lymphoid tissue specimens that had been collected in RNAlater (Ambion) and stored at -80°C were homogenized in Trizol reagent (Invitrogen) using silica beads and a bead beater. Chloroform was added to the homogenate, and the aqueous phase was collected after centrifugation. An equal volume of 70% ethanol was added to the aqueous phase and the total RNA was purified using the RNeasy kit (Qiagen). RNA quantification was carried out using the Ribogreen kit (Invitrogen).

Real time RT-PCR was carried out in an ABI 7700 thermal cycler using one step RT-PCR master mix and gene specific primers and FAM-labeled TaqMan probes. *In vitro* transcription of the SIVmac251 gag gene, TNF-alpha, and RPL13A were carried out using their respective plasmid constructs. Based on the molecular mass of the transcripts, copy numbers were determined and the standards for real time PCR were generated by serially diluting known copy numbers of the transcripts. RPL13A was used as internal control and the copy numbers of SIVmac251 gag and TNF-alpha were determined by using 100-500 ng of total RNA.

Statistical analyses

A two-tailed, nonparametric Mann-Whitney test was used to compare the virus burden measured by *in situ* hybridization and real time RT-PCR between control animals and animals that received CART. Significant differences were assumed for probability values of $p < 0.05$.

Results

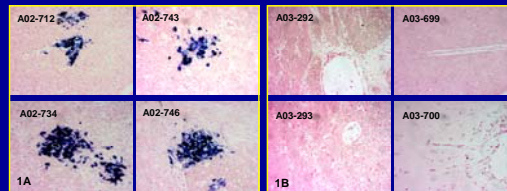


Figure 1. Relative brain virus burden in untreated control macaques vs. animals receiving CART. ISH for SIV RNA showing productively infected cells in brain parenchyma from four untreated control macaques (Panel A). The presence of viral RNA, identifying SIV-infected cells, is localized with the dark blue chromogen NBT/BCIP. SIV-infected cells are localized far less frequently in brain sections from animals that received CART (Panel B). Nuclear fast red counterstain. All sections are at 200x magnification.

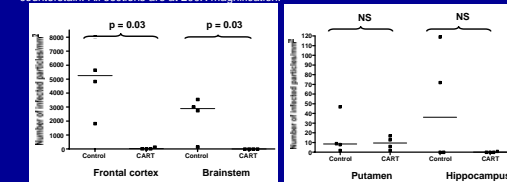


Figure 2. Computer image analysis of *in situ* hybridization for SIV RNA. Significantly fewer SIV+ cells are seen in frontal cortex and brainstem from animals that received CART as compared to untreated controls. However, the difference in numbers of infected cells in putamen and hippocampus are not significant between control and treated animals. Note that markedly fewer numbers of SIV infected cells in putamen and hippocampus in both control and antiretroviral agent treated animals. NS, not significant.

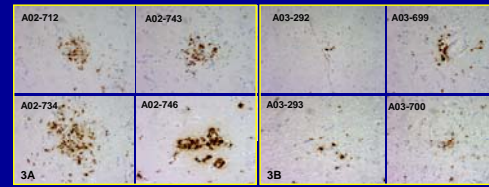


Figure 3. Fewer activated macrophages are localized in brain tissue from monkeys that received CART than in controls. IHC assays using an antibody that recognizes CD68, an antigen expressed on activated macrophages. Panel 3A shows large numbers of CD68+ macrophages in microglial nodules and perivascular infiltrates in brain sections from control animals. Fewer CD68 expressing cells are present in brain sections from animals treated with combination antiretroviral therapy (Panel 3B). DAB (brown) chromogen with hematoxylin counterstain. All sections are at 200x magnification.

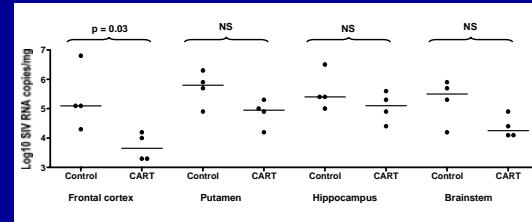


Figure 4. Quantification of virus burden in brain tissue by real time RT-PCR. Tissue virus burdens were lower in all compartments of brain tissue examined from animals that received CART; however, the differences were statistically significant only in frontal cortex. Horizontal bars represent median values for each data set. NS, not significant.

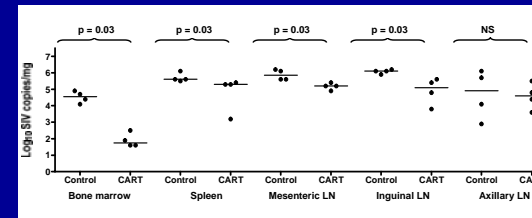


Figure 5. Quantification of virus burden in lymphoid tissues by real time RT-PCR. Tissue virus burdens are significantly lower in bone marrow, spleen, mesenteric lymph node, and inguinal lymph node from animals that received CART than from controls. Horizontal bars represent median values for each data set. NS, not significant.

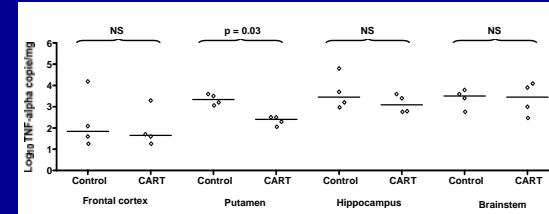


Figure 6. Enhanced expression of TNF-alpha in brain of control animals. Real-time RT-PCR with TNF-alpha specific TaqMan probe shows enhanced expression in all four regions of brain examined in control animals compared to animals that received CART. The levels of TNF-alpha expression are significantly lower only in putamen from monkeys that received CART. Horizontal bars represent median values for each data set. NS, not significant.

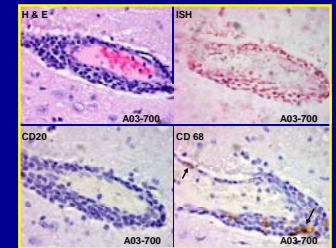


Figure 7. Brain lesions in animals that received CART. Sections of brain tissue from two of four animals that received CART show perivascular mononuclear cell infiltrates in multiple regions. However, SIV RNA was not detected in the cells of these infiltrates by ISH. The mononuclear infiltrates do not express the B-lymphocyte antigen, CD20. Few CD68 expressing macrophages are localized in the lesions (arrows).

Conclusions

- The virus burden was lower in all regions of brain tissue from monkeys that received CART as compared to control animals.
- The differences in virus burden between control animals and those that received CART were statistically significant in frontal cortex, bone marrow, spleen, mesenteric lymph node, and inguinal lymph node but not in the brain stem, hippocampus, putamen and axillary lymph node.
- The level of TNF-alpha expressed in all regions of brain tissue examined was lower in animals that received CART than in control animals.
- Although the virus burden was lower, mild encephalitis was present in two of four animals that received CART.
- A prolonged period of therapy may be required to reduce brain virus burden lymphoid tissue virus burden.
- By reducing the virus burden in the brain, antiretroviral agents may decrease the incidence and severity of encephalitis indirectly by reducing the expression of inflammatory cytokines.