High frequencies of resting CD4⁺ T cells containing integrated viral DNA are found in rhesus macaques during acute lentivirus infections

Yoshiaki Nishimura^a, Reza Sadjadpour^a, Joseph J. Mattapallil^{b,1}, Tatsuhiko Igarashi^a, Wendy Lee^a, Alicia Buckler-White^a, Mario Roederer^b, Tae-Wook Chun^c, and Malcolm A. Martin^{a,2}

^aLaboratory of Molecular Microbiology, ^bVaccine Research Center, and ^cLaboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

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We and others have reported that the vast majority of virus-producing CD4⁺ T cells during the acute infection of rhesus macaques with simian immunodeficiency virus (SIV) or CXCR4 (X4)-using simian/human immunodeficiency viruses (SHIVs) exhibited a nonactivated phenotype. These findings have been extended to show that resting CD4⁺ T lymphocytes collected from SIV- or X4-SHIV-infected animals during the first 10 days of infection continue to release virus *ex vivo*. Furthermore, we observed high frequencies of integrated viral DNA (up to 5.1×10^4 DNA copies per 10^5 cells) in circulating resting CD4⁺ T cells during the first 10 days of the infection. Integration of SIV DNA was detected only in memory CD4⁺ T cells and SHIVs preferentially integrated into resting naïve CD4⁺ T cells. Taken together, these results show that during the acute infection large numbers of resting CD4⁺ T cells carry integrated nonhuman primate lentiviral DNA and are the major source of progeny virions irrespective of coreceptor usage. Prompt and sustained interventions are therefore required to block the rapid systemic dissemination of virus and prevent an otherwise fatal clinical outcome.

HIV | integration | simian immunodeficiency virus | simian/human immunodeficiency virus

During the first 15 years of the AIDS epidemic, the prevailing dogma was that HIV and simian immunodeficiency virus (SIV) were unable to replicate in quiescent CD4⁺ T lymphocytes (1, 2). This failure to infect nonactivated T cells in culture was attributed to multiple mechanisms including inefficient reverse transcription (RT) (1, 3–5), impaired nuclear import of reverse transcripts (6, 7), and low intracellular dNTP pools (8). It was also believed that HIV was unable to integrate into the genome of resting CD4⁺ T cells (9). Because unintegrated DNA has been reported to be inefficiently transcribed (10–12), impaired integration could also contribute to the failure of lymphocytes in the G₀/1a stage of the cell cycle to support productive infections (13).

Although these results have been confirmed in numerous laboratories, studies conducted during the past decade have shown that resting T cells, recovered from human lymphoid tissue explants maintained *ex vivo*, release substantial quantities of progeny virions (14, 15). Another report demonstrated that resting CD4⁺ T lymphocytes, in specimens obtained from HIV-infected individuals or acutely SIV-infected macaques, are producing viral RNA (16). We reported that 32–88% of circulating naïve CD4⁺ T lymphocytes from rhesus monkeys infected with CXCR4-tropic (X4-tropic) simian/human immunodeficiency viruses (SHIVs) were productively infected by day 10 postinfection (PI) (17). Immunophenotyping by flow cytometry and combined *in situ* hybridization/immunohistochemistry revealed that the vast majority of the virus-producing CD4⁺ T lymphocytes collected from these SHIV-infected animals were not expressing activation markers.

It could be argued that the prodigious levels of virus production by quiescent naïve cells measured by endpoint dilution/

cocultivation reflected the activating effects induced by the monoclonal antibodies used for positive cell sorting and/or the subsequent incubation with MT4 cells (17). Accordingly, negatively-selected resting CD4⁺ T lymphocytes were purified from SIV-infected (day 7 PI) and SHIV-infected (day 10 PI) rhesus monkeys and cultivated in the presence of autologous serum and the absence of IL-2 for 4 days. Infectious virus was, in fact, detected in the supernatant medium from each *ex vivo* culture. In a separate series of experiments, the integration status of viral DNA in resting naïve and memory CD4⁺ T cells, recovered from SIV- and SHIV-infected macaques on days 7 and 10 PI, respectively, was determined by limiting dilution Alu-LTR PCR. High frequencies of integrated SIV DNA were detected in memory but not naïve CD4⁺ T cells, in agreement with the differential expression of the CCR5 coreceptor in these 2 lymphocyte subsets (18, 19). In animals inoculated with X4-tropic SHIV, 13–51% of the circulating naïve CD4⁺ T cells contained integrated DNA, assuming each cell carries a single integrated provirus. These results indicate that during the acute infection, integration of viral DNA occurs in a prototypical resting cell.

Results

In earlier experiments evaluating virus production during the acute infection of 3 rhesus monkeys with the highly-pathogenic X4-tropic SHIV_{DH12R}, we reported that resting naïve CD4⁺ T cells were massively infected and, as shown in Fig. 1, were the major source of infectious virus circulating in the blood on day 10 PI (17). In that study, activated CD8⁺ T lymphocytes were observed in the blood (CD69⁺, HLA-DR⁺, and Ki-67⁺) and lymph nodes (Ki-67⁺) by week 2 PI, but the activation status of CD4⁺ T cells recovered from these sites remained unchanged from their preinoculation resting state. Confocal microscopy of day 10 lymph node specimens, using SIV riboprobes and the anti-Ki-67 mAb, revealed that >96% of SHIV-producing cells did not express Ki-67. Taken together, these results indicated that the vast majority of virus-producing cells during the acute *in vivo* infection exhibited a nonactivated phenotype.

As a follow-up to this initial study, we wanted to answer 2 questions: (i) would virus production by resting CD4⁺ T lymphocytes, after their isolation from acutely infected animals, continue *ex vivo* and (ii) what was the integration status of viral DNA in resting virus-producing CD4⁺ T cells *in vivo*? Accordingly, individual rhesus monkeys were inoculated intravenously

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¹Present address: Department of Microbiology and Immunology, Uniformed Services University, Bethesda, MD 20814.

²To whom correspondence should be addressed. E-mail: malm@nih.gov.

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Fig. 1. Naive CD4⁺ T cell are the major source of infectious virus circulating in the blood during the acute infection of rhesus monkeys with SHIV_{DH12R}. The frequencies of productively-infected naive or memory cells collected on day 10 PI from 3 SHIV_{DH12R}-infected animals were determined by cocultivation with MT4 cells for 14 days as described (17).

with 1×10^4 TCID₅₀ SIV_{mac239} (macaque CN30) or 1×10^5 TCID₅₀ SHIV_{DH12R} (macaque WGG) and the levels of plasma viremia and circulating CD4⁺ T cells were determined during the first 10 days of infection (Fig. 2A). The SIV-infected animal experienced a more rapid increase of its plasma virus load compared with the SHIV-infected macaque and both monkeys sustained partial depletions of their peripheral blood CD4⁺ T cells. Peripheral blood mononuclear cells (PBMCs) were prepared from both monkeys on days 7 and 10 PI and a portion of

Fig. 2. Peripheral blood CD4⁺ T cell profiles and viral nucleic acid levels during the acute SIV and SHIV infections of macaques. (A) Circulating CD4⁺ T cell numbers and plasma viral RNA loads were measured in a SIV (CN30)-infected and a SHIV_{DH12R} (WGG)-infected rhesus monkey at the indicated times. (B) PBMC samples, collected on days 7 and 10 PI from macaques CN30 and WGG, were stained with anti-CD3, CD4, CD28, and CD95 mAbs to distinguish naive from memory CD4⁺ T cells and sorted by FACS for determinations of the cell-associated viral DNA copies per 10^5 cells by DNA PCR.

each PBMC sample was sorted by FACS for quantitative cell-associated viral DNA content in naive (CD95^{low}CD28^{high}) or memory (CD95^{high}CD28^{low and high}) CD4⁺ T lymphocytes. As shown in Fig. 2B, high levels (3.1 or 3.8×10^4 DNA copies per 10^5 cells) of memory CD4⁺ T cells from the SIV-infected macaque contained viral DNA on days 7 and 10 PI, whereas the frequency of infected naive cells was significantly lower. This difference is consistent with the expression levels of the CCR5 coreceptor used by SIV in these 2 CD4⁺ T lymphocyte subsets. In contrast, SHIV_{DH12R} DNA was detected in both naive and memory CD4⁺ T cells, reaching levels of 1.6×10^4 and 1.7×10^4 DNA copies per 10^5 cells, respectively, on day 10 PI, consistent with the surface expression of CXCR4 on both CD4⁺ T cell subsets (20).

Spontaneous Virus Production by Resting CD4⁺ T Cells ex Vivo. To preserve the nonactivated status of CD4⁺ T cells during purification, PBMC samples, collected on day 7 from SIV-inoculated macaque CN30 and on day 10 from SHIV-inoculated macaque WGG, were initially stained with non-CD4⁺ T cell markers (CD8, CD20, CD14, and CD16) and markers of activation (CD25, CD69, and HLA-DR). These collection times were selected because: (i) circulating memory CD4⁺ T lymphocytes had declined to low levels in the SIV-infected monkey (CN30) by day 10 PI; and (ii) larger numbers of SHIV_{DH12R}-infected cells were present in animal WGG on day 10 compared with day 7 PI. Lymphocytes, negative for non-CD4 and activation markers, were then sorted by FACS. The purity of the resulting CD4⁺ T cell populations from both animals was $>96\%$; these cells expressed minimal levels of CD25, CD69, or HLA-DR (Fig. 3A).

The negatively-sorted resting CD4⁺ T cell preparation from each animal was cultured in duplicate for 4 days in the absence of any stimulation, including exogenous IL-2, as described in *Methods*. The production of progeny virus was monitored daily by measuring RT activity released into the medium. No RT activity was measured in these culture supernatants. The detection limit of the ³²P-RT-assay used (21) ranged from 2.5×10^3 to 8×10^3 TCID₅₀/mL, based on RT measurements of SIV and SHIV stocks of known infectious titer. To amplify any infectious virus released into the medium, filtered supernatants, collected daily (days 1–4) from the RT-negative duplicate cultures of resting SIV- or SHIV-infected CD4⁺ T cells, were spinoculated onto MT-4 T cells, and the duplicate cultures of MT-4 T cells were maintained for an additional 14 days. As shown in Fig. 3B, RT activity was detected as early as day 1 (1 of 2 wells) in the supernatants from each of the original nonactivated CD4⁺ T cell cultures. Both duplicate wells were positive for SIV and SHIV production in supernatants collected on days 3–4. These results indicate that resting CD4⁺ T cells, collected from macaques during acute SIV and SHIV infections, continued to produce and release progeny virions during 4 days of ex vivo cultivation.

Integration Status of Viral DNA in CD4⁺ T Lymphocytes During Acute SIV and SHIV Infections. Because of the long-standing belief that integration of reverse transcripts did not occur in quiescent cells, we next investigated whether integrated DNA could be detected during the primary infection. A modified Alu-LTR PCR approach, using pairs of outward-facing PCR primers binding to conserved regions of human and rhesus macaque AluDNA sequences, in conjunction with a cloned cell line containing a single copy of integrated SIV DNA, were used to analyze genomic DNA prepared from sorted and endpoint-diluted naive and memory CD4⁺ T cells from acutely SIV- and SHIV-infected animals.

The 3D8 cell line, carrying 1 copy of integrated SIV DNA, was derived from chronically-infected CEM $\times$ 174 cells, 8 weeks after infection with SIV_{mac316}, as described in *SI Text*. Cells carrying viral DNA were initially identified by DNA PCR and then

Fig. 4. Quantitation of integrated viral DNA in naïve or memory $CD4^+$ T cells collected from acutely SIV or SHIV-infected macaques. (A) Genomic DNA from 3D8 cells was serially diluted 2-fold, in triplicate, starting with 500 cell DNA equivalents and analyzed by Alu-LTR PCR. (B) Genomic DNAs, purified from sorted naïve or memory $CD4^+$ T cells from SHIV_{DH12R}-infected macaque 95P005 on day 10 PI, were serially diluted, and integrated proviral DNA was measured by Alu-LTR PCR. (C) The frequencies of naïve or memory $CD4^+$ T cells carrying integrated SIV or SHIV DNA were determined by Alu-LTR PCR and normalized to the values obtained with the 3D8 cell line.

infected cells were immunophenotypically resting memory and naïve $CD4^+$ T lymphocytes, respectively (16, 17, 26). We have extended this observation in the current study and found that nonactivated virus-infected cells, recovered from acutely-infected animals, continue to release low levels of infectious progeny virions *ex vivo* without additional stimulation. In this regard, intriguing quantitative differences have been reported when HIV-1 RT and integration reactions are evaluated during *ex vivo* infections of $CD4^+$ T cells exhibiting a continuum of activation states. For example, when viral DNA generated by RT is measured in *ex vivo*-activated versus freshly-purified resting T cells, 30- to 100-fold more DNA copies per cell are detected in the former (24, 25). In contrast, the HIV-1 DNA content in “endogenously” activated $CD4^+$ T cells (negatively-sorted $CD4^+$ T lymphocytes expressing HLA-DR and CD69) was only 2-fold higher than that present in resting T cells. A similar hierarchy of relative integration frequencies (1:5:50) was observed for resting, endogenously-activated, and *ex vivo*-activated $CD4^+$ T cells, respectively. These results suggest that virus infectivity *in vivo*,

both in resting and activated $CD4^+$ T cells, may be far less robust than that measured *in vitro*.

HIV-infected resting $CD4^+$ T cells have been identified during both the acute and chronic phases of infection *in vivo*. Some reports (27, 28) have described the presence of integrated HIV-1 DNA and the recovery of virus from $CD45RA^+$ naïve $CD4^+$ T lymphocytes from chronically-infected individuals, particularly after coreceptor switching. In addition, latently HIV-1-infected, resting memory $CD4^+$ T cells have been detected in individuals receiving highly active antiretroviral therapy with extremely low levels of viremia (29–31). When resting memory $CD4^+$ T lymphocytes from viremic and nonviremic, latently-infected individuals were purified and cultured *ex vivo* in the absence of activating stimuli, only cells from viremic persons spontaneously released progeny virions (32).

In contrast to the chronic phase of the HIV-1 infection in which the frequency of latently-infected resting memory $CD4^+$ T cells with replication competent-integrated provirus is exceedingly low (33), high levels of virus-producing resting cells have

Table 1. Frequencies of integrated and total viral DNA in naïve or memory CD4⁺ T cells in SHIV-infected (day 10) or SIV-infected (day 7) animals

Animal ID	CD4 subset	Integrated copies/ 10 ⁵ cells	Total DNA copies/ 10 ⁵ cells	Integrated/ total DNA ratio
94E057 (SHIV)	Naïve	1.27×10 ⁴	7.25×10 ⁴	0.17
	Memory	6.34×10 ³	2.35×10 ⁴	0.27
95P005 (SHIV)	Naïve	5.08×10 ⁴	2.84×10 ⁵	0.18
	Memory	1.27×10 ⁴	1.07×10 ⁵	0.12
H482 (SHIV)	Naïve	1.27×10 ⁴	6.44×10 ⁴	0.20
	Memory	1.06×10 ⁴	5.67×10 ⁴	0.19
WGG (SHIV)	Naïve	2.11×10 ⁴	1.57×10 ⁴	1.35
	Memory	1.06×10 ⁴	1.71×10 ⁴	0.62
CN30 (SIV)	Naïve	*	1.51×10 ³	*
	Memory	6.34×10 ³	3.13×10 ⁴	0.20

*Below detection limit.

been reported during the acute infection. One study (16), using in situ hybridization in combination with staining by antibodies to activation and cell cycle markers, reported that 50% of the virus-infected cells in lymph node specimens from recently-infected individuals were HLA-DR⁺ and Ki-67⁺, the resting memory subset. In the more tractable SIV/macaque system, a majority of virus-positive cells in lymph node biopsies were shown to be HLA-DR⁺ and Ki-67⁺ on day 12 after intravaginal inoculation. Not unexpectedly, the intracellular levels of viral RNA measured in these experiments were 4- to 6-fold higher in activated cells present in the same microscopic fields. In a follow-up study using the same approach, >90% of SIV RNA-positive memory CD4⁺ T cells in the colon were found to be negative for CD69, CD25, and Ki-67 on day 10 PI (26).

In this study we have shown that both SIV and SHIVs are able to infect resting CD4⁺ T lymphocytes during the acute infection of rhesus monkeys and integrate DNA copies of their genomes into host cell chromosomal DNA. The observed high frequencies of resting CD4⁺ T cells carrying integrated viral DNA on day 7 PI (SIV) and day 10 PI (SHIV) is consistent with the reported high frequencies of virus-infected cells using other approaches (17, 34). In the case of acutely SHIV-infected macaques, 13–51% of the circulating naïve CD4⁺ T cells contained integrated viral DNA, which is in good agreement with the reported 30–90% frequency of naïve CD4⁺ T cells releasing progeny virions (17). The results of the integration assays also reflected the differential levels of coreceptor expression in the CD4⁺ T lymphocyte subsets targeted by SIV and X4-SHIVs (20). Integration of SIV DNA was observed only in memory CD4⁺ T cells and the X4 SHIVs preferentially integrated into naïve versus memory CD4⁺ T cells. Thus, during the acute infection of macaques, resting CD4⁺ T cells are the major targets of nonhuman primate lentiviruses, irrespective of their coreceptor usage.

Based on available evidence, the very low levels of virus production, observed in ex vivo cultures of resting CD4⁺ T cells from acutely SIV- and SHIV-infected monkeys (Fig. 3B), are difficult to reconcile with the high levels of plasma viremia ($\approx 10^7$ RNA copies per mL) contemporaneously measured in these animals. A plausible explanation of this apparent paradox is that: (i) the high frequency of circulating infected, nonactivated CD4⁺ T lymphocytes is reflective of the extraordinarily large number of similar productively infected cells in lymphoid tissues and/or effector sites; (ii) the latter population produces small amounts of virus per cell; and (iii) the net effect of the slow release of small quantities of progeny virions by an enormous fraction of the total CD4⁺ T cell population would result in high SIV or SHIV virus loads, as suggested (26). In this regard, it has recently been reported that SIV-infected resting cells may generate

70–93% of peak virus production, based on Bayesian statistical analyses (35).

A related and currently-unresolved issue concerns the mechanism responsible for the prodigiously rapid rate of virus spread throughout a sizable fraction of total body CD4⁺ T cells during the initial weeks of the infection. It would seem unlikely that cell-free virus transmission alone could account for the rapid increase of virus-infected cells *in vivo*. The high concentrations of tightly-packed juxtaposed infected and uninfected resting CD4⁺ T cells *in vivo* suggest alternative mechanisms for virus dissemination such as cell-to-cell spread, dendritic cell-mediated transfer, and cytokine-augmented dispersal of progeny virions all may contribute to this process. The extremely-rapid systemic spread exhibited by SIV and SHIVs in macaques and presumably, by HIV-1 in humans, during the acute infection clearly requires prompt, potent, and sustained interventions to prevent the establishment of a chronic infection and an otherwise fatal clinical outcome. The rapid pace of virus dissemination presents a formidable challenge for the development of an effective prophylactic HIV vaccine.

Methods

Virus and Animals. The origin and preparation of the tissue culture-derived SHIV_{DH12R} and molecular clone-derived SIV_{mac239} stocks have been described (36). Rhesus macaques (*Macaca mulatta*) were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals and were housed in a biosafety level 2 facility; biosafety level 3 practices were followed (36).

Plasma Viral RNA Quantitation. Viral RNA levels in plasma were determined by real-time RT-PCR (ABI Prism 7700 Sequence Detection System; Applied Biosystems) as described (20).

Lymphocyte Immunophenotyping and Live Cell Sorting. EDTA-treated blood samples were stained for flow cytometric analysis as described (20). The preparation of positively-sorted naïve and memory CD4⁺ T cells negatively sorted resting and CD4⁺ T cells are described in [SI Text](#). The purity of the resulting CD4⁺ T cell populations, negative for non-CD4 and activation markers (CD25, CD69, and HLA-DR), from both animals was >96%.

Calibration of Cell Numbers for Quantitative DNA and Alu-LTR PCR. Cell numbers in each genomic DNA sample were determined by quantitative PCR of albumin genes, by using an ABI Prism 7700 Sequence Detection System. The rhesus macaque albumin primer and probe sequences were AlbF, TGCATGAGAAAACGCCAGTAA; AlbR, ATGGTCGCCTGTTCACCAA; and AlbP, AGAAAGTCACCAATGCTGCACGGAATC, as described (20). The standard used was rhesus macaque genomic DNA present in known numbers of PBMCs, as determined by serial limiting dilution.

Cell-Associated DNA Levels in Naïve or Memory CD4⁺ T Lymphocytes. Cell-associated viral DNA was measured by a quantitative PCR assay for SIV *gag* by using a ABI Prism 7700 Detection System as described (20). Cell numbers

analyzed in each reaction had been determined by quantitative PCR of albumin DNA in each sample.

Resting CD4⁺ T Lymphocytes Culture and Amplification of Infectious Virus in Cultured T Cell Lines. Negatively-sorted resting CD4⁺ T cells (2×10^6 cells), were treated with Pronase (Roche; 20 mg/mL), and cultured for 4 days in the absence of any stimulation in RPMI medium 1640 supplemented with 15% autologous serum, collected from each animal before infection. Culture supernatants were assayed for RT activity to measure progeny virus production (21). To amplify progeny virions in supernatants of resting CD4⁺ T cell cultures, filtered supernatants, collected daily (days 1–4) were spinoculated (37) onto MT-4 T cells in duplicate. Virus production was assessed by measuring the RT activity released into the culture medium on day 14.

Alu-LTR PCR to Detect Integrated Viral DNA. The isolation of a cloned cell line (3D8) containing a single integrated copy of SIV DNA is described in *SI Text*. Genomic DNA was prepared from FACS-sorted naïve and memory CD4⁺ T cells as described (17). To detect only integrated lentiviral genomes, the described Alu-LTR PCR technique (29) was modified for use with rhesus macaque Alu sequences. To improve the sensitivity of detecting integrated SIV DNA situated near genomic Alu sequences in either orientation, pairs of outward-

facing PCR primers, binding to conserved regions of human and rhesus macaque AluDNA sequences were used in combination with an SIV LTR reverse primer, as described (38, 39). For the first-round PCR, genomic DNA was serially diluted in 2-fold dilutions (500 to 2 cell equivalents), and each dilution was divided into triplicate pools. The first-round rhesus Alu-SIV LTR PCR primers were: RS-Alu-1 (+), 5'-TACTCAGGAGGCTGAGGCAGGAG-3'; RS-Alu-2 (-), 5'-GCCTCCCAAAGTACTAGGATTACAG-3', and SIV25T3 (-, 5'-CAAACCTCCATGCTAGAACCTCTCCC-3'. The primers used for the nested PCR were SIV.LTR-315–338 (+), 5'-GGAAGAAGGCATCATACCAGATTG-3' and SIV.LTR.427–448 (-), 5-AATGCTCCTCATCCTCTGTGC-3'. PCR conditions for both the first-round and nested PCRs have been described (29). SIV LTR nested PCR products were visualized on 2% agarose TAE gels (SeaKem ME Agarose; Cambrex Biosciences) with ethidium bromide (0.5 µg/mL).

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