Abstract: A typical person infected with the retrovirus human T-lymphotropic virus type 1 (HTLV-1) carries tens of thousands of clones of HTLV-1-infected T lymphocytes, each clone distinguished by a unique integration site of the provirus in the host genome. However, only 5% of infected people develop the malignant disease adult T cell leukaemia/lymphoma, usually more than 50 years after becoming infected. We review the host and viral factors that cause this aggressive disease.
How does HTLV-1 cause adult T-cell leukaemia/lymphoma (ATL)?

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Abstract

A typical person infected with the retrovirus human T-lymphotropic virus type 1 (HTLV-1) carries tens of thousands of clones of HTLV-1-infected T lymphocytes, each clone distinguished by a unique integration site of the provirus in the host genome. However, only 5% of infected people develop the malignant disease adult T cell leukaemia/lymphoma, usually more than 50 years after becoming infected. We review the host and viral factors that cause this aggressive disease.

Introduction

HTLV-1 is a complex deltaretrovirus [1]. Its genome (Figure 1) includes components of simple retroviruses, as well as additional regulatory genes. The gag, pr (Protease), and pol (Polymerase and Integrase) genes are encoded from a single transcript, as a result of ribosome frameshifts. The env gene encodes a glycoprotein that mediates binding to the target cell receptors glucose transporter 1 and neuropilin; viral entry is also promoted by heparan sulphate proteoglycans [2]. The genome also has a unique set of regulatory genes, which encode Tax and Rex proteins, that regulate transcriptional initiation and nuclear RNA export, respectively [3]. Other alternatively spliced RNAs encode the p21, p12, p13, and p30 proteins, important for virus propagation in vivo [4-6]. The p12 protein, or its proteolytic product p8, promotes T cell activation, virus transmission, and escape from CTL and NK cell killing [4,7]. The p30 protein is a latency maintenance factor that cooperates with c-Myc in oncogenesis [8]. Unlike other retroviruses, HTLV-1 also encodes an anti-sense transcript that produces the HBZ, helix-basic loop zipper protein [9].

Three to five percent of infected individuals develop HTLV-1-associated myelopathy (HAM), characterized by lower limb spasticity, bowel and bladder disturbances, and progressive neurological decline over several years [10]. HTLV-1 infection is also associated with other inflammatory clinical...
conditions, including uveitis, arthropathy, myopathy, and pneumopathy [11,12]. These disorders are associated with a high virus load and overstimulation of dendritic cells, resulting in chronic production of high levels of interferon-stimulated gene products [13]. A further 5% of infected individuals develop a CD4+ T-cell leukaemia or lymphoma, designated adult T-cell leukaemia/lymphoma (ATL), that occurs almost exclusively in individuals who acquired HTLV-1 as a result of breast feeding, although four or five decades generally elapse before development of disease [14]. ATL is characterized by frequent blood, bone marrow, and brain involvement, hypercalcaemia, and lytic bone lesions. The acute forms have a median survival of about one year, despite intensive chemotherapy. Studies of arsenic and interferon treatment show potential clinical benefit, which may be due to inhibition of leukaemia cell initiating activity [15]. This effect may be enhanced by a synthetic retinoid, ST1926 [16]. Recent data suggest an improved response with the addition to combination chemotherapy of an antibody to CCR4, a chemokine receptor overexpressed on ATL cells responsible for targeting leukocytes to the central nervous system and endothelial cells involved in angiogenesis [17].

HTLV-1 varies little in sequence compared with HIV-1, and the genotype of HTLV-1 in cases of ATL or HAM/TSP is not distinct from that in asymptomatic carriers of the virus. The different outcomes of HTLV-1 infection must therefore be due to differences in the host. The innate immune response [18] to HTLV-1 has been understudied until recently. In patients with HAM/TSP, both the frequency and the lytic activity of natural killer (NK) cells is significantly lower than in asymptomatic carriers or uninfected subjects [19-22]. This ill-understood phenomenon deserves further research. Type 1 (beta) interferon impairs HTLV-1 replication in vitro [23], but its role in chronic HTLV-1 infection is not known. HTLV-1 Tax protein induces strong expression of Type 2 (gamma) interferon in infected cells [24]. Gene expression microarray analysis of freshly isolated peripheral blood mononuclear cells suggests [21] that chronic expression of interferons – probably both Type 1 and Type 2 – contributes to the inflammatory tissue damage in HAM/TSP. Sustained treatment with type 1 (alpha) interferon in combination with AZT is effective [25] in some cases of ATL, especially chronic ATL. It is believed that this benefit derives from the antiproliferative effects of the two agents, rather than their antiviral actions, but the mechanism remains unclear.

The acquired immune response to HTLV-1 has been intensely studied. HTLV-1 infection frequently elicits a very high titre of specific antibody; antibody can confer some protection against infection [26], but its role (if any) in the chronic phase of infection is unclear. HTLV-1 also elicits abundant, chronically activated CD8+ cytotoxic T lymphocytes (CTLs) [27-29]. The efficiency or “quality” of the specific CTL response to HTLV-1 is a major determinant of the proviral load and the risk of inflammatory disease [30]. Since the risk of ATL also increases with proviral load, it is likely that CTL quality also determines
ATL risk, but this has not been directly demonstrated. The chief factors that determine a high quality anti-HTLV-1 CTL response [30], and therefore a low risk of associated diseases, are the host genotype in the HLA Class 1 [31,32] and killer immunoglobulin-like receptor (KIR) loci [33] (respectively HLA-A*02 and -Cw*08, and KIR2DL2, in southern Japan), and the ability to present epitopes from the HBZ antigen to CTLs [34].

HTLV-1-specific CD4+ T cells are more abundant in HAM/TSP than in asymptomatic carriers, and may contribute to inflammation [35]. HTLV-1 Tax stimulates CD4+ T cells to produce the chemokine CCL22 [36]. CCL22 in turn maintains a high frequency of regulatory CD4+ T cells (Treg) that express the characteristic proteins FoxP3, CD25, GITR and the CCL22 receptor, CCR4. Treg that are not HTLV-1-infected may impair the quality of the CTL response to HTLV-1 [37] and so increase the proviral load and the risk of disease. Clonal primary ATL cells also frequently express these Treg proteins, as do some non-malignant HTLV-1-infected cells. However, in HTLV-1-infected Treg the expression and actions of FoxP3 in the infected cell are impaired by the products of the tax [38] and HBZ [39] genes, and HTLV-1-infected FoxP3+ cells do not themselves exert regulatory functions [37]. We conclude that, although ATL may arise in a Treg clone, ATL is not necessarily a malignancy of Treg [40,41].

**Mechanisms of oncogenesis**

Both Tax and HBZ are implicated in oncogenesis [42]. One model implicates Tax in tumour initiation, and HBZ in maintenance. Although HTLV-1 can infect a wide range of human cells in vitro, the specificity for immortalization of activated T cells appears to be determined at a post-entry step. Recent work suggests that suppression of Tax activity by TCF1 and LEF1, two Wnt transcription factors, is relieved by T-cell activation [43]. Tax has transcriptional effects through CREB/ATF proteins to upregulate virus expression by functioning as a coactivator for CREB-binding proteins, CBP and p300 [44]. In addition, Tax interactions with CREB binding proteins promotes chemotaxis and transmission of HTLV-1 through enhanced expression of a Ras family member known as Gem [45]. Tax also promotes the ability of NFAT, AP-1, and NFkB pathways to upregulate expression of cellular genes that mediate lymphocyte proliferation and resistance to apoptosis [46]. Transgenic expression of Tax results in NK and T cell malignancies, depending on the promoter used. Overexpression of NF-kB pathway genes is a common property of these models [47-49]. Effects of Tax on NF-kB appear to be mediated by direct binding to IKKgamma and p100 as well as alterations of ubiquitination of NF-kB regulatory proteins, such as TRAF6 [46]. Tax-mediated K63-ubiquitination of TRAF6 also promotes interaction with anti-apoptosis protein Mcl-1, and survival of transformed cells [50]. IL-17RB is a NF-kB product expressed...
in ATL cells, that provides a feed-forward autocrine loop to further activate the NF-kB pathway [51]. Tax can also directly interact with proteins to block cell cycle inhibitors (p16, p15, Rb) and activate cyclins and cyclin-dependent kinases [1]. Moreover, Tax binding to PDZ-containing proteins upregulates PI3K-Akt-mTOR signalling. Lastly, Tax induces genetic instability, evidenced by its ability to produce multinucleation, chromatin bridges, aneuploidy, telomere attrition, and clastogenic DNA damage [52]. These effects are mediated by generation of reactive oxygen species and dysfunctional DNA repair processes, as a result of direct interactions with MAD1, PCNA, DNA topoisomerase, DNA polymerase beta, mini-chromosome maintenance MCM2-7 helicase, Chk2, and Ku80 [53-56]. In addition, Tax functionally inactivates p53 [21,57].

However, Tax is often repressed once tumours develop [58]. In contrast, HBZ is ubiquitously expressed in ATLL cells and HTLV-1 infected cells in vivo [59]. In mouse models, HBZ also induces inflammatory disorders with elevated CD4+Foxp3+ T_reg cells in the intestines, skin, and lung [60]. However, these HBZ-transgenic cells tend to lose FoxP3 expression, resulting in increased interferon gamma expression and a pro-inflammatory phenotype. In addition, HBZ induces T cell neoplasia in vivo, but at a slower rate and lower frequency than that seen in Tax transgenic models. HBZ promotes proliferation of HTLV-1-infected cells [61,62] and minimizes the effect of repeated mitosis on cellular ageing by enhancing expression of the host genes telomerase reverse transcriptase (hTERT) and JunD [63]. Interactions between Tax and HBZ on HTLV-1-infected cells are complex, and in some cases opposite (Figure 3). For example, HBZ represses AP-1, NFAT, and CREB activities [64]. Conversely, Tax inhibits TGFbeta signaling, whereas HBZ activates it. Tax activates both the canonical and non-canonical NFkB pathways, whereas HBZ inhibits only the canonical NFkB pathway [65]. Tax promotes cell proliferation but also induces cellular senescence by induction of p21 and p27, whereas HBZ prevents senescence by inhibiting p65 [66]. Lastly, HBZ suppresses the canonical Wnt pathway, but enhances the non-canonical pathway, suggesting that HBZ modulates the intra-cellular environment of peripheral T cells targeted by the virus [67].

Clonality

HTLV-1 stimulates infected T cells to proliferate in vivo, by persistent or intermittent expression of Tax and HBZ. This proliferation results in long-lived, abundant clones of infected T cells in the circulation [68], each clone distinguished by a unique site of integration of the HTLV-1 provirus in the host genome. The majority of infected clones carry a single proviral copy [69]; about 10% of ATL clones contain 2 copies, some of which may be defective [70].
The proviral integration site of HTLV-1 in the host genome [71] is not random, but is strongly biased towards certain transcription factor binding sites, notably those that bind STAT1, TP53, and HDAC6 [72]. Integration within 10kb of a known oncogene confers a survival advantage on the clone in vivo, but does not appear to contribute directly to leukaemogenesis [70]. Although no hot-spot of integration is associated with ATL, there is evidence that integration within 15kb upstream of host genes that are frequently dysregulated in other leukaemias occurs significantly more frequently than expected by chance; such clones account for about 6% of ATL cases in southern Japan [70].

There is recent evidence that the HTLV-1 provirus has the potential to alter host chromatin structure (Satou et al, submitted). This observation raises the possibility that HTLV-1 disrupts host gene regulation either in the immediate vicinity of the provirus or by altering long-range chromatin interactions.

Underlying the small number of highly expanded clones in the circulation, it is now clear that there is a very large number of lower-abundance HTLV-1-infected T cell clones (Figure 2) [71]: the median number of clones in the circulation is around 10,000 in an asymptomatic carrier and around 30,000 in a patient with HAM/TSP, exceeding previous estimates by 2 orders of magnitude [73]. The lower-abundance HTLV-1+ clones make up the majority of the HTLV-1 proviral load, and the proviral load – which determines the risk of both inflammatory and malignant disease - correlates with the total number of clones in the individual, not with the degree of oligoclonal proliferation [71].

The lower-abundance clones express Tax more frequently than high-abundance clones [72], and Tax-expressing cells turn over faster than non-expressing cells in vivo [74]. The rate of spontaneous Tax expression by a given clone is influenced by the genomic integration site of the provirus in that clone: specifically, the distance and transcriptional orientation of the provirus relative to the nearest host gene [72]. Since the risk of malignant transformation in many cell types is proportional to the total number of cell divisions [75], persistent HTLV-1-driven proliferation may increase the risk of ATL arising from these low-abundance clones. Several recent lines of evidence support this notion that ATL may arise from low abundance clones rather than the oligoclonally proliferated, high-abundance clones [76]. First, the risk of ATL rises with increased proviral load, and in turn the proviral load correlates with the total number of clones in the circulation, but not with the degree of oligoclonality [71]. Second, the attributes of the genomic integration site in ATL clones resemble those of the low abundance clones, but differ significantly from those of the intermediate-abundance, oligoclonally proliferated clones [70]. Finally, the closely related virus HTLV-2 produces an oligoclonality profile in peripheral blood similar to that seen in ATL, but with a total number of infected clones about 10-fold lower than seen in HTLV-1.
infection [77]. However, despite this strong oligoclonal proliferation, HTLV-2 does not cause malignant disease.

The host and viral factors that contribute to the development of ATL are summarized in Figure 3.

**Prospects and suggestions for future work**

There remains a gap in our understanding of the events occurring during clinical latency that lead to ATL and HAM from longitudinal clinical studies. In particular, we need to quantify the contributions of infectious HTLV-1 spread (new clone formation) and mitotic spread (proliferation of existing clones) to the maintenance of the proviral load.

The evidence summarized here suggests that every HTLV-1-infected clone has a potential to undergo malignant transformation, and that the risk of ATL is determined primarily by the number of HTLV-1-infected T-cell clones, which in turn is determined by the efficacy of ‘quality’ of the immune response. There are instances of “clonal succession” in ATL, in which the spontaneous disappearance of one malignant clone is followed by the appearance of a second, independent ATL clone [78]. Work is needed on strategies to prevent ATL in high-risk individuals, i.e. those with a proviral load >4%. It may be effective to reduce the total number of HTLV-1-infected clones (and therefore reduce the proviral load), for example with anti-CCR4 monoclonal antibody; to reduce the turnover rate of infected cells; and to boost the innate and acquired immune response.

Exciting new developments with immunodeficient mice, reconstituted with human hematopoietic stem cells, provide opportunities to study virus replication and tumour initiation in vivo [79,80]. These models will also be useful to test new chemopreventive agents and targeted therapies [81].

Ground-breaking work with immunotherapies has provided novel approaches in a wide range of malignancies. Chimeric antigen receptor-expressing T cells have proven activity in lymphoid leukaemias [82]. Immune checkpoint inhibitors that block costimulatory T cell receptor signaling have provided unexpected activity in a wide range of solid tumours and lymphomas, and may open the door to new clinical trials for ATL [83].
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**Figure 1.** Genome structure of the HTLV-1 provirus. The *gag, pr, pol,* and *env* genes are shown flanked by long terminal repeat (LTR) sequences. The pX regions includes regulatory genes encoding Tax, Rex, p21, p12, p13, and p30, as well as the antisense gene encoding HBZ (modified from [1]).
**Figure 2.** Frequency distribution of HTLV-1-infected T-cell clones in different manifestations of HTLV-1 infection. Each pie-chart shows the clones identified in 10μg of genomic DNA from the peripheral blood mononuclear cells of a single representative host. The size of each sector represents the relative abundance of that clone in the individual's proviral load. The oligoclonality index (OCI) (also known as the Gini index) is a measure of the non-uniformity of the frequency distribution (70). OCI = 0 implies that all clones have the same frequency; OCI = 1 implies that only a single clone is present. These data were reported in Figure 2A of Gillet et al 2011 (70).
Figure 3. Factors that determine the risk of ATL. These factors may be divided into three categories: host-specific factors, clone-specific, and stochastic factors. The host-specific factors – host genotype, age at infection, and certain co-infections [84], especially Strongyloides stercoralis – are independent of the proviral integration site. Persistent replication of long-lived infected clones, driven by Tax and HBZ, may lead to the accumulation of replicative mutations [75], whose frequency is strongly correlated with the risk of malignant transformation. This may explain the observed association between ATL and infection with HTLV-1 in early childhood.

The clone-specific oncogenic factors depend on the proviral integration site: spontaneous proviral expression is influenced by the flanking host genome [72]; and the HTLV-1 gene products, especially Tax and HBZ, promote the proliferation and longevity of existing clones and genetic instability. It is possible that insertional mutagenesis contributes to oncogenesis, by activation or disruption of host genes flanking the provirus or via long-range chromatin interactions. Finally, infected T cell clones that are specific to persistent and abundant antigens, such as CMV or Strongyloides antigens, may enjoy an additional proliferative advantage.

It is widely assumed [85] that secondary mutations – the stochastic factors - increase the proliferative or survival advantage of an HTLV-1+ clone, and contribute to malignant transformation. Direct evidence to substantiate this assumption is needed from whole-genome sequencing of ATL clones. Preliminary work along this line has already begun, with evidence that mutations in three signalling pathways – NF-κB, T-cell receptor and CCR4 – are associated with ATL [86].

* Tax expression is lost in the malignant clone in ~50% of cases of ATL.
Clone-specific factors

Each clone has its own pattern and intensity of expression of HBZ and tax genes:

- CREB
- AP-1
- Classical NFkB
- TGFβ
- Telomerase
- Wnt

↑ proliferation
↑ survival

- genetic instability
- ? insertional mutagenesis

2nd, 3rd, ... hits

Anti-Tax CTLs → Loss of Tax expression

→ ATL
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