Proliferating Cellular Nuclear Antigen Expression as a Marker of Perivascular Macrophages in Simian Immunodeficiency Virus Encephalitis

Kenneth Williams,* Annette Schwartz,[†]
Sarah Corey,* Marlene Orandle,^{†‡}
William Kennedy,[†] Brendon Thompson,[†]
Xavier Alvarez,^{†‡} Charlie Brown,[‡]
Suzanne Gartner,[§] and Andrew Lackner^{†‡}

From the Department of Medicine,* Harvard Medical School, Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, Boston, Massachusetts; the Division of Comparative Pathology,[†] New England Regional Primate Research Center, Harvard Medical School, Southboro, Massachusetts; the National Institutes of Health,[‡] Rockville, Maryland; and the Department of Neurology,[§] Johns Hopkins Medical School, Baltimore, Maryland

Brain perivascular macrophages are a major target of simian immunodeficiency virus (SIV) infection in rhesus macaques and HIV infection in humans. Perivascular macrophages are distinct from parenchymal microglia in their location, morphology, expression of myeloid markers, and turnover in the CNS. In contrast to parenchymal microglia, perivascular macrophages are continuously repopulated by blood monocytes, which undergo maturation to macrophages on entering the central nervous system (CNS). We studied differences in monocyte/macrophages *in vivo* that might account for preferential infection of perivascular macrophages by SIV. *In situ* hybridization for SIV and proliferating cellular nuclear antigen (PCNA) immunohistochemistry demonstrated that SIV-infected and PCNA-positive cells were predominantly found in perivascular cuffs of viremic animals and in histopathological lesions that characterize SIV encephalitis (SIVE) in animals with AIDS. Multilabel techniques including double-label immunohistochemistry and combined *in situ* hybridization and immunofluorescence confocal microscopy revealed numerous infected perivascular macrophages that were PCNA-positive. Outside the CNS, SIV-infected, PCNA-expressing macrophage subpopulations were found in the small intestine and lung of animals with AIDS. While PCNA is used as a marker of cell proliferation it is also strongly expressed in non-dividing cells undergoing DNA synthesis and repair. Therefore, more specific markers for cell proliferation including Ki-67, topoisomerase II α , and bromodeoxyuridine (BrdU) incorporation were used

which indicated that PCNA-positive cells within SIVE lesions were not proliferating. These observations are consistent with perivascular macrophages as terminally differentiated, non-dividing cells and underscores biological differences that could potentially define mechanisms of preferential, productive infection of perivascular macrophages in the rhesus macaque model of neuroAIDS. These studies suggest that within CNS and non-CNS tissues there exist subpopulations of macrophages that are SIV-infected and express PCNA. (Am J Pathol 2002, 161:575–585)

Infection of the central nervous system (CNS) by HIV or simian immunodeficiency virus (SIV) can result in neurological disease and AIDS dementia complex (ADC).¹ The best histological correlate of ADC is inflammatory macrophages in the CNS, some of which are infected.² We have demonstrated that perivascular macrophages as opposed to parenchymal microglia, are a major component of CNS lesions in rhesus macaques infected by SIV.³ Perivascular macrophages are the primary cell productively infected at peak viremia (2 weeks p.i.) and terminal AIDS in SIV encephalitis (SIVE).³ We and others have shown that productive CNS infection by HIV and SIV is transient, occurring at peak viremia then subsiding until late stage disease.^{3–7} Mechanisms for the preferential infection of subpopulations of tissue macrophages or CNS perivascular macrophages by SIV have not been addressed.

Brain perivascular macrophages, as transient CNS residents, are continuously replaced by bone marrow-derived monocytes.^{8–11} Approximately 30% of perivascular macrophages turnover in normal rodents within a 3–4 month period.⁸ In contrast, turnover of parenchymal microglia in the normal or inflamed CNS of rodents and humans is negligible.^{8–13} Similar to other human¹⁴

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Tulane Regional Primate Research Center, Tulane University Health Sciences Center, Covington, LA (M.O., X.A., A.L.).

Address reprint requests to Dr. Kenneth C. Williams, Division of Viral Pathogenesis, RE-113, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215. E-mail: kenneth_williams@hms.harvard.edu.

or non-human primate macrophage populations, neither perivascular macrophages nor parenchymal microglia undergo significant, detectable proliferation.¹⁵ It is possible, however, that bone marrow and blood monocytes, destined to replace CNS perivascular macrophages, undergo limited cell division.¹⁴ We have previously hypothesized that preferential infection of perivascular macrophages and continued turnover of these cells in the CNS may, in part, account for the observed kinetics of viral neuroinvasion and subsequent disappearance and reappearance of productive infection in the CNS.^{3,5} We now further hypothesize that developmental differences between perivascular macrophages that are transient CNS residents, and parenchymal microglia that are present in the CNS at birth and are long-term CNS residents, may account for preferential infection of perivascular macrophages. Moreover, HIV and SIV might also preferentially infect populations of tissue macrophages outside of the CNS, in a developmentally regulated manner.

Macrophages and CD4⁺ T lymphocytes differ with respect to HIV and SIV infection.^{16–18} In contrast to lymphocytes, macrophage infection is not dependent on cell activation and productively infected macrophages can be long-lived.^{19–21} Human and nonhuman primate derived macrophages do not undergo significant proliferation outside the bone marrow. However, DNA metabolism by macrophages appears important for normal cell function. In fact, macrophages have high rates, compared to other cell types, of DNA metabolism, resulting in part from their low levels of deoxynucleotides.^{22,23} It is therefore possible that high levels of DNA repair and recycling may facilitate lentiviral infection of macrophages.²³ In addition to differences in the biology of T lymphocyte *versus* macrophage infection by HIV, differential viral infection and infectability of macrophage subpopulations is also evident.^{24,25} Thus, the ability of HIV or SIV to infect macrophage subpopulations, and to become integrated within host DNA, may be regulated developmentally and controlled not only by surface receptors such as CD4, CXCR4, and CCR5, but by DNA metabolism.

We investigated a series of developmentally regulated myeloid markers and markers of DNA metabolism and report that PCNA, an antigen expressed by proliferating cells in the S phase of cell cycle^{26–29} and non-dividing cells undergoing DNA repair^{30,31} is selectively expressed by CNS perivascular macrophages early after infection and terminally in animals with AIDS and SIVE. Using more specific markers of cell proliferation including Ki-67, topoisomerase II α , and BrdU incorporation, we demonstrate that PCNA-positive perivascular macrophages do not undergo detectable cell division. These data suggest that PCNA-positive cells undergoing DNA synthesis and repair could facilitate productive SIV infection, and/or that PCNA expression is induced in these cells as a result of such infection. Similar to the CNS, we report that populations of macrophages in the small intestine and lung of animals with AIDS are SIV-infected and PCNA-positive. The short half-life of PCNA expression *in vitro*²⁹ supports the notion that the development of SIVE and perhaps HIVE, as seen by the accumulation of infected and non-

infected macrophages and MNGC, is a late event in AIDS pathogenesis, occurring concomitant or following dysfunction of the peripheral immune system. The expression of PCNA in macrophage populations within the brain, small intestine, and lung suggest similarities in the biology of these cells which might define, in part, developmental and regulatory factors contributing to their susceptibility for SIV and HIV infection.

Materials and Methods

Animals and Viral Infection

CNS tissues from 16 adult rhesus macaques were used for studies to characterize PCNA expression. Eight of these animals were infected with SIVmac251 (20 ng of SIV p27) by intravenous injection and killed at peak viremia (14 days p.i., $n = 3$) or terminal AIDS with SIVE ($n = 5$). Acutely infected animals (eg, 14 days p.i.) can have mild encephalitis characterized by perivascular cuffs of mononuclear cells but do not have multinucleated giant cells (MNGC) which are the hallmark of SIVE.^{32–35} One of the animals with terminal AIDS and SIVE was injected with bromodeoxyuridine (BrdU), 60 mg/kg, two hours before death. Gut tissues from this animal served as BrdU control tissues. Control CNS tissues ($n = 2$) consisted of brains obtained from normal, age-matched, and non-infected animals. In addition, to study the expression of PCNA in tissue macrophages outside the CNS, lung, small intestine and lymph node from animals at peak viremia ($n = 2$), terminal AIDS ($n = 2$), and uninfected controls ($n = 2$) were examined. All animals were housed at Harvard University's New England Regional Primate Research Center in accordance with standards of the American Association for Accreditation of Laboratory Animal Care.

Tissue Collection and Processing

All animals were anesthetized with ketamine-HCl and euthanized by an intravenous pentobarbital overdose and exsanguinated. CNS tissues were either collected in 10% neutral buffered formalin, and embedded in paraffin, or were blocked in optimal cutting temperature compound (O.C.T., Miles Inc., Elkhart, IN) and snap frozen. Formalin-fixed, paraffin-embedded tissues were sectioned at 6 μ m and used for immunohistochemistry or counterstained with hematoxylin and eosin by routine techniques. Frozen tissues were sectioned at 6 μ m and used for immunohistochemistry and immunofluorescence studies.

Immunohistochemistry

Routine, single-label immunohistochemistry was performed on formalin-fixed, paraffin-embedded sections using an avidin-biotin-complex method (ABC Standard, Vector Laboratories, Burlingame, CA) with diaminobenzidine (DAB) as the chromogen. Antibodies used included those with specificity for PCNA (M0879, DAKO Corp,

Carpinteria, CA), CD68 (KP-1, M0814, DAKO), BrdU (M074, DAKO), topoisomerase II α (M3532, DAKO) and Ki67 (MIB-1, M0505, DAKO). For all immunohistochemical studies, isotype-matched irrelevant immunoglobulins (DAKO) were used as negative controls.

Immunophenotype of PCNA-Positive Cells

Double-label immunohistochemistry and immunofluorescence were performed as previously described, to define the immunophenotype of PCNA-positive cells.^{3,36,37}

Immunohistochemistry

Double-label immunohistochemistry for PCNA and CD68 was performed to determine whether the PCNA immunoreactive cells were macrophages. For this, an anti-PCNA mAb was applied first with DAB as the chromogen as described above. An anti-CD68 antibody was then applied and detected using an alkaline phosphatase system (LSAB+, DAKO) and Vector Blue (Vector Laboratories) as the chromogen.

Immunofluorescence

Double-label immunofluorescence was performed on paraffin-embedded tissue sections using the anti-PCNA mAb (IgG1) and anti-glial fibrillary acidic protein (GFAP)-Cy3 (G-A-5, Sigma, St Louis, MO) for detection of astrocytes, or a rabbit polyclonal antibody specific for glucose transporter enzyme-1 (Glut-1, Chemicon) to identify CNS endothelial cells. For double labels using PCNA and GFAP conjugated to Cy3, a goat anti-mouse Alexa 488 (Molecular Probes, Eugene, OR) was used following the PCNA mAb. For double-label using PCNA and Glut-1, indirect immunofluorescence was performed and following primary antibodies, anti-mouse Alexa 488 was used to detect PCNA, while anti-rabbit Alexa 568 (Molecular Probes) was used to detect Glut-1. After immunofluorescence labeling, tissue sections were treated with 50 mmol/L CuSO₄ ammonium buffer for 15 minutes at room temperature to quench autofluorescence.³⁸

Assessment of SIV and PCNA Localization with SIVE Lesions

To further identify PCNA-positive cells and to determine their anatomical location relative to cells infected by SIV, confocal microscopy was used. Formalin-fixed, paraffin-embedded tissue sections were pretreated in a microwave for 20 minutes at high power with Vector antigen unmasking solution (Vector) according to the manufacturer's instructions. Thereafter, sections went through sequential washes, and were prehybridized at 45°C with hybridization buffer (containing 50% formamide with denatured herring sperm DNA and yeast tRNA at 10 mg/ml each). SIV digoxigenin-labeled riboprobes (provided by Drs. V. Hirsch and C. Brown, NIH, Rockville, MD)³⁹ were used at a concentration of 10 ng/slide in hybridization

buffer and hybridized overnight at 45°C. After stringency washes and blocking solution, sheep anti-digoxigenin (Roche) was applied to detect the probe, and slides were developed using HNPP/Fast Red TR (Roche). The HNPP/Fast Red substrate produces visible precipitates that fluoresce red when exposed to a 568-nm wavelength laser. Indirect immunofluorescence for PCNA was performed as described above using anti-mouse Alexa 488. In select tissue sections, SIV, PCNA, and cells undergoing programmed cell death were studied using *in situ* hybridization for SIV, immunofluorescence for PCNA, and *in situ* end-labeling (ApoTag, Invitrogen, Gaithersburg, MD) according to the manufacturer's instructions. To-Pro3 (a nuclear dye, Molecular Probes) was used in some sections to differentiate between individual cells. In this case To-Pro3 at 1 μ g/ml was incubated on tissue sections for 5 minutes and tissues were washed in PBS.

Confocal Microscopy

Confocal microscopy was performed using a Leica TCS SP laser scanning microscope equipped with 3 lasers (Leica Microsystems, Exton, PA). Individual optical slices represent 0.2 μ m, and 32 to 62 optical slices were collected at 512 $\times$ 512 pixel resolution. The fluorescence of individual fluorochromes was captured separately in a sequential mode, after optimization to reduce bleed-through between channels (photomultiplier tubes) using Leica software. NIH Image v1.62 and Adobe Photoshop v6 software were used to assign correct colors of up to four channels collected (3 fluorochromes: FITC or Alexa 488 (green), Cy3, Alexa 568 (red), and To-Pro3 (blue)), and the differential interference contrast image (gray scale).

Results

CNS Perivascular Macrophages are PCNA Immunoreactive following SIV Infection

In this study we sought to define differences in the biology of CNS perivascular macrophages versus parenchymal microglia which might result in preferential infection of perivascular macrophages. We hypothesized that developmental differences including levels of DNA repair between perivascular macrophages that are short-lived, and parenchymal microglia may play a role. We focused on PCNA a protein that is abundant in actively proliferating cells in S phase of the cell cycle and in non-dividing cells undergoing DNA synthesis and repair.²⁹⁻³¹ PCNA was not detected in the CNS of normal, non-infected animals, but a few PCNA-positive cells were found within perivascular cuffs of acutely viremic animals. PCNA was readily detected in the CNS of all animals with terminal AIDS and SIVE (Figure 1A). The majority of PCNA-positive cells in the CNS of animals with terminal AIDS and SIVE were located within perivascular cuffs and aggregates of mononuclear cells and MNGC characteristic of SIVE.

Figure 1. PCNA expression in the CNS of SIV infected animals is associated with macrophages in SIV lesions. **A:** Immunoperoxidase for PCNA in the brain of an SIV-infected macaque with terminal AIDS and encephalitis (SIVE). The majority of the PCNA-positive cells appear to be macrophages within an SIVE lesion. **B:** CD68 (blue) and PCNA (brown) immunohistochemistry within a mild perivascular cuff of an SIV-infected macaque with AIDS and SIVE. Within this panel there are CD68-positive macrophages which are PCNA-positive. **C:** CD68 (blue) and PCNA (brown) immunoreactivity in an SIVE lesion. PCNA expression is detectable within the SIVE lesion and appears to be associated with CD68-positive macrophages. Data presented here are representative of $n = 2$ macaques at primary viremia and $n = 5$ macaques with SIVE. Multiple tissue sections from different brain regions were examined.

To better define PCNA -positive cell types in the CNS, double-label immunohistochemistry for PCNA and macrophages (CD68) was performed on acutely viremic animals and those with SIVE. Within perivascular cuffs of viremic animals and SIVE lesions, numerous CD68-positive macrophages were detected, many of which were PCNA-positive (Figure 1, B and C). CD68-positive and

Figure 2. CD14 and PCNA within SIVE lesions. **A:** CD14 perivascular macrophages are the major cell type within SIVE lesions. **B:** Several of the perivascular macrophages are PCNA-positive. Data presented here are representative of $n = 5$ macaques with SIVE. Multiple tissue sections from multiple brain regions were examined.

SIV-infected macrophages are identical in location and morphology to CD14-positive perivascular macrophages that are the major cell infected within the CNS of macaques with SIVE.³ Immunohistochemistry on frozen CNS tissues demonstrated CD14-positive perivascular macrophages are the major cell type accumulating in the SIVE lesions. The pattern of PCNA-positive cells appears to overlap with that of CD14-perivascular macrophages (Figure 2). We were unable to perform CD14 and PCNA double-label experiments because the anti-CD14 mAb only functions in frozen tissues while the anti-PCNA mAb optimally functions in paraffin-embedded tissues. In addition to PCNA-CD68-positive macrophages, a few scattered PCNA-positive, CD68-negative cells were identified within SIVE lesions. These CD68-negative, PCNA-positive cells could be CD8⁺ T lymphocytes that are a consistent but minor population (less than 5% of the inflammatory cells) identified within SIVE lesions.^{40,41}

In some areas within encephalitic brains, it appeared that rare CNS astrocytes or endothelial cells were also PCNA reactive. Double-label confocal microscopy for PCNA and GFAP or Glut-1 confirmed that rare PCNA-positive cells were astrocytes (GFAP⁺) or endothelial cells (Glut-1⁺) (Figure 3). The majority of PCNA immuno-

Figure 3. Confocal images of double-labeled immunofluorescence to further define the immunophenotype of PCNA-positive cells in the CNS of macaques with SIVE. **A:** PCNA (green), GFAP (red) expression in an SIVE lesion. The majority of PCNA-positive cells are GFAP-negative, indicating that they are not astrocytes. Few scattered PCNA-positive cells are GFAP-positive astrocytes. Side panels are single-color images of PCNA (green), GFAP (red), and differential interference contrast image (DIC). **B:** PCNA (green) and Glut-1 (red) expression around a CNS vessel from an animal with SIVE. Several Glut-1-positive endothelial cells are PCNA-positive. Side panels are PCNA (green), Glut-1 (red) and DIC. Data presented here are representative of $n = 3$ macaques with SIVE. Multiple tissue sections from different brain regions were examined.

reactive cells were CD68-positive perivascular macrophages.

To characterize the expression of PCNA in tissue macrophages outside the CNS, double-label CD68 and PCNA immunohistochemistry was done in lung, small intestine and lymph node. In sections of lung from uninfected and viremic animals (Figure 4A), there were abundant CD68-positive macrophages within the interstitium, but PCNA expression was minimal, and double-positive cells were rare. In contrast, in sections of lung from animals with AIDS (Figure 4B), there were abundant CD68-positive interstitial macrophages as well as macro-

phages within alveolar spaces. These CD68⁺ cells were frequently PCNA-positive. In addition, in the lung of AIDS animals, there were numerous CD68-PCNA-double positive MNGC. In the small intestine of all animals examined, the lamina propria of most villous tips contained aggregates of CD68-positive macrophages. However, PCNA staining of these CD68-positive cells was variable in uninfected and viremic animals (Figure 4C), whereas in terminal AIDS animals (Figure 4D), most CD68-positive cells were PCNA-positive. In all animals, the majority of crypt of Lieberkuhn epithelial cells were PCNA-positive. In lymph nodes of control animals, PCNA staining was

Figure 4. PCNA expression in macrophages outside the CNS (lung, small intestine, lymph node). **A:** Double-label immunohistochemistry for PCNA (brown) and CD68 (blue) in the lung of an animal at peak viremia shows scattered CD68-positive cells, but no PCNA staining. **B:** In the lung of an SIV-infected macaque with terminal AIDS, the majority of macrophages (CD68+, blue) are also PCNA-positive including a multinucleated giant cell (center). **C:** Small intestine from a macaque at peak viremia shows PCNA-positive cells within the villous tip lamina propria but many CD68-positive cells are not PCNA-positive. **D:** In the small intestine from a macaque with terminal AIDS, most CD68-positive cells in the villous tip are also PCNA-positive. **E:** In the germinal center of a lymph node from an uninfected control there is abundant PCNA staining with scattered CD68-positive cells which do not stain for PCNA. **F:** In the medullary sinusoids of a lymph node from a macaque with terminal AIDS, PCNA-positive nuclei are present within a CD68-positive MNGC.

predominantly found in germinal centers (Figure 4E) with scattered positive cells in the medulla. A small number of CD68-positive cells were scattered within lymphoid follicles, and there was abundant staining of macrophages within medullary sinusoids. CD68- and PCNA-double-positive cells in control lymph nodes were rare. In viremic and terminal AIDS animals, lymph node staining was similar to that of the uninfected controls, however increased numbers of double-labeled cells were identified within the subcapsular sinuses and medullary sinusoids. In the lymph nodes of animals with terminal AIDS, there were scattered MNGC which were both PCNA- and CD68-positive (Figure 4F).

PCNA-Immunoreactive Cells in the CNS of SIV-Infected Macaques Are Not Undergoing Cell Proliferation

Since PCNA expression is associated with cell proliferation as well as DNA synthesis and repair,^{29–31} we sought to determine whether the PCNA-positive cells were proliferating. This was achieved using more specific cell proliferation markers including Ki-67 and topoisomer-

Figure 5. PCNA-reactive cells do undergo significant cell proliferation. Immunohistochemistry for BrdU incorporation (**A, B**), and labeling for Ki-67 (**C, D**) or topoisomerase II α (**E, F**) in brains of animals with SIVE (**A, C, E**) or control tissues (**B, D, F**). **A:** BrdU immunohistochemistry in the CNS tissue of an animal with AIDS and SIVE that was pulsed with BrdU before sacrifice (as described in the material and methods). No positive cells were present. **B:** BrdU incorporation within the gut of an SIV infected macaque with terminal AIDS and SIVE. **D:** Ki-67 within the spleen of an animal with SIVE. **C:** Ki-67 immunohistochemistry of the CNS of the same animal as shown in **D**. There were few scattered cells in the parenchyma but no detectable Ki-67 expression within SIVE lesions. **F:** Topoisomerase II α immunoreactivity within tonsil from an animal with SIVE. **E:** A few scattered cells in brains of animals with SIVE are positive for topoisomerase II α . These data suggest the PCNA-positive cells in SIVE are not proliferating. Data presented here are representative of $n = 5$ macaques with SIVE (for Ki-67 and topoisomerase II) and $n = 1$ macaque with SIVE (for BrdU). Multiple tissue sections from different brain regions were examined.

ase II α as well as BrdU incorporation. Few scattered cells in the CNS of animals with SIVE were immunoreactive for Ki-67, topoisomerase II α , or BrdU, although these markers were easily detected in lymphoid tissues of the same animals (Figure 5). These data suggest PCNA-immunoreactive macrophages in the perivascular cuffs and SIVE lesions are not undergoing significant cellular proliferation.

PCNA Immunoreactivity of Perivascular Macrophages Correlates with SIV Lesions

Our observations indicate that PCNA expression in the CNS occurs with the development of SIVE where the majority of PCNA immunoreactive cells are located within SIVE lesions. To determine whether viral infection alone drives PCNA expression, double- and triple-label confocal microscopy studies were performed to co-localize viral RNA with PCNA and cell type-specific markers. In these experiments we confirmed that the majority of PCNA expressing cells in SIVE lesions were macro-

Figure 6. Confocal microscopy for SIV and PCNA. Fluorescent *in situ* hybridization for SIV RNA followed by immunofluorescence for PCNA demonstrates the following. **A:** PCNA expression (green) in several nuclei of an SIV RNA (red)-positive MNGC. Side panels are SIV RNA (red), PCNA (green), and DIC. **B:** PCNA (green) and SIV RNA (red) within an SIVE lesion. Within this image many PCNA-positive cells are also SIV RNA-positive. There also appear to be PCNA-positive cells that are not SIV RNA-positive and SIV RNA-positive cells which are not PCNA-positive. Panel demonstrates PCNA (green) confined within an individual nucleus. Data presented here are representative of $n = 3$ macaques with SIVE. Multiple tissue sections from different brain regions were examined.

phages. Several of these PCNA-positive cells were also SIV RNA-positive (Figure 6). However, not all of the SIV RNA-positive cells had detectable levels of PCNA. Conversely, not all of the PCNA-positive cells had detectable SIV RNA (Table 1). These results indicate many, but not all of the PCNA-positive cells are productively SIV-infected as demonstrated by the presence of SIV RNA. When we compared double label immunohistochemistry results for SIV protein (p28) and PCNA, we found a higher

Table 1. Percentage of SIV RNA and PCNA Single and Double Positive Cells in the CNS of Animals with SIVE

Animal number	PCNA ⁺ SIV ⁻	SIV ⁺ PCNA ⁻	PCNA ⁺ SIV ⁺
A92-620	12.9	7.0	35.8
A97-6	12.6	8.6	47.3
A98-41	14.7	12.0	40.5
A97-76	5.0	17.6	11.8

Cell counts were acquired using brain sections from at least two different brain regions, examining 10 SIVE lesions per animal. MNGC were counted as one cell. PCNA⁺/SIV⁻, PCNA⁻ expressing non-SIV-infected cell; SIV⁺/PCNA⁻, SIV-infected non-PCNA-expressing cell; PCNA⁺/SIV⁺, PCNA-expressing and SIV-infected cell. SIV infection was assessed using *in situ* hybridization with SIV riboprobes as described in Materials and Methods.

percentage of cells that were SIV-infected and PCNA-positive (data not shown).

In addition to the demonstration of SIV- and PCNA-positive cells within classical SIVE lesions containing MNGC, on several occasions, we also detected PCNA-positive cells adjacent to CNS vessels (Figure 7). These SIV-infected, PCNA-positive cells are of particular interest because they may be cells that have recently migrated to the CNS.

Apoptosis of SIV RNA and PCNA-Positive Perivascular Macrophages

Perivascular macrophages are replaced by blood monocytes but the fate of the replaced perivascular macrophages is not defined. It has been proposed that they leave the CNS,⁴² but in different experimental conditions they remain in the CNS for years.⁴³ It also is possible that perivascular macrophages die in the CNS, where the rate of cell death could be augmented by lentiviral infection. To begin to examine these issues, we performed double- and triple-label immunofluorescence and confocal microscopy studies detecting SIV RNA, ApoTag, and PCNA. Within normal and non-infected CNS, we detected scattered ApoTag-positive cells around vessels that had morphology consistent with perivascular macrophages. Within SIVE lesions, we found CD68-positive macrophages which were PCNA-positive, but few of these cells were ApoTag-positive. ApoTag-positive cells were easily and preferentially detected in the majority of SIVE lesions examined. These observations underscore that perivascular macrophages within SIVE lesions have accentuated DNA repair, but suggest that few of these cells are committed to programmed cell death.

Discussion

Infection of the CNS of humans by HIV or macaques by SIV results in neurological disease. The best-known histological correlate of this disease is the presence and number of inflammatory macrophages. In macaques, the primary cell type that is productively infected *in vivo* is the perivascular macrophage as opposed to parenchymal microglia.³ Differences between perivascular macro-

Figure 7. PCNA expression in SIV-positive cells adjacent to a blood vessel in the brain of a macaque with terminal AIDS and SIVE. SIV RNA (red), PCNA (green), and To-Pro3 (blue, DNA) immunofluorescence demonstrating 3 cells adjacent to a CNS vessel. Two of these cells are PCNA-positive and also SIV-positive. This figure suggests that PCNA-positive cells which have recently trafficked to the CNS are SIV-infected. Side panels are SIV-RNA (red), PCNA (green), and To-Pro3 (blue) stained nuclei.

phages and parenchymal microglia, which could contribute to the observed selective infection *in vivo*, have not been studied. Both perivascular macrophages and parenchymal microglia have appropriate surface receptors (CD4, CXCR4, CCR5) that enable them to be infected. We sought to determine whether markers of monocyte/macrophage maturation might also play a role. In this report we demonstrate PCNA, a marker of cell proliferation and DNA repair, is expressed at high levels in the perivascular aggregates of mononuclear cells and MNGC that define SIVE. The PCNA-positive cells appear primarily to be perivascular macrophages based on location, morphology immunohistochemical phenotype and the fact that they were often SIV-infected.³ The absence of labeling with other, more specific markers of cell proliferation including BrdU incorporation indicates that these cells are not dividing. In addition to the brain, SIV-infected macrophages, which were PCNA-positive, were found in the small intestine and lung of viremic animals but mainly animals with AIDS. These observations raise the possibility that subpopulations of macrophages in gut, lung and CNS have similar biological characteristics with regard to SIV infection and PCNA

expression. It is interesting to note that similar to the CNS, tissue macrophages in the gut and lung can be a major target of SIV and HIV in humans and non-human primates with AIDS where susceptibility of macrophages to infection can parallel and perhaps drive histopathology.^{2,18,24,44}

PCNA, despite its name, is strongly expressed *in vitro* by non-dividing cells that have been irradiated and undergo DNA repair.^{29–31} PCNA is also strongly expressed in non-dividing macrophages in bone marrow of AIDS patients but is not found in bone marrow macrophages of patients with dysplastic disease.⁴⁵ Although an absolute correlation of SIV RNA with PCNA expression was not found on a cell-to-cell basis, PCNA-positive macrophages were often SIV RNA-positive and in several sections they accounted for more than 50% of cells in a lesion. It is possible that we have underestimated the number of SIV RNA-positive cells which are PCNA-positive because we only examined 10- to 30- μ m-thick sections. Given that lesions are considerably larger, we might have missed one marker or both that were positive above or below portions of lesions examined. Similarly, we have investigated SIV RNA-positive cells using a ri-

boprobe cocktail, consisting of seven different SIV probes spanning the majority of the SIVmac239 genome.³⁹ It is possible, however, that if we used riboprobes for particular genes (eg, *vpr*) the association could be increased. Additionally, stages of virus life cycle could be important for PCNA expression. Lastly, the kinetics of PCNA protein expression *in situ* are not defined, but expression *in vitro* lasts for approximately 24–48 hours.^{29,46} Thus, depending on how recently the cells arrived, stages of viral life cycle, and protocols used the percentage of labeled cells might change.

Our observations *in situ* suggest that macrophages in SIV-infected brains do not undergo significant and detectable proliferation. These observations are consistent with those in humans of limited proliferative capacity for monocyte/macrophages outside the bone marrow.¹⁴ This is in contrast to rodent monocyte/macrophages, which divide, *in vitro* and *in vivo*. It is possible, however, that bone marrow-derived monocyte/macrophages, which are programmed to traffic to the CNS and replace perivascular macrophages, could be PCNA-positive as they enter. Thus, PCNA expression may predispose perivascular macrophages to viral infection, or alternatively, PCNA expression might be found uniquely in perivascular macrophages, as a result of viral infection. Similar characteristics of viral infection and PCNA expression seem to function in the gut and lung where monocytes from the bone marrow traffic to and become macrophages in these tissues. One can envision a scenario in which monocytes infected in the bone marrow traffic to the lung, gut, or brain and arrive as infected cells. Infection of monocytes in the marrow and blood is considered to be low and difficult to detect. It is thought that cellular differentiation of myeloid cells parallels susceptibility to viral infection, and as these cells migrate and become macrophages, viral gene expression is detected.^{25,47} Moreover, select populations of tissue macrophages are infected by HIV and SIV. In the case of the lung, gut and brain, histopathology appears related to susceptibility of local macrophages to viral infection and replication.^{18,24} Precise mechanisms responsible for this are not defined, but our data suggest that PCNA expression by macrophages in these different tissues is a common feature and may relate to susceptibility of viral infection by HIV and SIV. Whether PCNA expression in the bone marrow precedes and facilitates viral infection or whether viral infection induces PCNA expression by monocyte/macrophages is not known but merits further study. Similarly, the analysis of PCNA expression developmental by monocyte and macrophage subpopulations seems important. Overall, these observations underscore the notion that perivascular macrophages in the CNS and subpopulations of macrophages in the gut and lung might be similar with regard to their rate of infection and PCNA expression.

HIV and SIV infection of macrophages, which are terminally differentiated cells, appears to be dependent, in part, on auxiliary genes including *vpr* and *vpu*. These genes are involved in cell cycle checkpoints and cell proliferation^{48,49} and may simultaneously push macrophages toward cell proliferation and cell death.^{49,50} We

found SIV RNA-positive cells, which are also PCNA-positive, but few of these cells are ApoTag-positive. Apoptosis of macrophages and T lymphocytes plays a role in the resolution of inflammation in rodents.^{51,52} In HIV and SIV infection, the picture is less clear. While viral infection can result in lymphocyte and macrophage death, it can also result in the prolonged life of infected cells as has been observed for HIV-infected macrophages *in vitro*.⁵³ Whether infection of perivascular macrophages in the CNS inhibits basal rates of perivascular macrophage apoptosis remains speculative. Differences in the cell biology of parenchymal microglia that are long-term residents of the CNS, and perivascular macrophages that are short-term residents, play a role in differential infection of perivascular macrophages over parenchymal microglia warrants further study.

After the initial entry of productively infected macrophages into the CNS, as demonstrated in perivascular cuffs of SIV-infected macaques and HIV-infected humans, productive viral infection disappears until the development of AIDS.^{4–7} The fate of the productively infected cells has not been addressed. It is possible that the virus becomes latent, and the cells long-lived. Alternatively, perivascular macrophages might leave the CNS or die. Studies describing the expression of PCNA in tissues do not exist, but it is likely that such expression occurs over days. Thus, our observation of PCNA-positive macrophages in the CNS of animals with terminal AIDS and SIVE suggest, similar to reports in humans^{5,6} that these cells have recently immigrated to the CNS. Overall, these data support the notion that SIVE and HIVE, as defined by the presence of perivascular aggregates of infected mononuclear cells and MNGC, are not chronic progressive diseases. Instead, SIVE and HIVE most likely occur terminally, with the onset of AIDS and immune suppression.

Mechanisms for the selective retention and/or accumulation of SIV infected- and non-infected perivascular macrophages at terminal AIDS are not known. These cells could accumulate in the CNS as a result of viral infection. They might have a basal rate of apoptosis in non-infected CNS that increases following viral infection. Alternatively, viral infection might result in an increase in the life span of infected macrophages, resulting from a decrease in their basal apoptotic levels. Regardless of mechanisms of PCNA expression, these data point to potential molecular differences between perivascular macrophages and parenchymal microglia, which may account for the observed preferential infection of the perivascular cells.

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References

- Price RW, Brew B, Sidtis J, Rosenblum M, Scheck AC, Cleary P: The brain in AIDS: central nervous system HIV-1 infection and AIDS dementia complex. *Science* 1988, 239:586–592
- Glass JD, Fedor H, Wesselingh SL, McArthur JC: Immunocytochemical quantitation of human immunodeficiency virus in the brain: correlations with dementia. *Ann Neurol* 1995, 38:755–762
- Williams KC, Corey S, Westmoreland SV, Pauley D, Knight H, deBakker C, Alvarez X, Lackner AA: Perivascular macrophages are the primary cell type productively infected by simian immunodeficiency virus in the brains of macaques: implications for the neuropathogenesis of AIDS. *J Exp Med* 2001, 193:905–915
- Price RW: The two faces of HIV infection of cerebrospinal fluid. *Trends Microbiol* 2000, 8:387–391
- Gosztonyi G, Artigas J, Lamperth L, Webster HD: Human immunodeficiency virus (HIV) distribution in HIV encephalitis: study of 19 cases with combined use of in situ hybridization and immunocytochemistry. *J Neuropathol Exp Neurol* 1994, 53:521–534
- Sinclair E, Gray F, Ciardi A, Scaravilli F: Immunohistochemical changes and PCR detection of HIV provirus DNA in brains of asymptomatic HIV-positive patients. *J Neuropathol Exp Neurol* 1994, 53:43–50
- Zink MC, Clements JE: The two faces of HIV infection of cerebrospinal fluid. *Trends Microbiol* 2000, 8:390–391
- Hickey WF, Vass K, Lassmann H: Bone marrow derived elements in the central nervous system: an immunohistochemical and ultrastructural survey of rat chimeras. *J Neuropathol Exp Neurol* 1992, 5:246–256
- Hickey WF, Kimura H: Perivascular microglial cells of the CNS are bone marrow-derived and present antigen. *Science* 1988, 239:290–292
- Lassmann H, Hickey WF: Radiation bone marrow chimeras as a tool to study microglia turnover in normal brain and inflammation. *Clin Neuropathol* 1993, 12:284–285
- Lawson LJ, Perry VH, Gordon S: Turnover of resident microglia in the normal adult mouse brain. *Neuroscience* 1992, 48:405–415
- Unger ER, Sung JH, Manivel JC, Chenggis ML, Blazar BR, Krivit W: Male donor-derived cells in the brains of female sex-mismatched bone marrow transplant recipients: a Y chromosome specific in situ hybridization study. *J Neuropathol Exp Neurol* 1993, 52:460–470
- Lassmann H, Schmied M, Vass K, Hickey WF: Bone marrow derived elements and resident microglia in brain inflammation. *Glia* 1993, 7:19–24
- van Furth R, Raeburn JA, van Zwet TL: Characteristics of human mononuclear phagocytes. *Blood* 1979, 54:485–500
- Williams K, Barr-Or A, Ulvestad E, Olivier A, Antl JP, Yong WV: Biology of adult human microglia in culture: comparisons with peripheral blood monocytes and astrocytes. *J Neuropathol Exp Neurol* 1992, 51:538–549
- Weinberg JB, Matthews TJ, Cullen BR, Malim MH: Productive human immunodeficiency virus type I (HIV-1) infection of nonproliferating human monocytes. *J Exp Med* 1991, 174:1477–1482
- Levy JA: The transmission of AIDS: the case of the infected cell. *JAMA* 1988, 259:3037–3038
- Narayan O, Clements JE: Biology and pathogenesis of lentiviruses. *J Gen Virol* 1989, 70:1617–1639
- Igarashi T, Brown CR, Endo Y, Buckler-White A, Plishka R, Bischofberger N, Hirsch V, Martin MA: Macrophage are the principal reservoir and sustain high virus loads in rhesus macaques after the depletion of CD4+ T cells by a highly pathogenic simian immunodeficiency virus/HIV type 1 chimera (SHIV): implications for HIV-1 infections of humans. *Proc Natl Acad Sci USA* 2001, 98:658–663
- Gallay P, Hope T, Chin D, Trono D: HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. *Proc Natl Acad Sci USA* 1997, 94:9825–9830
- Heinzinger NK, Bukrinsky MI, Haggerty SA, Ragland AM, Kewalramani V, Lee MA, Gendelman HE, Ratner L, Stevenson M, Emerman M: The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proc Natl Acad Sci USA* 1994, 91:7311–7315
- Terai D, Wasson BD, Carrera CJ, Carson DA: Dependence of cell survival on DNA repair in human mononuclear phagocytes. *J Immunol* 1991, 147:4302–4306
- Perno CF, Yarchoan R, Cooney DA, Hartman NR, Gartner S, Popovic M, Hao Z, Gerrard TL, Wilson YA, Johns DG: Inhibition of human immunodeficiency virus (HIV-1/HTLV-III_{Ba}-L) replication in fresh and cultured human peripheral blood monocytes/macrophages by azidothymidine and related 2', 3'-dideoxynucleosides. *J Exp Med* 1988, 168:1111–1125
- Stevenson M, Gendelman HE: Cellular and viral determinants that regulate HIV-1 infection in macrophages. *J Leukocyte Biol* 1994, 56:278–288
- Gendelman HE, Narayan O, Molineaux S, Clements JE, Ghotbi Z: Slow, persistent replication of lentiviruses: role of tissue macrophages and macrophage precursors in bone marrow. *Proc Natl Acad Sci USA* 1985, 82:7086–7090
- Takasaki Y, Deng JS, Tan EM: A nuclear antigen associated with cell proliferation and blast transformation. *J Exp Med* 1981, 154:1899–1909
- Bravo R, Macdonald-Bravo H: Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites. *J Cell Biol* 1987, 105:1549–1554
- Bravo R, Macdonald-Bravo H: Changes in the nuclear distribution of cyclin (PCNA) but not its synthesis depend on DNA replication. *EMBO J* 1985, 4:655–661
- Kelman Z: PCNA: structure, functions and interactions. *Oncogene* 1997, 14:629–640
- Celis JE, Madsen P, Nielsen S, Celis A: Nuclear patterns of cyclin (PCNA) antigen distribution subdivide S-phase in cultured cells—some applications of PCNA antibodies. *Leukemia Res* 1986, 10:237–249
- Toschi L, Bravo R: Changes in cyclin/proliferating cell nuclear antigen distribution during DNA repair synthesis. *J Cell Biol* 1988, 107:1623–1628
- Westmoreland SV, Halpern E, Lackner AA: Simian immunodeficiency virus encephalitis in rhesus macaques is associated with rapid disease progression. *J Neurovirol* 1998, 4:260–268
- Lackner AA: Pathology of simian immunodeficiency virus induced disease. *Current Topics in Microbiology and Immunology: Simian Immunodeficiency Virus*. Edited by RC Desrosiers, and N Letvin. Berlin, Springer Verlag, 1994, pp 35–64
- Lane JH, Tarantal AF, Pauley D, Marthas M, Miller CJ, Lackner AA: Localization of simian immunodeficiency virus nucleic acid and antigen in brains of fetal macaques inoculated in utero. *Am J Pathol* 1996, 149:1097–1104
- Smith MO, Heyes MP, Lackner AA: Early intrathecal events in rhesus macaques (*Macaca mulatta*) infected with pathogenic or nonpathogenic molecular clones of simian immunodeficiency virus. *Lab Invest* 1995, 72:547–558
- Wykrzykowska JJ, Rosenzweig M, Veazey RS, Simon MA, Halvorsen K, Desrosiers RC, Johnson RP, Lackner AA: Early regeneration of thymic progenitors in rhesus macaques infected with simian immunodeficiency virus. *J Exp Med* 1998, 187:1767–1778
- Klein RS, Williams KC, Alvarez-Hernandez X, Westmoreland S, Force T, Lackner AA, Luster AD: Chemokine receptor expression and signaling in macaque and human fetal neurons and astrocytes: implications for the neuropathogenesis of AIDS. *J Immunol* 1999, 163:1636–1646
- Schnell SA, Staines WA, Wessendorf MW: Reduction of lipofuscin-like autofluorescence in fluorescently labeled tissue. *J Histochem Cytochem* 1999, 47:719–730
- Hirsch VM, Adger-Johnson D, Campbell B, Goldstein S, Brown C, Elkins WR, Montefiori DC: A molecularly cloned, pathogenic, neutralization-resistant simian immunodeficiency virus, SIVsmE543–3. *J Virol* 1997, 71:1608–1620
- von Herrath M, Oldstone MB, Fox HS: Simian immunodeficiency virus (SIV)-specific CTL in cerebrospinal fluid and brains of SIV-infected rhesus macaques. *J Immunol* 1995, 154:5582–5589
- Lackner AA, Smith MO, Munn RJ, Martfeld DJ, Gardner MB, Marx PA, Dandekar S: Localization of simian immunodeficiency virus in the central nervous system of rhesus monkeys. *Am J Pathol* 1991, 139:609–621
- Broadwell RD, Charlton HM, Ebert PS, Hickey WF, Shirazi Y, Villegas J, Wolf AL: Allografts of CNS tissue possess a blood-brain barrier: II. Angiogenesis in solid tissue and cell suspension grafts. *Exp Neurol* 1991, 112:1–28
- Kida S, Steart PV, Zhang ET, Weller RO: Perivascular cells act as scavengers in the cerebral perivascular spaces and remain distinct

- from pericytes, microglia and macrophages. *Acta Neuropathol* 1993, 85:646–652
44. Williams K, Hickey WF: Central nervous system damage, monocytes and macrophages, and neurological disorders in AIDS. *Annu Rev Neurosci* 2002, 25:537–562
 45. Titius BR, Thiele J, Schaefer H, Kreipe H, Fischer R: Ki-S1 and proliferating cell nuclear antigen expression of bone marrow macrophages: immunohistochemical and morphometric study including reactive (inflammatory) myelitis, secondary aplastic anemia, AIDS, myelodysplastic syndromes and primary (idiopathic) osteomyelofibrosis. *Acta Haematol* 1994, 91:144–149
 46. Yerly-Motta V, Pavy JJ, Bosset SF, Arbez-Gindre F, Herve P: Validity of PCNA immunostaining in normal and tumor cells: comparison with Ki-67 labelling. *J Histotechnol* 1999, 22:287–293
 47. Gendelman HE, Orenstein JM, Baca LM, Weiser B, Burger H, Kalter DC, Meltzer MS: The macrophage in the persistence and pathogenesis of HIV infection. *AIDS* 1989, 3:475–495
 48. Rogel ME, Wu LI, Emerman M: The human immunodeficiency virus type 1 vpr gene prevents cell proliferation during chronic infection. *J Virol* 1995, 69:882–888
 49. Goh WC, Rogel ME, Kinsey CM, Michael SF, Fultz PN, Nowak MA, Hahn BH, Emerman M: HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo. *Nat Med* 1998, 4:65–71
 50. Gummuluru S, Emerman M: Cell cycle- and Vpr-mediated regulation of human immunodeficiency virus type 1 expression in primary and transformed T-cell lines. *J Virol* 1999, 73:5422–5430
 51. Nguyen KB, McCombe PA, Pender MP: Macrophage apoptosis in the central nervous system in experimental autoimmune encephalomyelitis. *J Autoimmun* 1994, 7:145–152
 52. Nguyen KB, McCombe PA, Pender MP: Increased apoptosis of T lymphocytes and macrophages in the central and peripheral nervous systems of Lewis rats with experimental autoimmune encephalomyelitis treated with dexamethasone. *J Neuropathol Exp Neurol* 1997, 56:58–69
 53. Berman MA, Zaidivar F, Jr., Imfeld KL, Kenney JS, Sandborg CI: HIV-1 infection of macrophages promotes long-term survival and sustained release of interleukins 1 α and 6. *AIDS Res Hum Retrovir* 1994, 10:529–539