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Clonal expansion of HTLV-1 infected cells depends on the CD4 versus CD8 phenotype

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1. ABSTRACT

As other deltaretroviruses HTLV-1 replication *in vivo* includes a first short step of reverse transcription that is followed by the persistent clonal expansion of infected cells. *In vivo* these cells include the CD4+ and CD8+ lymphocytes yet the virus induces adult T cell leukemia/lymphoma (ATLL) that is regularly of the CD4+ phenotype. Cloned infected cells from individuals without malignancy possess a dramatic increase in spontaneous proliferation, which predominated with CD8+ lymphocytes and depends on the amount of *tax* mRNA. In fact, the clonal expansion of HTLV-1 positive CD8+ and CD4+ lymphocytes relies on two distinct mechanisms: infection prevented cell death in the former whereas recruiting the latter into the cell cycle. Furthermore infected *tax*-expressing CD4+ lymphocytes cumulate cellular defects characteristic of genetic instability. Therefore, HTLV-1 infection establishes a preleukemic phenotype that is restricted to CD4+ infected clones. Investigating the mechanisms underlying apoptosis, cell cycling and DNA repair in cloned cells from naturally infected individuals will permit to deciphering the molecular pathogenesis of HTLV-1 infection.

2. INTRODUCTION

Retroviruses are unique as they exist as DNA and/or RNA species. Their polymerases are reverse transcriptases devoided of 3' exonucleolytic activity and genetic variability is thereby a part of their way of life (1, 2). Among retroviruses, deltaretroviruses possess an additional mechanism of replication that accompanies an original way of genetic variability. In addition to reverse transcriptase that generates an error rate in the same range as those of other retroviruses, these lymphotropic viruses encode regulatory proteins that interfere with many host cell pathways including cell cycle, apoptosis and DNA repair (3). This results in the persistent clonal expansion of infected cells and generates a significant level of genetic variability resulting from somatic mutations of the proviral sequence (4-6).

Deltaretroviruses include human T-cell leukemia viruses type -1 (7) and -2 (HTLV-1 and 2) (8), the recently discovered HTLV-3 (9) and -4 (10), simian T-cell leukemia viruses (STLV) (11), and the bovine leukemia virus (BLV) (12). They infect vertebrates and cause leukemia and lymphoma. Two steps characterize the course of

deltaretroviruses infection *in vivo*, including a brief period of primary infection that is followed by the chronic and persistent infection (5, 6, 13, 14). After experimental infection, primary infection starts with viral contamination and, at least for HTLV-1 in squirrel monkey (*Saimiri sciureus*) and BLV in sheep, finishes 1-6 months later, as soon as both humoral and cellular antiviral host immune responses have been mounted (14, 15). The second phase of the infection encompasses the remaining lifespan of infected organisms. It could be clinically latent or associated with the development of inflammatory or malignant diseases. HTLV-1 infects CD4+ and CD8+ lymphocytes *in vivo*. In a recent work, we found that the cell-associated dissemination of the virus *in vivo* pertains for both lymphocytes subtypes but relies on specific mechanisms with respect to the cellular phenotype. Clonal expansion of HTLV-1 infected cells was found to result from the accumulation of CD8+ lymphocytes or from the proliferation of CD4+ cells. Here we review the main virological and pathogenic consequences of these findings.

3. CD4+ AND CD8+ T LYMPHOCYTES ARE THE MAJOR HTLV-1 RESERVOIR

In vitro, HTLV-1 infects a wide range of cells (16) including several non lymphoid tumor cell lines such as human osteogenic sarcoma cells (17), lung cells, cervical carcinoma cells (HeLa) (18), human gastric HGC-27 cells (19), human promyelocytic leukaemia HL60 cells (20) as well as primary endothelial cells (21), monocyte, microglial cells (22), and mammary epithelial cells (23). *In vivo*, HTLV-1 intercellular passage, i.e. horizontal, reverse-transcription-based replication, requires a close cell-to-cell contact *via* the formation of the recently described "virological synapse" between the infected and target cells (24). In addition *in vivo*, HTLV-1 is found primarily in CD4+ and CD8+ (25) T lymphocytes and less frequently in other cell types such as monocytes, endothelial cells and dendritic cells (26, 27).

Recently the glucose transporter GLUT1 was found as a receptor for HTLV-1 (16), mediating viral binding and entry. *In vitro*, GLUT-1 is found on all mammalian cell lines (28) whereas *in vivo*, its expression characterizes a restricted number of cell types including activated T cells (29). This contributes to explain the HTLV-1 tropism *in vivo*. In addition to a cell-specific expression of its receptor, the HTLV-1 tropism might depend on post-entry events. After cell entry, retrovirus envelope interacts with its specific receptor and thereby blocks further superinfection. This could also alter the function of the receptor. Indeed, the interaction between the HTLV-1 encoded GP46 with GLUT-1 negatively interferes with glucose entry and therefore could compromise cell viability, especially in cells having a high metabolic rate. Accordingly it is possible that *in vivo*, HTLV can initially spread with a large tropism but with the subsequent elimination of cells having a high metabolic rate (16). Conversely, after proviral integration, a reduced level of envelope expression might subsequently permit glucose intake and thereby help cell viability and persistent infection.

Neuropilin 1 (NRP-1) is also involved in HTLV-1 entry and may be an additional cell receptor for the virus (30). NRP1 is expressed on a broad range of cell lines from various origins *in vitro* and over-expressed upon T-cell activation (30). In contrast with GLUT1, NRP1 specifically concentrates in "virological synapses" but currently, the respective contributions of GLUT1 and NRP1 to the binding and fusion steps of the HTLV-1 entry process remain unknown. Other molecules on the cell surface may be critical for HTLV-1 env-mediated binding and/or fusion such as heparan sulfate proteoglycans and certain integrins, including ICAM-1, ICAM-3 and V-CAM (31, 32). These latter may act as cofactors for HTLV-1 induced cell fusion whereas HSPGs have been reported to play a role in the binding of HTLV-1 to target cells and to contribute to HTLV-1 infection of primary CD4+ T cells (33, 34).

4. CLONAL EXPANSION OF HTLV-1 INFECTED T CELLS

Originally it was thought that HTLV-1 integration was polyclonal in HTLV-1 associated myelopathy (HAM-TSP) (35). Thereafter, it was shown also by Southern Blotting that TSP/HAM was accompanied by occasional oligoclonal expansion of infected cells (36). Another team examined variations in the T cell receptor (TCR) Vα and Vβ chains in peripheral blood mononuclear cells (PBMC) derived from patients with HAM/TSP and evidenced the proliferation of anti-HTLV-1 specific CD8+ T lymphocytes without distinguishing infected from uninfected cells (37). Finally, by using sensitive PCR-derived techniques such as linker mediated PCR or inverse PCR (38-40), it was possible to provide evidence of clonal expansion of HTLV-1 bearing T cells in all infected individuals. This route of replication was found to pertain at all stages of the infection and to characterize both CD4+ and CD8+ infected T lymphocytes (41, 42). As 2^n proviral copies are generated for a given clone after n cell divisions, few cell-associated replication cycles are sufficient to mount elevated proviral loads. This contributes to explain the combination of high proviral loads observed in infected individuals with the very low cell-to-cell transmission rate and the apparent low genetic drift of the virus (40, 43).

5. PROVIRAL GENETIC VARIABILITY PARALLELS CLONAL EXPANSION

HTLV-1 genetic variation results mainly from post-integration events that consist in somatic mutations of the proviral sequence occurring during clonal expansion (4). Surprisingly, the frequency of somatic mutations was much higher than that expected as the 3'RU5 mutation frequency was found to be 600 times higher than that for the HTLV-1 reverse transcription (4). At steady state, 60% of HTLV-1 positive clones include 8% to 80% of infected cells harboring a mutated HTLV-1 provirus, without evidence for reverse transcription-associated mutations. Interestingly both the provirus and the cellular genome display the same process of somatic mutations (4). The experimental infection of squirrel monkeys (*Saimiri sciureus*) and sheep with HTLV-1 and BLV, respectively, evidenced that deltaretrovirus infection is a two-step

process that includes an early and transient phase of reverse transcription followed by the persistent multiplication of infected cells by clonal expansion (6, 14). For the first time, RT-dependent substitutions could be evidenced for a deltaretrovirus by analyzing BLV replication during the first weeks following experimental sheep infection (6). The two main diseases related to HTLV-1 are TSP/HAM and ATLL. ATLL is malignant CD4+ monoclonal lymphoproliferative disease (44) while TSP/HAM is a neurological disorder characterized by the infiltration of CD4+ and CD8+ T cells in the thoracic spinal cord (45). Both clonal expansion and somatic mutations have been found to participate to the pathogenesis of these conditions.

6. PATHOGENICAL AND CLINICAL IMPLICATIONS IN ATLL

ATLL occurs after a prolonged period of clinical latency lasting, on average, greater than 30 years (46). As in other lymphoid malignancies, malignant ATLL cells frequently infiltrate skin, bone, bone marrow, and other organs *in vivo* (44). This results from the cellular transport of the integrated provirus to the corresponding sites, as confirmed by molecular studies (41). The tumor clone is usually monoclonal for HTLV-1 integration (47) and is accompanied by a background of polyclonally expanded and untransformed cells (41). During the period of clinical latency preceding tumor onset, infected cells express the HTLV-1-encoded Tax oncoprotein (48, 49), which is known for possessing a pleiotropic effect on cellular metabolism, particularly on cell cycle, DNA repair and apoptosis (49). We recently investigated this preleukemic period of the infection in an animal model, the sheep model of BLV infection, which permits to reconstitute the clonal history of preleukemic cells, from the experimental infection to the tumor stage. Premalignant clones, identified by the integration site of the provirus, are early and clearly distinguished from other virus-exposed cells on the basis of their degree of clonal expansion and genetic instability (5). Detectable as early as 15 days after the beginning of virus exposure, premalignant cells display a two-step pattern of extensive clonal expansion together with a mutation load strongly higher than that of other virus-exposed cells that remain untransformed during the lifespan of animals. There is no fixation of somatic mutations over time, suggesting that they regularly lead to cellular death, partly contributing to maintain a normal lymphocyte count during the prolonged premalignant stage. This equilibrium is regularly broken after a period of 18.5 to 60 months of clinical latency, when a dramatic decrease in the genetic instability of premalignant cells coincides with a rapid increase in lymphocyte count and lymphoma onset.

7. PATHOGENICAL AND CLINICAL IMPLICATIONS IN TSP/HAM

HAM/TSP is a chronic debilitating inflammatory disease of the central nervous system, characterized by axonal damage and demyelination, most pronounced in the midthoracic spinal cord. HAM/TSP is characterized by high proviral loads that result from the extensive proliferation of a restricted number of infected clones (50). One of the main pathological features of the disease is a chronic inflammatory process that predominates in the lateral and posterior columns of the spinal cord in the thoracic region. Lesions include CD4+ and CD8+ cellular infiltrates that correspond to infected and uninfected cells. As infected lymphocytes sharing the same flanking sequences are regularly detected in both the cerebrospinal fluid and the peripheral blood of patients with HAM/TSP, it was concluded that HTLV-1 crosses the blood-brain barrier by way of the migration of HTLV-1 infected lymphocytes (51). Similarly, HTLV-1 infected T cells infiltrate the aqueous humor, the synovial fluid, and the alveolar liquid of patients with uveitis, arthropathy, and alveolitis, respectively, with the evidence of a cell associated transport of the virus in each of these situations (Wattel E, unpublished works). Similar results have been obtained with various organs in the Squirrel Monkey experimentally infected with HTLV-1 positive cells (14). Together these data indicate that the clonal expansion of infected cells helps these cells to migrate in body compartment where they contribute to trigger inflammatory damage.

8. WHICH MECHANISMS UNDERLIE CLONAL EXPANSION?

From the studies detailed above, it appears that high provirus loads resulting from clonal expansion of HTLV-1 bearing T cells are associated with viral dissemination, inflammatory diseases and preleukemic states. As a corollary, the somatic mutation rate parallels the level of clonal expansion and culminates at the symptomatic stages of the infection (5, 6). Therefore, clonal expansion and somatic substitutions play a critical role in the pathogenesis of HTLV-1 associated diseases. Knowing the mechanisms that underlie these two processes will help to understand and target HTLV-1 diseases.

Schematically, the 2 main mechanisms contributing to clonal expansion are cell accumulation and cell proliferation. The former relies on the regulation of the cell cycle and the latter on the regulation of apoptosis. We recently investigated these two cell processes in CD4+ and CD8+ cells (52). This was done by developing a cellular model of HTLV-1 replication through clonal expansion, by T-cell limiting dilution cloning of cells derived from HLTV-1 infected patients without malignancy (52). Cloning uninfected and naturally infected CD4+ and CD8+ T cells deriving from the same infected individuals permitted to clearly compare the effects of the infection on CD4+ and CD8+ cells. Results indicated that the clonal expansion of HTLV-1 positive cells relies on 2 clearly distinct mechanisms for CD4+ and CD8+ lymphocytes. Indeed HTLV-1 infection propels CD4+ infected T cells into the cell cycle while preventing cell death in CD8+ infected cells. In addition, infected *tax*-expressing CD4+ lymphocytes cumulate cellular defects characteristic of genetic instability.

In *vivo*, HTLV-1 infected CD4+ and CD8+ lymphocytes display the same pattern of clonal expansion (52). The higher degree of *in vivo* clonal expansion in infected CD4+ lymphocytes might well contribute to

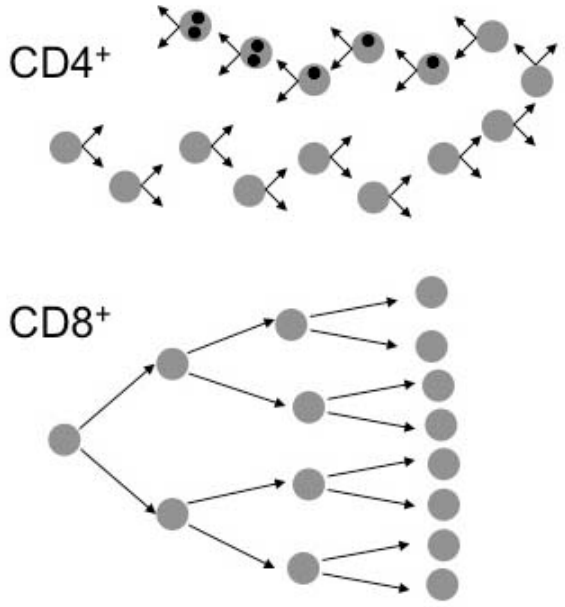

Figure 1. Clonal expansion of HTLV-1 positive cells result from the proliferation of CD4+ lymphocytes versus the accumulation of CD8+ cells. Two clones harboring the same number of cells are represented. The figure assumes a 50% programmed cell death for CD4+ lymphocytes versus 0% for CD8+ cells. Accordingly, to mount an identical number of cells, the number of CD4+ cells division is significantly higher than that of CD8+ cells. Together with the negative impact of the virus on the DNA damage repair response, this helps the infected CD4+ clone to acquire genetic rearrangements (block circles).

selecting malignant events, as a high level of clonal expansion of deltaretrovirus-infected cells is the signature of premalignant clones *in vivo* (5, 6, 14). Furthermore, *ex vivo*, cloned CD4+ HTLV-1+ lymphocytes accumulate numerous cellular defects, including multinuclearity, chromatin bridges and nuclear abnormalities that characterize genetic instability (53-59). One can propose that, together with these cellular abnormalities and the extensive oligo/polyclonal expansion observed *in vivo*, cell cycling, which is restricted to HTLV-1+-CD4+ untransformed clones in a *tax*-dependent manner, is implicated in promoting HTLV-1 associated lymphoid malignancies and establishes a preleukemic phenotype that is restricted to CD4+ infected cells. By contrast, the CD8+-restricted inhibition of apoptosis, which is independent of tax expression, might favor CD8+-dependent control of the infection (60) and inflammatory processes involved in the pathogenesis of TSP/HAM, uveitis or infective dermatitis. The figure 1 summarizes the behaviors of CD4+ versus CD8+ cells upon HTLV-1 infection. As upon infection CD4+ cells proliferate while CD8+ cells accumulate, the number of cell divisions necessary for obtaining the same number of cells appears significantly higher for generating and maintaining a CD4+ clone when compared to a CD8+ clone having the same cell count. As these infected CD4+ cells seem impaired for the reparation of DNA damages (61), this excess of mitosis might expose the clone for malignant transformation.

9. MOLECULAR CAUSES UNDERLYING CLONAL EXPANSION IN VIVO

Numerous studies have investigated the interplay between HTLV-1, cell cycling, apoptosis, and genetic instability. Although some conflicting data have been published, it appears that this virus is antiapoptotic, promoting cell cycling and impairing DNA repair (61). However these effects have been evidenced in cell lines or in cells expressing virus-encoded proteins. Having observed that the effect of the infection on apoptosis, cell cycling and genetic instability depends on the lymphocyte phenotype, we now investigate the molecular mechanisms underlying these three precancerous processes in CD4+ versus CD8+ cells deriving from naturally infected individuals. We hypothesize that by taking into account these phenotype-dependent effects of the infection on cell behavior *in vivo*, the proposed experimental strategy will permit to gather pertinent data for better understanding HTLV-1 pathogenesis and for targeting pertinent defects.

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