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Is human T-cell lymphotropic virus type I really silent?

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The role of the cellular immune response to human T-cell lymphotropic virus type I (HTLV-I) is not fully understood. The low level of HTLV-I protein expression in peripheral blood lymphocytes has led to the widely held belief that HTLV-I is transcriptionally silent *in vivo*. However, most HTLV-I-infected individuals mount a strong and persistently activated cytotoxic T-lymphocyte (CTL) response to the virus; this observation implies that there is abundant chronic transcription of HTLV-I genes. Here we show that HTLV-I Tax protein expression rises quickly in freshly isolated peripheral blood lymphocytes, but that expressing cells are rapidly killed by CTLs. Mathematical analysis of these results indicates that the CTL response is extremely efficient and that the half-life of a Tax-expressing cell is less than a day. We propose that HTLV-I protein expression in circulating lymphocytes is undetectable by current techniques because of the efficiency of the CTL-mediated immune surveillance *in vivo*.

Keywords: cytotoxic T lymphocyte; latent; HTLV-I; half-life; turnover

1. INTRODUCTION

Human T-cell lymphotropic virus type I (HTLV-I) was the first replication-competent human retrovirus to be isolated (Poiesz *et al*. 1980). Approximately 10–20 million people are thought to be infected wordwide. HTLV-I is highly prevalent in southern Japan, the Caribbean Islands, Central Africa and South America. The majority (about 95%) of infected individuals develop no associated disease; however, in a small percentage of individuals, infection results in one of two types of disease. About 2–3% develop adult T-cell leukaemia (ATL); a further 2–3% develop one or more of a range of inflammatory diseases—the most common of which is HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), an inflammatory disease of the central nervous system (CNS). While cases of ATL and HAM/TSP have been reported in the same individual, the frequency of occurrence is no more than would be expected by chance and the pathogenesis of ATL and inflammatory disease is thought to be independent. Infection with HTLV-I results in lifelong viral persistence, with rare possible exceptions described (Daenke *et al*. 1994).

Proviral load is remarkably high in HTLV-I infection. Typically 0.5–10% of peripheral blood mononuclear cells (PBMCs) are provirus positive. The most commonly infected cells are $CD4^+$ $CD45RO^+$ T lymphocytes (Richardson *et al*. 1990). A small proportion of $CD8^+$ lymphocytes are sometimes seen to be infected (Hanon *et al*. 2000*b*; Koyanagi *et al*. 1993; Cho *et al*. 1995; Gessain *et al*. 1990). Median viral load in individuals with HAM/TSP is significantly higher than in healthy carriers (HCs), although the ranges are broad and overlap (Nagai *et al*. 1998).

The $CD8^+$ cytotoxic T-lymphocyte (CTL) response to HTLV-I infection is very strong, with up to 10% of $CD8^+$ cells being specific for a single epitope (Bieganowska *et al*. 1999; Jeffery *et al*. 1999), and directed almost entirely against a single viral protein—Tax. Despite this strong, persistently activated CTL response it is widely believed that HTLV-I is latent. Here we summarize evidence for viral latency, contradictory evidence for persistent transcription and new results that suggest a way in which the contradictory data can be reconciled. We present the first estimate of the rate of turnover of HTLV-I-infected cells and discuss how this affects the view of the role of CTLs in HTLV-I infection.

2. EVIDENCE FOR LATENCY

The current view of HTLV-I is of a rather inactive infection. The virus is thought to be transcriptionally silent, replicating almost entirely by mitosis of the host cell. The evidence for viral latency is as follows.

(i) Viral mRNA is not detectable in the majority of infected PBMCs (Richardson *et al*. 1997).
(ii) Little or no viral protein is detectable in PBMCs (Moritoyo *et al*. 1999).
(iii) There is very little cell-free virus in the plasma. Almost all viral genetic material resides, in DNA form, integrated within the host genome of infected cells (Cann & Chen 1996).
(iv) The viral genome is highly genetically stable. Reverse transcriptase is devoid of any proofreading capabilities, so replication via reverse transcription would be expected to result in many errors. The fact

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that there is so little intrapatient nucleotide sequence diversity (< 0.5% in HTLV-I (Wattel *et al*. 1996) compared with *ca*. 20% in some regions of HIV-l; Pedroza Martins *et al*. 1992) suggests that there is little active viral replication.

In this view of HTLV-I infection, the turnover of provirus-positive cells would be expected to be low, since HTLV-I is not thought to be intrinsically cytopathic, and the rate of lysis by CTLs would be low since the absence of viral protein synthesis would render the virus invisible to the immune response. If the virus is latent it could be argued that a strong CTL response is inappropriate. Indeed, the action of specific CTLs is suggested as one of the possible causes of HAM/TSP; this point is discussed in more detail later.

The prevailing view of HTLV-I dynamics can therefore be summarized as follows.

(i) HTLV-I is largely latent.
(ii) As a consequence of viral latency, CTLs are ineffective at controlling viral load.
(iii) The strong, specific CTL response is inappropriate and potentially damaging.

3. EVIDENCE FOR PERSISTENT TRANSCRIPTION

Despite the evidence for viral latency summarized in § 2, there are some elements of HTLV-I infection that are difficult to reconcile with a latent virus.

(i) There is a strong, persistently activated immune response directed against HTLV-I. High levels of specific CTLs (Bieganowska *et al*. 1999; Jeffery *et al*. 1999) and antibodies (Kira *et al*. 1992) are found in the majority of infected individuals. It is not clear how such a strong immune response could be raised and maintained by a transcriptionally silent virus.
(ii) A longitudinal study has identified an accumulation of mutations with time in a single individual (Niewiesk *et al*. 1996). Replication via cellular mitosis is thought to have an error rate of the order of 1 in 10^9—far too low to account for the observed mutations. This suggests that at least in this individual, infectious transmission may be occurring.
(iii) The cytosine analogue lamivudine causes a temporary reduction in viral load (Taylor *et al*. 1999). As lamivudine inhibits reverse transcriptase activity this result again suggests that at least some infectious transmission is occurring.
(iv) Viral budding and reverse transcriptase activity have been detected *in vitro* in long-term IL-2-dependent cell lines (Gessain *et al*. 1990).
(v) *tax/rex* mRNA has been detected in a minority of infected cells (Kinoshita *et al*. 1989; Gessain *et al*. 1991).

4. UNDERSTANDING HTLV-I 'LATENCY': INFECTION IS HIGHLY DYNAMIC

Here we present new experimental and theoretical work that helps to resolve the contradictory evidence regarding HTLV-I latency.

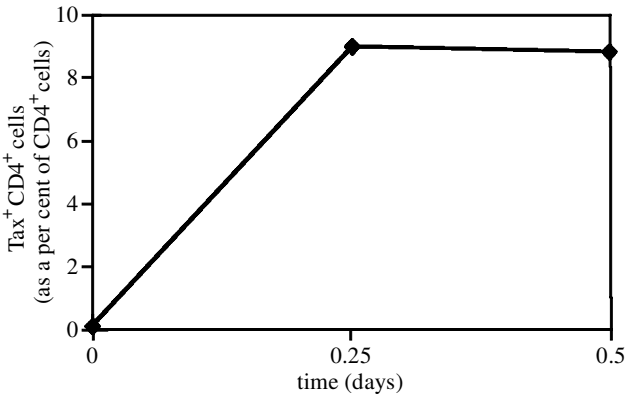

Figure 1. Increase of Tax-positive $CD4^+$ cells (as a percentage of $CD4^+$ cells) with time *ex vivo*.

(a) *Experimental design*

Fresh PBMCs were obtained from HTLV-I-infected patients and the expression of the intracellular viral protein Tax (against which most of the cellular immune response is directed) was quantified using a sensitive flow cytometric technique (Hanon *et al*. 2000*a*). The number of $CD4^+$ cells expressing Tax was measured against time under a number of different circumstances:

(i) in cultivated PBMC;
(ii) in cultivated whole blood (anticoagulated);
(iii) in PBMC that had been depleted of $CD8^+$ cells.

Details of the experimental procedures are published in Hanon *et al*. (2000*a*). In all cases cells were cultured in the absence of IL-2 and mitogens.

(b) *Results*

(i) *Tax expression in PBMCs*

In all five individuals studied, there was a rapid increase during the first 6–12 h of *in vitro* culture in the number of cells expressing Tax. Typically, in freshly isolated PBMCs, the level of Tax expression was very low—often undetectable (< 0.1% of PBMCs). The level of Tax expression then rose to a peak at about 6 h; the result from a representative experiment is shown in figure 1.

The increase in Tax-expressing cells could not be accounted for by proliferation of cells during the short-term *in vitro* culture (Hanon *et al*. 2000*a*).

Both the existence of and the increase in viral mRNA was confirmed by reverse transcriptase polymerase chain reaction (PCR).

Intracellular antibody staining for p24, a protein encoded by the HTLV-I gag gene demonstrated that a large proportion (51%) of Tax-positive cells also expressed p24 after *in vitro* culture for 24 h.

Comparison of the per cent of Tax-expressing cells with the proviral load (estimated by semi-quantitative PCR) indicates that 10–80% of provirus-positive cells start to express Tax *in vitro* within 6–12 h (Hanon *et al*. 2000*a*).

These data are consistent with results (Gessain *et al*. 1990) showing p53, p24 and p19 positivity by Western blot; p15 and p19 positivity by indirect immunofluoresence; a high density of retroviral particles and some budding (by electron microscopy); and a low level of reverse transcriptase activity in HTLV-I infected cells *in vitro*. It should be noted that the results of Gessain *et al*.

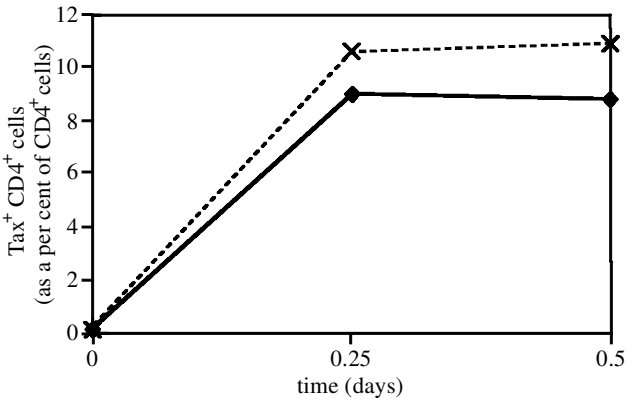

Figure 2. Increase of Tax-positive $CD4^+$ cells (as a percentage of $CD4^+$ cells) with time *ex vivo*. Crosses denote PBMCs from which $CD8^+$ cells were depleted; filled diamonds denote whole PBMCs.

(1990), unlike the ones presented here, were obtained using IL-2-dependent, phytohaemagglutinin-stimulated long-term cell lines.

(ii) *Tax expression in whole blood*

One possible explanation for the increase in Tax expression in PBMCs observed during short-term culture was that by isolating PBMCs, serum factors that normally suppressed antigen induction *in vivo* (Tochikura *et al.* 1985) were being removed. We tested this hypothesis by assaying Tax expression in whole blood that had been anticoagulated with either heparin or EDTA. If anything, the fraction of $CD4^+$ cells expressing Tax was increased in whole blood compared with PBMCs. Similar results were obtained whether the blood was treated with heparin or EDTA. Furthermore, this result also rules out an increase in Tax expression *ex vivo* due to mitogenic properties of the fetal calf serum in which the PBMCs were cultivated. All other experiments described here and in Hanon *et al.* (2000*a*) were obtained using the same batch of foetal calf serum.

(iii) *Tax expression in PBMCs depleted of $CD8^+$ cells*

Another possible explanation for the high levels of Tax expression that are seen *in vitro* but not *in vivo* was that the culture condition led to an increase in the spatial separation of infected cells and effector CTLs, thus reducing the rate of lysis of infected cells *in vitro*. This possibility was tested by reducing the density of $CD8^+$ cells and observing the effect on Tax expression.

In samples from which $CD8^+$ cells had been depleted there was an increase in Tax expression in $CD4^+$ cells. A typical data set is shown in figure 2.

To confirm that $CD8^+$ cells caused lysis of infected cells (rather than reduced Tax expression via some other inhibitory mechanism) two sets of experiments were conducted; the first tested the effects of concanamycin A (CMA) and the second tested the effects of γ-interferon (IFN-γ).

CMA was added to whole PBMCs. CMA is known to inhibit perforin-mediated lysis by destroying intracellular perforin (Kataoka *et al.* 1996). On addition of CMA to whole PBMCs, the frequency of Tax-expressing cells rose to levels similar to those caused by selective depletion of $CD8^+$ cells (Hanon *et al.* 2000*a*). Staining of PBMCs with propidium iodide after short-term *in vitro* culture showed that Tax-positive cells were 40 times more likely to die than Tax-negative cells during this period of culture; this ratio was reduced tenfold by the addition of CMA (Hanon *et al.* 2000*a*).

When excess neutralizing IFN-γ mAbs were added to whole PBMCs, there was no effect on the level of Tax expression (E. Hanon, unpublished observation). When recombinant IFN-γ was added to purified $CD4^+$ cells, this again had no effect on the level of Tax expression (E. Hanon, unpublished observation).

These results demonstrate that the observed reduction of Tax expression in the presence of $CD8^+$ cells *in vitro* is more likely to be caused by CTL-mediated lysis than by the action of IFN-γ produced by the $CD8^+$ cells.

(c) *Rate of lysis of Tax-expressing cells*

The above results suggested to us that the low levels of viral protein expression detected in freshly isolated PBMCs were not due to viral latency but rather to the elimination of Tax-expressing cells by an efficient CTL response. This led us to try to quantify the rate of lysis of Tax-expressing cells *in vitro*.

The experimental data obtained over the first 12 h *ex vivo* were fitted to the following simple model:

$$\frac{dy}{dt} = c - \varepsilon z y.$$

y represents the number of Tax-expressing $CD4^+$ cells (measured as a fraction of all $CD4^+$ cells). c represents the rate of increase of Tax-expressing cells in the absence of lysis by $CD8^+$ cells; this was assumed to be constant within each of the 6-h culture periods 0–6 h and 6–12 h; intermediate data points (not shown) suggest that this is a reasonable assumption. ε denotes the rate of lysis of Tax-expressing cells by $CD8^+$ cells and z denotes the number of HTLV-I specific CTL. εz was assumed to be constant within each of the 6-h periods. The model was fitted separately to the data from 0–6 h and from 6–12 h and an average εz calculated over 12 h. This approach was adopted, rather than attempting to fit the model continuously, because of a paucity of data points and an incomplete understanding of the underlying dynamics. The results obtained are summarized in table 1.

The limited number of data points available made it impossible to calculate confidence intervals on the estimates of εz, so instead we considered the impact of changes in the assumptions made in the model, and errors in the experimental data. The hypothesis that Tax expression is seen *in vitro* but not *in vivo* because of a reduced rate of CTL-mediated lysis *in vitro*, implies that these estimates of εz will be underestimates of the *in vivo* value. We were therefore interested in obtaining a lower bound on εz. If Tax expression rises to a peak before 6 h, then a lower estimate of εz will be obtained. For this reason we also calculated εz making the highly conservative assumption that Tax expression reaches its peak after only 1 h and then remains constant; values of εz calculated under this assumption are shown in brackets in table 1. The limited data on Tax expression that we have suggests that it does not rise so rapidly. The values in brackets therefore represent an approximate lower bound on εz.

Table 1. *Estimates of death rate (due to CTL lysis) and half-life of Tax-expressing cells*

	εz (day^{-1})	half-life of infected cells (days)[a]
patient 1 (TAU)	4.1 ± 0.5 (3.4)	0.2
patient 2 (TW)	1.0 ± 0.2 (0.5)	0.7
patient 3 (HT)	1.5 ± 0.2 (0.8)	0.5
patient 4 (TBA)	0.8 ± 0.3 (0.5)	0.9
patient 5 (HAP)	0.5 ± 0.3 (0.3)	1.4
mean	1.6	0.7
standard deviation	1.5	0.5

[a] Half-life calculated considering CTL clearance only; natural death and direct impact of infection on death rate are not included.

The errors shown on εz are those that would result from a 5% error in the measurement of Tax expression at 6 and 12 h. This and further error analysis demonstrates that these estimates of εz are highly sensitive to experimental error.

At present there is no accurate and reliable means of estimating the total number of HTLV-I specific CTLs so it was not possible to estimate ε separately.

The sensitivity to error and shortage of data indicate that these figures should be considered only as rough estimates. They indicate that the half-life of Tax-positive cells *in vitro* is in the order of days. Our postulate that the Tax expression seen *in vitro* is due to reduced CTL efficiency implies that the half-lives calculated here will be overestimates of the *in vivo* half-lives.

These calculations represent the first attempt to quantify the rate of CTL-mediated lysis in HTLV-I infection. The death rate of Tax-expressing cells due to CTLs is *ca*. 2 day^{-1}. Comparing this with the death rate of 0.008 day^{-1} of CD4^{+} T lymphocytes in uninfected individuals (Hellerstein *et al*. 1999) suggests that CTLs have a profound effect on the life span of Tax-expressing cells, reducing it from about 125 days to less than a day. It is likely that CTL-mediated lysis of infected cells occurs almost exclusively in solid lymphoid tissue since there will be a much higher rate of contact between infected cells and specific CTLs there.

If these data are representative of the rate of CTL-mediated lysis *in vivo*, what is the estimated rate of turnover of HTLV-I-infected cells *in vivo*? In a typical HTLV-I-infected person, proviral load ranges between 0.5 and ten copies of proviral DNA per 100 PBMCs. Assuming one copy of proviral DNA per cell, this indicates that 0.5–10% of PBMCs are infected. Out of these infected cells *ca*. 10–80% express Tax within one day. If the level of Tax-expression, HTLV-I infection frequency and CTL lytic activity in peripheral lymphocytes is assumed to be roughly representative of all lymphocytes in the body (*ca*. 10^{12} cells), then cell turnover due to CTL-mediated lysis can be estimated at 10^{9}–10^{11} cells day^{-1}. This must be regarded as a preliminary estimate since it is based on a relatively small amount of *in vitro* data.

(d) *Interpretation of results*

The ability of a large number of infected cells to spontaneously express viral proteins *ex vivo* indicates that HTLV-I may not be latent *in vivo*. Of course caution must be exercised in extrapolating *in vitro* results and further *in vivo* confirmation of persistent viral gene expression and elevated cell turnover is needed. The evidence for the very efficient lysis of Tax-expressing cells by CTLs *in vitro* provides a possible explanation for the low level of viral proteins detected in fresh PBMCs. We speculate that the strong anti-Tax CTL response *in vivo* eliminates infected cells as viral proteins start to be expressed. That is, we suggest that efficient immune surveillance rather than viral latency is the reason that only very low levels of viral mRNA and viral proteins can be detected in fresh PBMCs. Similarly, the low level of sequence diversity could arise not because the virus is latent, but because the level of infectious cell–cell transmission is limited by an efficient CTL response and perhaps by an intrinsically low rate of infectivity of the virus (Cann & Chen 1996). An additional factor that might cause the low rate of infectious transmission has been suggested (Wodarz *et al*. 1999).

We suggest that the high levels of Tax expression that are seen *ex vivo* are because culture conditions have reduced CTL efficiency by increasing spatial separation of target and effector cells, so increasing the number of Tax-expressing cells that escape lysis.

5. SPECIFIC CTL IN HTLV-I INFECTION

As mentioned earlier, CTLs are generally thought to be ineffective at controlling HTLV-I infection and possibly to play a role in the pathogenesis of HAM/TSP. The most likely mechanisms for CTL damage are as follows.

(i) Inflammatory cytokines such as tumour necrosis factor-α (TNF-α) or IFN-γ produced by activated, HTLV-I-specific CTLs in the CNS might cause demyelination (Biddison *et al*. 1997).
(ii) CTLs that cross-react between an HTLV-I antigen and a self-antigen might destroy cells in the CNS.
(iii) HTLV-I might infect cells of the CNS, which are then destroyed by the specific CTL response.

The results presented here support a different view of the effects of HTLV-I-specific CTLs. While not disproving a part for CTLs in the pathogenesis of HAM/TSP, they do suggest that CTLs limit the viral burden in infected individuals. Because a high proviral load of HTLV-I is correlated with disease, CTLs may help to reduce the risk of disease. This is in line with work that demonstrates that an efficient CTL response may therefore help to protect against HAM/TSP. The evidence for a protective role for CTLs is summarized below.

(i) It has been reported (Jeffery *et al*. 1999) that, given HTLV-I infection, the class I allele HLA-A2 halves the odds of developing HAM/TSP. Among healthy carriers (HCs), individuals who are A2 positive have a proviral load only one-third of that of individuals who are A2 negative. It was therefore postulated that individuals who are A2 positive have a more efficient antiviral CTL response that is able to reduce proviral load and help to prevent disease.
(ii) The ratio of coding changes (nucleotide changes that alter the amino-acid sequence) to non-coding

changes in *tax* is significantly higher in HCs than in HAM/TSP (Niewiesk *et al*. 1994, 1996). This indicates that there is a higher selection intensity for amino-acid change in HCs than in HAM/TSP. We suggest that the most significant force for amino-acid change is anti-Tax CTLs, so this result implies that CTLs are actually exerting a stronger selection pressure on virus in HCs than in HAM/TSP. Again this suggests that HCs have a more efficient CTL response than individuals with HAM/TSP.

(iii) A theoretical model has been proposed (Nowak & Bangham 1996), which explains the protective role of CTLs in persistent viral infections. This model states that an efficient CTL response (defined as one in which there is a high rate of proliferation of CTLs on meeting its target antigen) lowers the virus load. Furthermore, it demonstrates that there need not be a large difference in the magnitude of the CTL response (i.e. in the number of specific CTLs) between an efficient responder and an inefficient responder.

6. T-CELL TURNOVER AND IMMUNE SUPPRESSION

Despite the apparently high rate of destruction of $CD4^+$ T lymphocytes, HTLV-I-infected individuals do not develop the severe immune suppression seen in AIDS, although there is some evidence (Marsh 1996; Mueller & Blattner 1997; Jacobson *et al*. 1990) of limited immune suppression in HTLV-I-infected individuals. Since the development of AIDS is incompletely understood, it is not possible to give a full explanation for this different outcome in HTLV-I and human immunodeficiency virus-1 (HIV-1) infection, but some tentative suggestions can be made. HTLV-I infection does not result in the decline in $CD4^+$ T-cell numbers that is seen in HIV-1 infection, even though the destruction of infected cells appears to proceed at a similar rate in both infections. This suggests that the input of $CD4^+$ T cells in HTLV-I infection is greater than in HIV-1 infection. There are at least two possible factors that could lead to greater CD4 T-cell production in HTLV-I-infected individuals than in HIV-1-infected individuals. First, there is evidence that the production of new, naive T cells is impaired in HIV-1 infection (Douek *et al*. 1998), i.e. that the generation of lymphocyte precursors in the bone marrow and/or thymic function is adversely affected by HIV-1 infection. Second, HTLV-I infection stimulates the proliferation of both infected and uninfected bystander $CD4^+$ T cells (Wucherpfennig *et al*. 1992) and is associated with protection against apoptosis (Copeland *et al*. 1994); conversely HIV-1 infection frequently results in apoptosis (Amiesen & Capron 1991) or anergy (Pinching 1988) of $CD4^+$ cells. So it would seem that the production of new T cells from the thymus and/or by proliferation of existing T cells is greater in HTLV-I infection than in HIV-1 infection. This may help to explain why a decrease in the $CD4^+$ T-cell population is not observed in HTLV-I infected individuals. Furthermore, HIV-1 infection is known to impair the function of infected helper cells (Lane *et al*. 1985; Miedema *et al*. 1988), which could affect the generation of new helper cells and CTLs, further damaging the immune system.

7. SUMMARY

The data presented here indicate that HTLV-I might be a far more dynamic infection than previously thought. If the very short half-life of Tax-expressing cells *in vitro* is representative of the *in vivo* situation, then infected cells are turning over at a rate of approximately 10^9–10^{11} infected cells per day. Furthermore, CTLs, far from being ineffective, significantly reduce viral load *in vitro* and possibly *in vivo*. We conclude that the apparent rate of HTLV-I protein expression is low not because HTLV-I is latent, but because the rate of production and the rate of clearance of cells expressing viral proteins is closely matched, so the equilibrium level of actively reproducing virus is low.

To summarize, our results suggest the following.

(i) HTLV-I is not latent. A large fraction of provirus-positive cells start to express viral proteins within one day.

(ii) A highly efficient CTL response rapidly eliminates infected cells once they start to express viral proteins. For this reason, cell-free virus, viral mRNA and viral protein are often below detection limits and infectious transmission is limited (this explains the relative lack of sequence diversity).

(iii) High levels of Tax expression are seen *ex vivo* because culture conditions reduce CTL efficiency (by increasing spatial separation of target and effector cells) so increasing the number of Tax-expressing cells that escape lysis.

(iv) CTLs reduce the proviral load *in vitro* and possibly *in vivo*. This is supported by work showing that an individual with an efficient CTL response is less likely to develop disease than an individual with a weaker CTL response.

(v) HTLV-I is a highly dynamic infection with infected cells turning over at a rate of approximately 10^9–10^{11} cells per day.

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