Early Spatial and Temporal Events of Human T-Lymphotropic Virus Type 1 Spread following Blood-Borne Transmission in a Rabbit Model of Infection

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Human T-lymphotropic virus type 1 (HTLV-1) infection causes adult T-cell leukemia/lymphoma (ATL) and is associated with a variety of lymphocyte-mediated disorders. HTLV-1 transmission occurs by transmission of infected cells via breast-feeding by infected mothers, sexual intercourse, and contaminated blood products. The route of exposure and early virus replication events are believed to be key determinants of virus-associated spread, antiviral immune responses, and ultimately disease outcomes. The lack of knowledge of early events of HTLV-1 spread following blood-borne transmission of the virus in vivo hinders a more complete understanding of the immunopathogenesis of HTLV-1 infections. Herein, we have used an established animal model of HTLV-1 infection to study early spatial and temporal events of the viral infection. Twelve-week-old rabbits were injected intravenously with cell-associated HTLV-1 (ACH-transformed R49). Blood and tissues were collected at defined intervals throughout the study to test the early spread of the infection. Antibody and hematologic responses were monitored throughout the infection. HTLV-1 intracellular Tax and soluble p19 matrix were tested from ex vivo cultured lymphocytes. Proviral copy numbers were measured by real-time PCR from blood and tissue mononuclear leukocytes. Our data indicate that intravenous infection with cell-associated HTLV-1 targets lymphocytes located in both primary lymphoid and gut-associated lymphoid compartments. A transient lymphocytosis that correlated with peak virus detection parameters was observed by 1 week postinfection before returning to baseline levels. Our data support emerging evidence that HTLV-1 promotes lymphocyte proliferation preceding early viral spread in lymphoid compartments to establish and maintain persistent infection.
were tested at defined intervals throughout 8 weeks after viral exposure. Our data indicate that HTLV-1 targets primary lymphoid and gut-associated lymphoid compartments to establish infection. Additionally, early HTLV-1 infection was associated with a transient lymphocytosis that correlated with peak virus detection. Our findings support emerging data that indicate active virus-mediated lymphocyte proliferation is a prelude for early HTLV-1 viral spread to promote establishment and maintenance of the persistent infection.

MATERIALS AND METHODS

Cell lines and inoculation procedures to establish HTLV-1 infection. A CD4+/CD25+ rabbit lymphocyte line (R49) containing three integrated copies of the HTLV-1 ACH clone was used to establish HTLV-1 infection in New Zealand White rabbits as described previously (4). The derivation, maintenance, and infectious properties of the full-length, wild-type HTLV-1 proviral clone (ACH) used to generate the R49 cell line has been previously reported (4). JURKAT T cells, CD4+/CD25+ and HTLV-1 negative (clone E6-1; American Type Culture Collection catalog number TIB-152) used to inoculate control rabbits were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 10% penicillin-streptomycin (100 μg/ml), and 10% glutamine (0.03 mg/ml) at 37°C in 5% carbon dioxide.

We obtained 28 outbred and specific-pathogen-free female 12-week-old New Zealand White rabbits from a commercial source (Harlan, Indianapolis, IN). Seven groups composed of one control rabbit and three HTLV-1-inoculated rabbits were necropsied at 1, 3, 5, 7, 14, 21, and 56 days postinfection. Rabbits were housed and maintained in accordance with an approved Ohio State University Animal Care and Use Protocol. On the day of inoculation, the infected rabbit group was inoculated with 1 x 10⁷ R49 cells in the lateral ear artery. The control rabbits were inoculated with 1 x 10⁷ HTLV-1-negative JURKAT T cells in the lateral ear vein. The rabbits were evaluated daily for any overt signs of clinical disease.

Serological and hematologic analysis. Reactivity to specific viral antigenic determinants was detected using a commercial HTLV-1 Western immunoblot assay (GeneLabs Diagnostics, Singapore) adapted for rabbit plasma by use of alkaline phosphatase-conjugated goat anti-rabbit IgG (1:1,000 dilution; Chemicon, Temecula, CA). Rabbit plasma was diluted 1:100 for Western immunoblot analysis. Plasma showing reactivity to HTLV-1 Gag (p24 or p19) and Env (p21 or gp46) antigens was classified as positive for HTLV-1 seroreactivity. Complete hematological analysis from 500 x 10⁶ of whole blood was performed by automated cell counting (VetScan; Abaxis), and cell differential counts were confirmed by counting at least 100 leukocytes from blood smears.

Blood and tissue sampling. Blood was collected at defined intervals throughout the study, and HTLV-1-infected or control animals were necropsied at 1, 3, 7, 14, 21, and 56 days postinfection (PI). Peripheral blood mononuclear cells (PBMC) were isolated from 10 to 20 ml of whole blood from the lateral auricular artery at each time point indicated. Percoll (Sigma-Aldrich Corp., St. Louis, MO) was added to diluted 1:100 for Western immunoblot analysis. Plasma showing reactivity to HTLV-1 Gag (p24 or p19) and Env (p21 or gp46) antigens was classified as positive for HTLV-1 seroreactivity. Complete hematological analysis from 500 x 10⁶ of whole blood was performed by automated cell counting (VetScan; Abaxis), and cell differential counts were confirmed by counting at least 100 leukocytes from blood smears.

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Detection of proviral copy number by PCR. To quantify the proviral copy number, genomic DNA was isolated from rabbit PBMC and tissue-derived mononuclear leukocytes using the manufacturer’s protocol (Qiagen). DNA concentration and quality were determined (Nanodrop Technologies). HTLV-1 polymerase gene (pol) primers were used at a final concentration of 200 nM for both forward and reverse primers. The forward primer, 5’-CCC TAC AAT CCA ACC AGC TCA G-3’, and the reverse primer, 5’-TTT TGG GCT AGT GAA GTG GTG-5’, were used to amplify genomic DNA. Standard curves were generated using DNA from a molecular clone of HTLV-1, the ACH plasmid, and assayed concurrently with test samples. Forward rabbit glyceraldehyde 3-phosphate dehydrogenase (rGAPDH) primer 5’-TGG CCA AGT CGT TCA AGT CGT TT-3’ and reverse primer 5’-TCT TCG TCG TGT GTC GTG TC-5’ were used at a final concentration of 250 nM to amplify genomic DNA. The standard curves for rGAPDH were generated by subcloning a 780-bp fragment from the rGAPDH gene into the pCR4-Topo vector (Invitrogen) and run concurrently with test samples. For each run, a standard curve was generated from triplicate samples of 10⁻³ dilutions of plasmid DNA in DNase/RNase-free water. The HTLV-1 proviral copy number was expressed as the number of infected cells per 1 x 10⁷ PBMC and was calculated using the following formula: [(HTLV-1 pol copy number)/(rabbit GAPDH copy number)] x 10,000. Using this method, we detected ~3 proviral copies per R49 cell, confirming data previously reported by Collins et al. (4).

Statistical methods. Linear mixed models, which are ideal for modeling continuous longitudinal data, were employed to model correlated multiple observa-
Hematologic parameters in infected rabbits. Rabbit serum samples were tested for reactivity to specific viral antigenic determinants by an HTLV-1 Western immunoblot assay. All R49-inoculated rabbits seroconverted by 4 weeks after exposure and remained positive through the 10-week study (data not shown). In the absence of a suitable uninfected rabbit CD4⁺ lymphocyte cell line, control rabbits were inoculated with Jurkat T cells. Jurkat T cells represented a transformed T-cell line without expression of HTLV-1. The control rabbits inoculated with Jurkat T cells had no virus-specific reactivity throughout the study.

Automated complete blood counts and differential cell counts were performed to test early alterations of hematologic parameters during early HTLV-1 infection. One week after inoculation there was a statistically significant increase in the number of total leukocytes and lymphocytes detected in HTLV-1-infected rabbits. The mean (± standard deviation [SD]) total leukocyte count in HTLV-1-infected rabbits at 1 week postinfection, $11.9 \pm 5.2 \times 10^6$ cells/ml, was significantly increased ($P = 0.0005$) compared to $3.9 \pm 1.3 \times 10^6$ cells/ml in Jurkat T-cell-inoculated control rabbits (Fig. 2A). One week after inoculation there was also a statistically significant increase ($P = 0.0009$) in the absolute lymphocyte counts for HTLV-1-infected rabbits: $9.2 \pm 4.9 \times 10^6$ cells/ml compared to Jurkat T-cell-inoculated rabbits, $2.8 \times 10^6 \pm 1.0$ cells/ml (Fig. 2B). The remaining leukocytes (heterophils, monocytes, basophils, and eosinophils) were not statistically different from the control rabbits (data not shown). Cytologic examination of blood smears from rabbits with elevated lymphocyte counts at 1 week postinoculation revealed increased reactive and atypical lymphocytes (Fig. 3).

Soluble p19 matrix and intracellular Tax detection from blood and tissue-derived mononuclear leukocyte cultures. To monitor changes in HTLV-1-infected rabbits for the number of cells that were capable of producing soluble p19 in culture, their blood and tissue-derived mononuclear leukocytes were cultured ex vivo for 24 h in complete medium and supernatants were tested by using a commercial ELISA for p19 MA production. Spleen and MLN have been previously identified to contain HTLV-1 in rabbit and monkey models of infection (14, 17). Intraepithelial lymphocytes were tested as representative of induction sites of the gut-associated lymphoid tissue.

Blood and tissue-derived mononuclear leukocytes isolated from the MLN and spleens, cultured for 24 h ex vivo, produced the highest amount of p19 MA 1 week postinfection, except for
GALT-derived IEL, which produced the greatest amount of p19 MA at the 56-day PI time point (Fig. 4). The mean (± SD) p19 MA production was highest for mononuclear leukocytes derived from the MLN and spleen at 1 week postinfection: 347 (± 163) and 330 (± 214) pg/ml, respectively (Fig. 4).

One key viral protein (Tax) known to stimulate lymphocyte proliferation was tested. We analyzed intracellular Tax in lymphocytes (cultured for 24 h ex vivo) from the PBMC and mononuclear leukocytes isolated from the spleen, MLN, and IEL. Detection of Tax-positive cells was optimized using serial dilution of Jurkat (Tax⁻) and MT-2 (Tax⁺) cells (Fig. 5). The highest mean (± SD) intracellular Tax-positive mononuclear leukocytes (19%) was seen in the MLN 3 days PI (Fig. 6). The Tax-positive mononuclear leukocytes detected at 1 and 3 days postinfection were likely from the R49 inoculum but illustrate that following intravenous exposure virus-expressing cells traffic to mucosal-associated lymphoid compartments. Lower percentages (1 to 3%) of Tax-positive mononuclear leukocytes were also detected from ex vivo cultured leukocytes from the spleen, MLN, IEL, and PBMC at later sampling times postinfection. PBMC had the lowest mean (± SD) percentage of intracellular Tax-positive mononuclear leukocytes throughout the study period.

Proviral copy number in blood and tissue-derived mononuclear leukocytes. We used real-time PCR analysis to determine the proviral copy number in PBMC and in tissue-derived mononuclear leukocytes isolated from the spleen, MLN, and IEL (Fig. 7). In contrast to p19 MA production, the highest

FIG. 3. Reactive and atypical lymphocyte morphologies. Complete hematological analysis from 500 μl of whole blood was performed by automated cell counting, and cell differential counts were confirmed by counting at least 100 leukocytes from blood smears. Lymphocytes from blood smears were evaluated morphologically based on size and nuclear and cytoplasmic characteristics as normal, reactive (increased overall size and nuclei), and atypical (increased size with cleaved nuclei). Representative lymphocytes from stained blood smears from rabbits with elevated lymphocyte counts at 1 week postinoculation are shown. (A) Normal lymphocyte; (B) reactive lymphocyte; (C and D) atypical lymphocytes with cleaved nuclei. Magnification, ×100 with oil immersion. Bar (panel C), 10 μm.

FIG. 4. Soluble p19 MA production from PBMC and mononuclear leukocytes from spleen, MLN, and IEL. Blood and tissue-derived mononuclear leukocytes were cultured ex vivo for 24 h in complete medium. Cell-free supernatants were tested by using a commercial ELISA for p19 MA production.
mean proviral copy numbers were detected at 2 weeks postinfection in PBMC and 8 weeks postinfection in mononuclear leukocytes from spleen, MLN, and IEL. PBMC consistently maintained the highest proviral copy numbers throughout infection, with the highest mean (± SD) proviral copy numbers at 2 weeks postinfection, 174 (±155) infected cells per 10⁴ PBMC, and continued at high levels through 56 days postinfection, 152 (±145) cells per 10⁴ PBMC (Fig. 7). During the early stages of infection (days 1 to 21), tissue-derived mononuclear leukocytes had lower overall proviral copy numbers than PBMC. Interestingly, at the later stage of infection (56 days PI), splenic and MLN-derived mononuclear leukocytes contained their highest mean (± SD) proviral copy numbers: 184 (±155) and 94 (±85) lymphocytes per 10⁴ PBMC, respectively (Fig. 7). Interestingly, intraepithelial lymphocytes had undetectable to low proviral copy numbers during the early course of infection, but we detected 281 (±203) per 10⁴ PBMC proviral copies at 56 days PI (Fig. 7).

**DISCUSSION**

To more completely understand the immunopathogenesis of HTLV-1 spread, knowledge is required of the early events of the persistent retrovirus infection. Here, we tested host and virological parameters during early infection after intravenous inoculation of HTLV-1-infected cells in an established rabbit model. Our experimental design simulated transmission of the virus following inoculation by contaminated blood products in patients, which remains a significant problem worldwide in regions that do not screen to eliminate HTLV-1 from the blood supply. Our data are the first to indicate that HTLV-1 is concentrated in primary lymphoid and gut-associated lymphoid compartments in our model of early infection. In our model, early HTLV-1 infection was associated with a transient lymphocytosis with reactive and atypical circulating lymphocytes. We also documented that peak virus detection parameters occur early in the course of infection at the time of expansion of these lymphocyte populations in the blood. Our data suggest that successful HTLV-1 viral spread is correlated with early-onset lymphocytosis, which would promote establishment and maintenance of the persistent infection.

The lack of knowledge of the earliest events of HTLV-1 infection in humans is primarily due to the difficulty of identifying patients immediately following exposure and the lack of adequate samples during this critical time period. Animal models have offered alternative approaches to study the early spatial and temporal events following infection. In a squirrel monkey model of infection following intravenous inoculation with HTLV-1-infected cells, primary lymphoid organs were also found to be reservoirs of infected lymphocytes (14). Our data extend these findings and indicate new information regarding active virus replication during the early stages of infection. Prior to the typical development of an active immune response against HTLV-1 (2 to 4 weeks), we found cells in the MLN and spleen that produced p19 in culture and contained intracellular Tax. This pattern of virus spread is likely a key determinant in allowing HTLV-1 to spread in tissue reservoirs and expand early target cells before being eliminated by cytotoxic T cells (3). Our group (5) and others (19) have reported that HTLV-1 sequences from infected human subjects are highly conserved, and thus it is unlikely that viral mutations or selection of unique viral species plays a major role in determining tissue spread, but this remains for future studies to examine, following early exposure of molecularly cloned viruses.

Our data indicate that infected lymphocytes derived from the MLN and spleen produce HTLV-1 p19 and Tax in short-term culture assays and suggest that these tissue compartments are favored reservoir tissues for the virus to establish a persistent infection (Fig. 8). Lymphocytes that express HTLV-1 pro-
proteins are readily killed by HTLV-1-specific CD8+ T lymphocytes (2, 28). The microenvironments of the MLN and the spleen are likely more conducive for expressing large amounts of virus without HTLV-1-specific CD8+ T-lymphocyte pressure during the early phase of HTLV-1 infection (e.g., during the first 2 weeks PI). Our data also indicate that determining proviral copy number from circulating lymphocytes is not correlative with virus detection in tissue reservoirs during early infection. In our model system we did not measure chronic HTLV-1 infections in rabbits, and it remains possible that the proviral copy number from circulating lymphocytes may equilibrate more closely with proviral copy numbers in lymphoid compartments over time. Our findings predict that, to be effective, early intervention strategies to block HTLV-1 early viral spread must be directed at these tissue reservoirs.

Lymphocytes isolated from the IEL expressed low amounts of virus and contained the fewest infected lymphocytes over the course of early infection, but they produced soluble p19 MA in ex vivo cultured leukocytes and had increased proviral copy numbers by 56 days PI. Thus, GALT appears to represent a primary long-term reservoir for HTLV-1, consistent with its natural route of transmission via oral exposure following breast-feeding. It will be important in future studies to reexamine the tissues we tested in our current study following oral transmission of HTLV-1 in animal models such as rabbits. It is likely that the dynamics of virus detection in tissues may change when the virus is transmitted across mucosal barriers during early virus spread compared to that following intravenous transmission. Tax-positive cells were detected at days 1 and 3 PI from ex vivo cultured leukocytes from the spleen and MLN, possibly from the inoculated R49 cells. These foreign cells would be subject to an intense innate immune response in our immunocompetent rabbits, which may have prevented
their detection in our p19 and PCR assays. Future studies using labeled virus particles or labeled virus-infected cells will be required to trace the fate of infected cells during early mucosal transmission.

Collectively, our study has provided new insights into HTLV-1 transmission from contaminated blood or whole blood products. Herein, we used an established animal model of HTLV-1 infection to demonstrate that intravenous infection with cell-associated HTLV-1 targets lymphocytes located in both primary lymphoid and gut-associated lymphoid compartments. Our findings support emerging evidence that HTLV-1 promotes lymphocyte proliferation and traffic of infected cells to selective tissue microenvironments such as GALT prior to establishing a persistent infection.

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