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Original Article

Whole body clonality analysis in an aggressive STLV-1 associated leukemia (ATLL) reveals an unexpected clonal complexity

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ABSTRACT

HTLV-1 causes Adult T cell Leukemia/Lymphoma (ATLL) in humans. We describe an ATL-like disease in a 9 year-old female baboon naturally infected with STLV-1 (the simian counterpart of HTLV-1), with a lymphocyte count over 10^{10}/L, lymphocytes with abnormal nuclear morphology, and pulmonary and skin lesions. The animal was treated with a combination of AZT and alpha interferon. Proviral load (PVL) was measured every week. Because the disease continued to progress, the animal was euthanized. Abnormal infiltrates of $CD3^{+}CD25^{+}$ lymphocytes and Tax-positive cells were found by histological analyses in both lymphoid and non-lymphoid organs. PVL was measured and clonal diversity was assessed by LM-PCR (Ligation-Mediated Polymerase Chain Reaction) and high throughput sequencing, in blood during treatment and in 14 different organs. The highest PVL was found in lymph nodes, spleen and lungs. One major clone and a number of intermediate abundance clones were present in blood throughout the course of treatment, and in organs. These results represent the first multi-organ clonality study in ATLL. We demonstrate a previously undescribed clonal complexity in ATLL. Our data reinforce the usefulness of natural STLV-1 infection as a model of ATLL.

Abbreviations: ATLL, Adult T cell Leukemia/Lymphoma; AZT, azidothymidine; CUIS, common unique integration site; HAM/TSP, Associated Myelopathy/Tropical Spastic Paraparesis; HTLV-1, Human T-Lymphotropic Virus type 1; HTS, high-throughput sequencing; IFN-α, interferon-α; LM-PCR, Ligation-Mediated Polymerase Chain Reaction; NHP, Non-Human Primates; OCI, oligoclonality index; PVL, proviral load; STLV-1, Simian T-Lymphotropic Virus type 1; UIS, unique integration site.

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Introduction

Human T-Lymphotropic Virus type 1 (HTLV-1) infects 5–10 million people worldwide [1] and causes a malignant lymphoproliferative disease called Adult T-cell Leukemia/Lymphoma (ATLL) [2,3], as well as a neurological condition named HTLV-1 Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP) [4,5]. ATLL is normally characterized by the proliferation of transformed CD4+CD25+ infected lymphocytes [6] and is usually described as a monoclonal clonal infection, i.e. with a single integration of the HTLV-1 proviral genome. Nevertheless, recent studies suggested that HTLV-1 clonality might be more complex in ATL [7]. ATLL has a poor prognosis, with a mean survival time of 6 and 13 months in the acute and lymphoma types respectively (for a review see [8]). A series of reports have however shown that antiviral therapy i.e. the use of azidothymidine (AZT) combined with interferon-α (IFN-α) improves the survival rate of ATL patients suffering from the acute and chronic/smoldering forms [8–10] even if mechanism(s) of action of those compounds is (are) still debated [11,12].

In vivo, integrated HTLV-1 genomes (i.e. proviruses) are mostly detected in $CD4^+$ T-lymphocytes, although other cell types are susceptible to viral infection [13,14]. In addition, a high proviral load (PVL), i.e. the percentage of circulating HTLV-1-infected lymphocytes, is strongly associated with a higher risk of developing an HTLV-1-related disease [15–17]. *In vivo*, the virus can propagate and increase the PVL by two routes: first, by *de novo* infection of cells by virions which then undergo reverse transcription and integrate in a new genomic site; second, by clonal expansion of the already infected cells in the absence of viral replication. During the chronic phase of infection, HTLV-1 replicates mostly *via* clonal expansion [18–20] and individual clones of infected cells can persist in patients for many years [19].

Simian T-Lymphotropic Virus type 1 (STLV-1) is the simian counterpart of HTLV-1 [21–25]. HTLV-1 and STLV-1 are almost identical at the nucleotide sequence level [21,22] and at least 31 old-world Non-Human Primates (NHP) species, including baboons, are naturally infected with STLV-1 [21,24,26–30]. After a period of clinical latency, some NHP may develop an STLV-1-associated disease characterized by CD4+ proliferation, which has been considered as STLV-associated lymphoma or as a simian version of ATLL [23,31–37]. The most common findings in baboon lymphomas were weight loss, generalized lymphadenopathy, overall weakness, dyspnea, hepatosplenomegaly, pneumonia and nodular skin lesion [33]. Upon necropsy, visceral involvement of lymph nodes was also noted. Histologically, there was infiltration of lymph nodes with sheets of neoplastic lymphocytes. In some animals, large pleomorphic lymphocytes were seen with prominent centrally located nucleoli [31].

The CNRS primate Center hosts a colony of males and females *Papio anubis* that are naturally infected with STLV-1. These animals live in semi-free ranging conditions. Among these animals, one female displayed symptoms that were reminiscent of human ATL and was therefore treated with combination of AZT and interferon-α. Blood samples were taken on a weekly basis for 18 weeks. Because the disease continued to progress and the proviral load remained high, the animal was euthanized. Histopathological analyses confirmed the presence of ATL with the presence of CD4+ T-cells infiltrates in organs, some of which expressed the viral oncoprotein Tax. STLV-1 clonality analysis was then performed in a series of 18 consecutive blood samples as well as in 14 organs. The same major clones and an expected high number of intermediate clones are shared by the blood and the organs but displayed an organ specific distribution. These results are interesting because they are different from the well accepted model of ATL. These data also suggest that infected clones of minor size may play a role in ATL pathogenesis.

Materials and methods

Animal

The *P. anubis* female (animal #V932E), 12 kg, belonged to a previously described cohort [38] of STLV-1 naturally infected *P. anubis* housed at the primate center of the Centre National de la Recherche Scientifique in Rousset sur Arc and cared for in compliance with French regulations and guidelines of the Federation of European Laboratory Animal Science Associations (FELASA).

Treatment

The animal received AZT that was embedded in food (Combivir, 1 tablet every day). A person working at the animal facility monitored the animal until he ate its food containing AZT. Alpha interferon (PEG-viraferon, 50 μg/week) was injected. The PEG-viraferon dose was even higher than in humans (1.5 μg/kg/week). Fever was not observed during this period. STLV-1 blood proviral load (PVL) was measured every week. Leukocyte count was also measured on a MS9-5V cell analyzer (Melet Schloesing laboratories).

Flow cytometry

Frozen PBMCs were thawed in complete RPMI and resuspended in PBS-2%FCS. CD3+ (clone SP34-2), CD4+ (clone L200) and CD8+ (clone RPA-T8) antibodies were purchased from BD Bioscience. Flow cytometry was performed using a Facscanto II with the FlowJo (version 9.9.3) software.

Histological analyses

Lymph node and skin biopsies were collected during treatment, while other organs were collected post-mortem. Formalin-fixed paraffin-embedded sections of each specimen were stained with HE (haematoxylin and eosin) and a panel of antibodies against the following antigens: CD3 (Dako), CD4 (Thermo scientific, 4B12), CD5 (Leica Biosystem, 4C7), CD7 (Leica Biosystem, LP15), CD20 (Dako, L26), CD25 (Leica Biosystem, 4C9), CD30 (Dako, BerH2), Ki67 (Dako, MIB-1), and anti-Tax LT-4 [39].

DNA purification

Genomic DNA was extracted, using the Nucleospin® Blood kit for blood samples and the NucleoSpin® Tissue kit for tissue biopsies, according to the manufacturer's instructions (Macherey–Nagel, Düren, Germany).

STLV-1 proviral load

Proviral load (PVL) was measured using previously described SK43 and SK44 Tax primers [40]. β-actin primers were previously published [41]. Quantitative PCR was performed using FastSYBR mastermix (Life technologies) with the Fast SYBRgreen thermal cycle protocol on a QuantStudio 7 Flex real-time PCR system (Life technologies). A CD4+ clone with a single integrated provirus, isolated from an HTLV-1 infected patient [42], was used as a standard [43].

Amplification and quantification of proviral integration sites

Identification of integration sites was performed via Ligation-Mediated Polymerase Chain Reaction (LM-PCR) on extracted genomic DNA, followed by high-throughput sequencing (HTS) as described previously [19]. LM-PCR primers (see Supplemental Table 1) were designed after sequencing of the *P. anubis* Long Terminal Repeat (accession number: KX683861) as previously described [44]. Libraries were deep-sequenced using an Illumina HiSeq. DNA sequences were aligned to the Baboon genome (papAnu2) and the STLV-1 genome (accession number: JX987040) sequences using an Eland implementation of CASAVA software (Illumina). The relative abundance of a given unique integration site (UIS) was calculated from its number of different shear sites and its absolute abundance (number of copy of the clone per 100 cells) was determined from the measurement of the proviral load. See Supplemental Table 2 for details of sequencing results.

Statistical analyses

Data analysis was carried out using R [45]. The VennDiagram package [46] was used to generate the Venn diagram. To characterize the clonal distribution, the oligoclonality index (OCI; Gini coefficient) index was calculated, as previously described [19], using the package reldist [47]. An oligoclonality index of 1 corresponds to a monoclonal distribution (one clone carries all the load) whereas an oligoclonality index of 0 describes a perfect polyclonal population (all clones have equal abundance).

Results

Aggressive ATL in a STLV-1 naturally infected P. anubis

During the surveillance of our naturally STLV-1-infected *P. anubis* cohort (n = 45), we identified a 9 year-old female baboon (#V932E) exhibiting dyspnea, marked emaciation, no