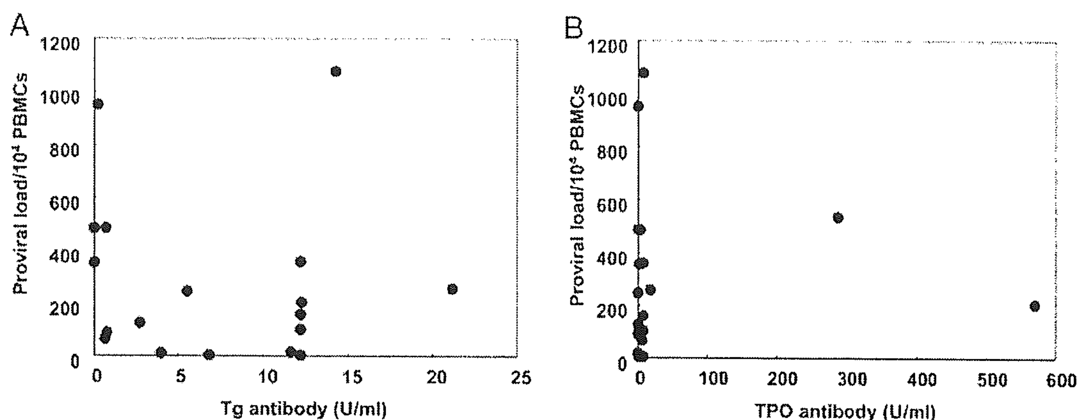

FIG. 2. Correlation between HTLV-I proviral load and antibody titer of Tg (A) or TPO (B) in HTLV-I-infected patients with HT. There was no significant correlation between copy number of HTLV-I proviral DNA and antibody titer of either Tg ($P = 0.6535$) or TPO ($P = 0.4703$).

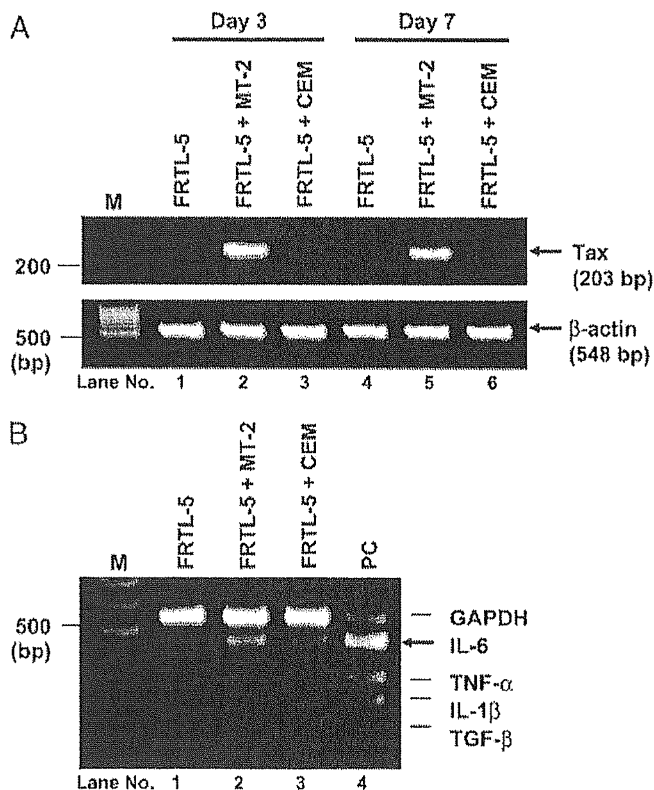

FIG. 3. HTLV-I can infect FRTL-5 cells and induce gene expression of IL-6. A, Detection of HTLV-I Tax mRNA in FRTL-5 cells by RT-PCR. FRTL-5 cells were cocultured with MMC-treated MT-2 or CCRF-CEM cells. At 3 and 7 d after cocultivation, FRTL-5 cells were harvested and then Tax mRNA expression was analyzed. Lane 1, cultured FRTL-5 cells at 3 d; lanes 2 and 3, FRTL-5 cells cocultured with MT-2 and CCRF-CEM cells at 3 d; lane 4, cultured FRTL-5 cells at 7 d; lanes 5 and 6, FRTL-5 cells cocultured with MT-2 and CCRF-CEM cells at 7 d. Human β-actin mRNA was used as a control. B, Induction of expression of IL-6 gene in FRTL-5 cells. Lane 1, cultured FRTL-5 cells at 3 d; lanes 2 and 3, FRTL-5 cells cocultured with MT-2 and CCRF-CEM cells at 3 d; lane 4, positive control. Rat GAPDH mRNA was used as a control.

Cytokine expression in FRTL-5 cells cocultured with MT-2 or CCRF-CEM cells

Tax activates not only the transcription of the viral genome but also the expression of various cellular genes. It is now clear that HTLV-I-infected T cells are capable of producing various cytokines through the transactivation of cytokine genes by the Tax protein (27). HTLV-I-infected nonlymphoid cells have also been reported to express various types of cytokines (28, 29). Therefore, we investigated the expression of inflammatory cytokines in FRTL-5 cells cocultured with MT-2 or CCRF-CEM cells by RT-PCR. RT-PCR was carried out with primer sets for IL-1β, IL-6, TNFα, and TGFβ as well as rat GAPDH. As shown in Fig. 3B, low levels of expression of IL-6 and TGFβ mRNA were detected in control FRTL-5 cells. The level of expression of IL-6 was increased in FRTL-5 cells cocultured with MT-2 cells but not in FRTL-5 cells cocultured with CCRF-CEM cells. Transcripts of IL-1β and TNFα were not detected in any of the samples.

Proliferation of FRTL-5 cells

It was reported previously that HTLV-I could infect synovial cells, resulting in their active proliferation (28). Finally, to investigate the relation of thyroid cell proliferation and HTLV-I infection, the proliferative response of FRTL-5 cells was examined by cocultivation with MT-2 cells and compared with that of FRTL-5 cells cocultured with CCRF-CEM cells using the WST-8 assay as an index of cell number. The proliferation of FRTL-5 cells at d 1, 3, and 5 was significantly increased by coculture with HTLV-I-infected T cells (Fig. 4). It was noted that MMC-treated MT-2 and CCRF-CEM cells could not proliferate.

Discussion

Although the etiology of autoimmune thyroid diseases has yet to be established, it appears to result from complex interactions between host genetic and environmental factors. The involvement of viral infection, particularly retrovirus infection, in the pathogenesis of autoimmune thyroid diseases has been demonstrated in animals and humans (30–34). However, the role of infection in precipitating autoimmune thyroid diseases remains largely hypothetical (34).

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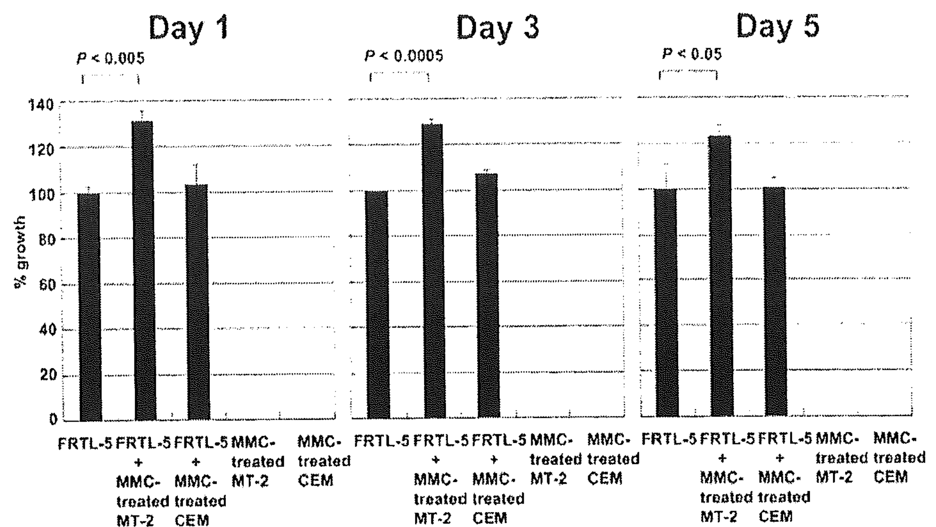

FIG. 4. Proliferation of FRTL-5 cells cocultured with MT-2 or CCRF-CEM cells. FRTL-5 cells were cultured in the presence or absence of MMC-treated MT-2 or CCRF-CEM cells for the indicated time periods. Four hours before terminating the culture, WST-8 was added to each well, and absorbance at 450 nm was measured. Data are expressed as percentage growth, compared with the uninfected FRTL-5 cells and represent the mean ± SD of triplicate measurements. MMC-treated MT-2 and CCRF-CEM cells could not proliferate.

Ciampolillo *et al.* (31) reported the presence of a HIV type 1-related DNA sequence in the thyroid and PBMCs of the patients with GD, but this finding was disputed by Humphrey *et al.* (35) and Tominaga *et al.* (36). Jaspan *et al.* (37) reported that over 85% of patients with GD have detectable serum antibodies against a human intracisternal type A retroviral particle. Furthermore, data from 35 members of three kindreds suggest that both human leukocyte antigen susceptibility and exposure to the retroviral particle are necessary for the development of GD (38). HTLV-I is considered to be implicated in the pathogenesis of autoimmune thyroid diseases in Japan, where this retrovirus is endemic, and epidemiological studies have shown an association between HTLV-I infection and thyroid disorders in Japan (12, 15–17).

The present study provides biological data suggesting the contribution of HTLV-I in the development of autoimmune thyroid diseases. Our results showed that: 1) the circulating HTLV-I proviral load was higher in HTLV-I-seropositive patients with HT or GD than asymptomatic HTLV-I carriers and lower than that in patients with HAM/TSP or ATL; 2) HTLV-I can be transmitted into thyroid cells from an HTLV-I-producing T cell line; 3) HTLV-I infection induced expression of IL-6 gene but not IL-1β, TNFα, and TGF-β in thyroid cells; and 4) HTLV-I-infected thyroid cells proliferated more actively than control cells.

The HTLV-I proviral load is thought to be a major determinant of HTLV-I-associated diseases. The proviral load is higher in the peripheral blood of patients with HAM/TSP than blood of asymptomatic carriers (21), as confirmed in the present study. It is also higher in the peripheral blood of patients with HTLV-I-associated uveitis and HTLV-I-seropositive patients with arthritis or connective tissue disease than asymptomatic carriers (39, 40). Similarly, we observed a significantly higher proviral load in HTLV-I-infected patients with either HT or GD than in HTLV-I asymptomatic carriers. Thus, a high proviral load might be involved in the pathogenesis of several other HTLV-I-associated inflammatory disorders in addition to HAM/TSP.

The unusually high proviral loads in HTLV-I infection results mainly from the Tax-driven activation and expansion of infected cells (41). The HTLV-I targets are mainly CD45RO-expressing CD4+ T lymphocytes, and the proviral load is reported to correlate with the number of memory T cells (42). Migration of HTLV-I-infected CD4+ T cells and HTLV-I-specific CD8+ cytotoxic T lymphocytes into the central nervous system is a critical step in the pathogenesis of HAM/TSP (43). Similarly, infiltration of lymphocytes plays a central role in the initiation and perpetuation of autoimmune thyroid diseases. Previous studies showed a good correlation between the degree of intrathyroidal lymphocytic infiltration and antithyroid antibody titer not only in HT (44) but also in GD (45). Although the accumulation of HTLV-I-infected T cells in the thyroid remains uncertain, HTLV-I proviral load did not correlate with antibody titer of either TPO or Tg in our study with HT. Further research using thyroid tissue from HTLV-I-infected patients is needed to support the hypothesis of the pathogenic involvement of HTLV-I-infected T lymphocytes.

HTLV-I might be transmitted from infiltrated lymphocytes to thyrocytes. We obtained evidence that thyroid cells can be infected by HTLV-I and that this infection induced gene expression of inflammatory cytokine IL-6 *in vitro*. HTLV-I Tax mRNA was detected in the FRTL-5 cells cocultured with MT-2 cells. Transcription of IL-6 is regulated by Tax protein in T cells and synovial cells (46, 47). Although the precise role of IL-6 in the pathogenesis of thyroid diseases is unknown, these results suggest the involvement of IL-6 expression in thyroid cells, which is related to Tax, in the development of inflammatory lesions caused by HTLV-I infection in the thyroid. To clarify the pathological association of thyroiditis with HTLV-I, we are attempting to detect HTLV-I proviral DNA and viral gene expression in the tissue of HTLV-I-associated thyroiditis.

The effect of HTLV-I infection on FRTL-5 growth was assessed by the WST-8 assay. Coculture with MT-2 cells increased the rate of cell proliferation. Because these effects were not observed in FRTL-5 cells cocultured with CCRF-CEM cells, they support the specific effect of HTLV-I infection on thyroid cell growth. Although several cytokines are known to modulate the proliferation of FRTL-5 cells, IL-6 had

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no significant effects on the cell growth (48). Because Tax can stimulate cell growth, the active proliferation of HTLV-I-infected thyroid cells may be related to Tax expression, and goiter in patients with autoimmune thyroid diseases may be regulated by HTLV-I infection.

HTLV-I might cause a systemic immune-mediated inflammatory disease potently involving tissues other than the central nervous system, HAM/TSP being only the major syndrome. The pathological association of HTLV-I with autoimmune thyroid diseases in HTLV-I carriers still remains to be clarified. It should be noted that HTLV-I infection is not the sole cause of autoimmune thyroid diseases because HTLV-I antibody was not present in the majority of the cases. Genetic factors, involved in autoimmune thyroid diseases, include human leukocyte antigen and cytotoxic T lymphocyte antigen-4 (CTLA-4) (49, 50). It has been shown that HTLV-I infection is not associated with CTLA-4 polymorphisms in either HT or controls (51). HTLV-I infection is not regulated by genetic factor such as CTLA-4 and may affect occurrence of HT as an independent, purely environmental factor. Further studies on the effects of HTLV-I infection of thyroid tissues should help elucidate the pathobiology and pathogenesis of HTLV-I-associated thyroid diseases.

Acknowledgments

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Cellular immune response to HTLV-1

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There is strong evidence at the individual level and the population level that an efficient cytotoxic T lymphocyte (CTL) response to HTLV-1 limits the proviral load and the risk of associated inflammatory diseases such as HAM/TSP. This evidence comes from host population genetics, viral genetics, DNA expression microarrays and assays of lymphocyte function. However, until now there has been no satisfactory and rigorous means to define or to measure the efficiency of an antiviral CTL response. Recently, methods have been developed to quantify lymphocyte turnover rates *in vivo* and the efficiency of anti-HTLV-1 CTLs *ex vivo*. Data from these new techniques appear to substantiate the conclusion that variation between individual hosts in the rate at which a single CTL kills HTLV-1-infected lymphocytes is an important determinant, perhaps the decisive determinant, of the proviral load and the risk of HAM/TSP. With these experimental data, it is becoming possible to refine, parameterize and test mathematical models of the immune control of HTLV-1, which are a necessary part of an understanding of this complex dynamic system.
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Keywords: cytotoxic T lymphocyte; HTLV-1; genetics; microarray; virological synapse; lymphocyte dynamics; HAM/TSP; host defence

Introduction

Three broad questions have driven research into the immune response to HTLV-1. First, how does HTLV-1 persist in the individual host? In particular, what is the role of the immune response in controlling or limiting viral persistence? Second, why do some HTLV-1-infected people develop a consequent inflammatory disease such as HAM/TSP, whereas the majority remain asymptomatic carriers of the virus? Is this difference in the outcome of infection due primarily to variation in the host or variation in the virus? Third, how is the inflammatory lesion in HAM/TSP initiated and maintained, and how can the inflammation be halted? In this review, we shall first summarize briefly the progress that has been made in answering each of these questions during the last two decades. We then review the more recent work that is attempting to resolve some of the outstanding issues. We conclude that the efficiency of a person's CTL response to HTLV-1 plays a dominant role in determining the proviral load of HTLV-1 and the risk of the associated inflammatory diseases.

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How does HTLV-1 persist in the host?

The apparent absence of HTLV-1 virions, mRNA or protein in circulating PBMCs in the majority of HTLV-1-infected people led to the conclusion that the proviral load of HTLV-1, which may reach more than 30% of peripheral blood mononuclear cells (PBMCs) and more than 50% of CD4$^+$ cells, is maintained mainly by proliferation of provirus-containing cells (Cavrois *et al.*, 1996; Etoh *et al.*, 1997; Eiraku *et al.*, 1998), rather than full-cycle virus replication mediated by reverse transcriptase. This conclusion was corroborated by the relative lack of sequence variation in HTLV-1 both within and between isolates (Daenke *et al.*, 1990; Kinoshita *et al.*, 1991; Komurian *et al.*, 1991; Slattery *et al.*, 1999), which appeared to exclude a major role of the error-prone reverse transcriptase in maintaining the proviral load.

However, in the absence of malignant transformation, continued mitosis of HTLV-1-positive cells must be driven by expression of HTLV-1 genes. Persistent expression of mRNA was detected in a low proportion (~1/5000) of cells in some individuals by RT–PCR (Gessain *et al.*, 1991), but the importance of such expression remained unclear. The discovery of a chronically activated cytotoxic T lymphocyte (CTL) response to HTLV-1, particularly against the Tax protein (Jacobson *et al.*, 1990; Kannagi *et al.*, 1991; Parker *et al.*, 1992; Goon *et al.*, 2004b), and a high titre of anti-HTLV-1 antibody, which often includes IgM (Nagasato *et al.*, 1991; Kira *et al.*, 1992; Ishihara *et al.*, 1994), strongly supported the idea that there was persistent expression of HTLV-1 proteins. Why, then, is HTLV-1 protein expression in fresh PBMCs low or undetectable (Moritoyo *et al.*, 1999; Hanon *et al.*, 2000a)? An important clue came from the demonstration by Hanon *et al.* (2000a) that CD4$^+$ T cells freshly isolated from an HTLV-1-infected person spontaneously express HTLV-1 proteins – initially Tax protein,

then Gag. This expression reaches a peak after about 12 h of incubation. Readdition of the CD8$^+$ T cells – which include CTLs – from the same blood sample reduced the expression of Tax in a dose-dependent manner (Hanon *et al.*, 2000a). This observation suggested that efficient surveillance by anti-HTLV-1 CTLs *in vivo* might suppress the frequency of Tax-expressing cells in the circulation to a level below the limit of detection by flow cytometry (Bangham, 2003).

The notion that HTLV-1-specific CTLs might play a decisive role in limiting HTLV-1 gene expression (and therefore the proviral load) *in vivo* was consistent with the finding of Niewiesk *et al.* (1994) and Niewiesk and Bangham (1996) that the Tax gene of HTLV-1 was subject to positive selection, especially in healthy asymptomatic carriers of the virus. Tax protein is the dominant target of HTLV-1-specific CTLs (Jacobson *et al.*, 1990; Kannagi *et al.*, 1991; Parker *et al.*, 1992; Goon *et al.*, 2004b), and the only plausible force that has been identified which might exert positive selection on the Tax gene is the strong anti-Tax CTL response. Indeed, naturally occurring sequence variants of HTLV-1 Tax encode epitopes that escape autologous CTL recognition (Niewiesk *et al.*, 1995), which is consistent with CTL-mediated selection on the Tax gene. Such CTL escape variants of Tax are, however, usually defective in Tax transcriptional activity (Niewiesk *et al.*, 1995).

The following picture of HTLV-1 persistence has emerged from recent experimental work (Asquith *et al.*, 2000; Overbaugh and Bangham, 2001; Bangham, 2003). Cells that carry HTLV-1 spontaneously start to transcribe the provirus *in vivo*; Tax expression drives mitosis of the infected CD4$^+$ T cells and maintains the proviral load. However, HTLV-1-expressing CD4$^+$ T cells are usually killed by autologous CTLs before they complete the replication cycle of HTLV-1. A small proportion of infected cells do complete the replication cycle; such cells can infect other T-cells directly through a cell-contact-triggered 'virological synapse' in a remarkable subversion of normal T-cell physiology (Igakura *et al.*, 2003). Transmission of HTLV-1 via this 'infectious' route may cause the observed preferential infection of T-cells that recognize HTLV-1 antigens (Hanon *et al.*, 2000b; Goon *et al.*, 2004a). Cells that express CTL escape variants of Tax have a survival advantage, but such cells do not outgrow the cells that express the wild-type Tax sequence because the CTL escape mutations also reduce the efficacy of Tax protein in transactivating the host cell pathways responsible for T-cell proliferation. In this way, an equilibrium is established (Nowak and Bangham, 1996) between the virus, which is persistently attempting to replicate, and the immune (largely CTL) counter-attack. If this picture of a dynamic equilibrium is correct, what are the implications? Here we suggest six such implications, and examine recent evidence for each:

(i) HTLV-1-specific CTLs must be highly active in killing HTLV-1-infected lymphocytes *in vivo*.
(ii) Individuals with a high frequency of HTLV-1-specific CTLs have a strong anti-HTLV-1 response.
(iii) Differences in proviral load – and associated differences in the risk of inflammatory diseases such as HAM/TSP – should be associated with differences between individuals in the efficiency of CTL-mediated surveillance against HTLV-1, that is, in the rate of CTL-mediated lysis of infected cells.
(iv) Individual differences in CTL efficacy are likely to be genetically determined, because each person appears to have their own 'set point' of proviral load. Therefore, genetic polymorphisms should be associated with individual differences in proviral load and/or in the risk of inflammatory diseases such as HAM/TSP.
(v) HTLV-1-infected T cells should have a short life expectancy *in vivo*.
(vi) In addition to frequent spontaneous onset of Tax transcription, there must also be a certain frequency of suppression of Tax transcription, albeit lower than the rate of onset of Tax transcription (see below).

HTLV-1-specific CTLs must be highly active in vivo

Following the first identification of HTLV-1-specific CTLs (Kannagi *et al.*, 1983, 1984), Jacobson *et al.* (1990) and subsequently Parker *et al.* (1992, 1994) showed that *ex vivo* CTLs were active against HTLV-1-infected cells. The specific prediction (Nowak and Bangham, 1996) that CTLs are especially active in individuals with a low proviral load, in whom CTL surveillance is putatively more effective, has recently been confirmed by Vine, Heaps and others (Vine *et al.*, 2004). These authors used DNA expression microarrays to study the expression of over 12 000 genes in unstimulated T cells taken *ex vivo* from HTLV-1 infected subjects and uninfected controls. A single small cluster of genes distinguished individuals with a low proviral load from those with a high load. This cluster contained between 9 and 40 genes (Figure 1) that were highly expressed in CD8$^+$ cells in individuals with a low proviral load. No other significant gene clusters were found either in CD4$^+$ cells or CD8$^+$ cells. Remarkably, this highly expressed gene cluster consisted mainly of genes that encode proteins known to be involved in the effector mechanisms of CTL-mediated lysis, including granzymes, perforin, granulysin and NKG2D. Thus, a high level of expression of lymphocyte lysis-related genes was associated with effective control of proviral load, both in asymptomatic HTLV-1 carriers and patients with HAM/TSP.

A high frequency of HTLV-1-specific CTLs should be associated with efficient suppression of HTLV-1 replication, that is, with a low proviral load

This apparently straightforward implication (Ogg *et al.*, 1998) is directly contradicted by the experimental data,

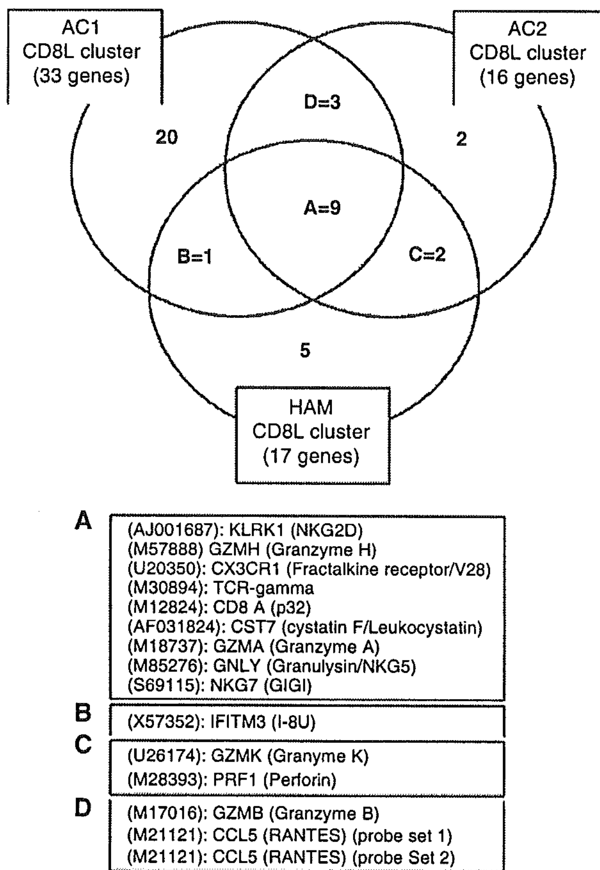


Figure 1 Venn diagram showing the number of genes that occurred in each CD8L cluster and the number and identity of genes that occurred in two or more CD8L clusters. In the three experiments, 27, 56 and 53% of genes present in the respective CD8L cluster occurred in this cluster in all three experiments: these shared genes are shown in the central white 'core' group ($n = 9$ genes). In both this core group and the genes ($n = 6$) shared by two experiments (gene groups B–D), there was a strong predominance of genes that encode proteins that mediate cellular cytotoxicity (granzymes, perforin, granulysin), T-cell Ag recognition (TCRγ, CD8a, NKG2D). Of the five remaining probe sets that appeared in the CD8L cluster in two of the three experiments, two (RANTES, NKG7) are also associated with the lytic granule of cytotoxic lymphocytes. Reproduced, by permission of the publisher, from Vine *et al.* (2004)

which show a zero or a weakly positive correlation between proviral load and the frequency of HTLV-1-specific CTLs (Kubota *et al.*, 2000a, b; Betts *et al.*, 2001; Wodarz *et al.*, 2001). This positive correlation is largely responsible for the presence of a higher frequency of HTLV-1-specific CTLs in patients with HAM/TSP than in asymptomatic carriers (Elovaara *et al.*, 1993; Greten *et al.*, 1998), although there may be a slightly higher frequency in HAM/TSP patients at a given proviral load.

However, on closer inspection, the logic of this implication is less clear (Bangham, 2003). A high proviral load will drive replication and activation of HTLV-1-specific CTLs, by providing a strong antigenic stimulus. However, the increased number and activity of CTLs will then more effectively kill HTLV-1-expressing cells, and thereby suppress HTLV-1 replication and lower the proviral load. In turn, the antigenic stimulus will diminish, and so the frequency and activation of specific CTLs will fall. In this case, neither mathematical models nor – still less – intuition can give a robust and reliable prediction where the equilibrium will be struck between the proviral load and CTL frequency. Only one clear conclusion can be drawn here: the frequency of specific CTLs is not a useful or reliable guide to the efficacy of the CTL response to a persistently replicating pathogen at equilibrium.

Differences between individuals in proviral load and the risk of HAM/TSP should be associated with differences in the efficiency of CTL-mediated surveillance

Until recently there has been no means to measure the efficiency of CTL-mediated lysis of virus-infected cells in a natural infection. The chief reason for this is that it has not been possible to obtain and detect both CTLs and autologous target (i.e. virus-infected) cells in or near their natural frequency and state of activation. Recently, however, Asquith *et al.* (2005) have attempted to resolve this difficulty in HTLV-1 infection, in which both the CTLs and HTLV-1-infected CD4$^+$ cells are frequently present at a sufficiently high frequency in peripheral blood that they can be enumerated without enrichment. Asquith *et al.* measured the loss of Tax-expressing CD4$^+$ cells in freshly isolated PBMCs after overnight incubation with different ratios of autologous CD8$^+$ cells. Neither cytokines nor any other stimulus were added to the cells. In principle, this approach amounts to a quantification of the assay developed by Hanon *et al.* (2000a).

The results (Figure 2a) showed that the per-CD8$^+$-cell rate of lysis of Tax-expressing CD4$^+$ cells not only varied widely between individuals but, most importantly, correlated negatively with the proviral load *in vivo*, both within asymptomatic HTLV-1 carriers and patients with HAM/TSP. This analysis is not subject to the difficult circular argument noted above in the correlation between CTL frequency and proviral load: the lysis assay developed by Asquith *et al.* (2005) gives an estimate of the CD8$^+$ cell lytic efficiency at the level of the single cell, not the population of cells. The finding of a significant negative correlation between the CTL lysis rate and proviral load *in vivo* gives strong credence to the idea that this *in vitro* measure reflects a physiologically meaningful activity of the CTLs.

These results indicate that CTLs do indeed reduce the proviral load of HTLV-1 *in vivo*. However by how much is the load reduced? The impact of CTLs has not been quantified in any natural persistent viral infection. Using the results of the *ex vivo* lysis assay, we can estimate the proportion of the observed variation between individuals in HTLV-1 proviral load that is explained by the measured variation in CD8$^+$ cell-mediated lytic rate. Surprisingly, the results show that this proportion lies between 30 and 50% (Asquith *et al.*, 2005). If this is

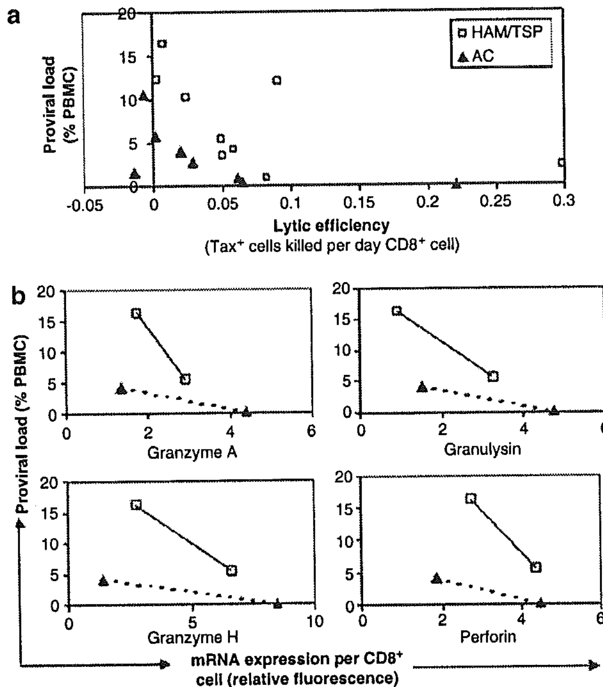


Figure 2 (a) A high rate of destruction of HTLV-1 Tax-expressing cells by $CD8^+$ cell-mediated lysis shows a statistically significant negative correlation with a low proviral load of HTLV-1 *in vivo* in both asymptomatic HTLV-1 carriers (AC) and patients with HAM/TSP. These results suggest that there is effective control of HTLV-1 replication *in vivo* by CTLs. Reproduced, by permission of the publisher, from Asquith *et al.* (2005). (b) The mean intensity of mRNA expression of genes that encode CTL lysis-associated proteins also varies inversely with the proviral load of HTLV-1 *in vivo*. The pattern of variation of mRNA expression intensity, both within and between the two groups of subjects, strongly resembles the pattern of variation in the rate of $CD8^+$ cell-mediated lysis shown in (a). Data on mRNA expression were taken from the DNA microarray study reported by Vine *et al.* (2004)

correct, it appears that the variation in the lytic rate of HTLV-1-specific $CD8^+$ lymphocytes is the largest single determinant of individual variation in proviral load and, therefore, in the outcome of infection with HTLV-1 (Asquith *et al.*, 2005; Bangham *et al.*, 1996). Second, if the measured rate of $CD8^+$ cell-mediated lysis *in vitro* is taken as an estimate of the rate of lysis *in vivo*, the data indicate that each HTLV-1-specific $CD8^+$ T cell kills about five HTLV-1-infected $CD4^+$ cells per day. Since a single CTL can lyse a target cell *in vitro* in 10 min before going on to kill another cell, this estimate seems plausible, even conservative. However, because of the high frequency of infected cells and CTLs, these figures imply that about 10^9 HTLV-1-infected cells are killed per day in a typical HTLV-1-infected host. This surprisingly high figure requires corroboration by an independent experimental technique.

Both this study of CTL-mediated lysis and the DNA expression array study noted above (Vine *et al.*, 2004) show that strong $CD8^+$ cell lytic activity is associated with a low proviral load in asymptomatic HTLV-1 carriers and in patients with HAM/TSP: see Figure 2b. However, a closer inspection of the results obtained by Asquith *et al.* (2005) (Figure 2b) reveals that, at a given rate of $CD8^+$ cell-mediated lysis, the proviral load is systematically higher in patients with HAM/TSP than in asymptomatic carriers. Possible causes of this CTL-independent difference in proviral load include epigenetic factors associated with the HTLV-1 provirus and host differences in the rate of proliferation of HTLV-1-infected $CD4^+$ T cells.

Host genetic polymorphisms determine the efficacy of an individual's immune response to HTLV-1

This hypothesis was tested in a large collaborative study of candidate gene polymorphisms in a population with endemic HTLV-1 infection in Kagoshima, southern Japan (Nagai *et al.*, 1998; Jeffery *et al.*, 1999, 2000; Vine *et al.*, 2002). The power of such candidate gene studies is constrained by the choice of candidate genes, whereas the effectiveness of genome-wide searches for genetic determinants is limited by the low statistical power after correction for multiple statistical tests. However, the results of this study were clear. The strongest identified genetic determinant of proviral was the HLA class 1 genotype (Vine *et al.*, 2002). Specifically, the genes *HLA-A*02* and *HLA-Cw*08* were independently and significantly associated with a lower proviral load and a lower risk of HAM/TSP (Jeffery *et al.*, 1999, 2000). Since the function of class 1 HLA proteins is to present antigenic peptides to CTLs, these results imply that individuals with *HLA-A*02* or *HLA-Cw*08* mount a particularly efficient CTL response against HTLV-1. These data are therefore consistent with the evidence from the functional assay (Asquith *et al.*, 2005, Figure 2a) and the DNA expression analysis (Vine *et al.*, 2004; Figure 1) that CTL efficacy is an important determinant, perhaps the decisive determinant, of HTLV-1 proviral load and therefore of the risk of inflammatory disease such as HAM/TSP.

The significance of the observed protection associated with *HLA-A*02* and *HLA-Cw*08* in Kagoshima lies less in the actual identity of these particular molecules than in the implication that CTLs play an important role in controlling HTLV-1 load. While the frequency of polymorphic alleles and therefore the strength of their influence on HTLV-1 infection will differ significantly between different populations, it seems highly improbable that the fundamental mechanisms of immune control will differ.

A further analysis by Furukawa *et al.* (2000) has cast more light on the protective effect given by *HLA-A*02*. Furukawa *et al.* showed that a minor sequence variation in HTLV-1 was associated with a significant difference in the prevalence of HAM/TSP in Kagoshima. Interestingly, *HLA-A*02* appeared to give protection against only one of the two prevalent sequence variants of HTLV-1, cosmopolitan subtype B, but not against subtype A. Recently, Sabouri *et al.* (2005) have confirmed the finding that *HLA-A*02* appears not to give protection against infection with cosmopolitan

subtype A in a population in Iran. Borducchi *et al.* (2003) found that *HLA-A*02* is associated with a lower prevalence of HAM/TSP in white subjects in Brazil, but the HTLV-1 genotype was not identified in this study.

In future studies of the influence of genetic polymorphisms on HTLV-1 infection, it will be important to examine the genes whose expression was associated with rapid and effective $CD8^+$ cell-mediated lysis of HTLV-1-infected cells in the DNA expression microarray study (Vine *et al.*, 2004; Figure 1).

HTLV-1-infected cells should have a short life span in vivo, *if they are indeed rapidly killed by CTLs*

Until recently, this implication has been difficult to test directly in natural HTLV-1 infection, because of the lack of a safe technique to label lymphocytes in humans. However, a suitable technique has been developed by Macallan *et al.* (1998, 2003), which involves quantifying the incorporation of the non-radioactive isotope deuterium (^{2}H) from glucose into the DNA of newly divided lymphocytes. A study is currently underway using this technique: preliminary results (Asquith *et al.*, unpublished) suggest that the lifespan of Tax^+ cells is indeed significantly shorter than that of Tax^- cells *in vivo*. It will be interesting to use similar techniques in patients with the HTLV-1-associated malignancy adult T-cell leukemia (ATL), to examine the lymphocyte dynamics and the impact of the CTL response on the control of the leukemic cell growth both before (Arnulf *et al.*, 2004) and after (Utsunomiya *et al.*, 2001; Harashima *et al.*, 2004) allogeneic bone marrow transplantation.

Tax expression must be suppressed in a proportion of infected cells

As described above, the proviral load of HTLV-1 appears to be maintained, when an equilibrium is established with the immune response, mainly by proliferation of HTLV-1-infected cells. To maintain the infection, these cells must proliferate faster than uninfected cells, because they are subject to CTL killing *in vivo* (Asquith *et al.*, 2005); cell survival might also be impaired by proviral integration into a critical region of the host genome or by direct toxic effects of the virus. Preferential proliferation of HTLV-1-infected cells requires the expression of HTLV-1 proteins, especially Tax. However, if HTLV-1 provirus-positive clones survived in the host only by persistent expression of the Tax protein, there would be a monotonic (one-way) increase in Tax expression over time: after some weeks or months, all infected cells would express Tax. Since this is not observed, it must be the case that either a proportion of cells that express Tax subsequently shut down Tax expression, or one or both daughter cells that arise by Tax-driven mitosis contain HTLV-1 proviruses that are initially silent. Recently, possible molecular mechanisms for such suppression of Tax expression have been identified, including both HTLV-1 factors – Rex (Hidaka *et al.*, 1988), the pX protein p30 II (Nicot *et al.*, 2004) and HBZ (Gaudray *et al.*, 2002) – and host factors – histone deacetylases (Lemasson *et al.*, 2004) and GLI-2/THP (Smith *et al.*, 2001). In each case, the evidence to date indicates a partial rather than complete reduction in proviral transcription, and the extent of suppression in natural HTLV-1 infection is not yet known. However, even partial suppression can give a significant survival advantage to an HTLV-1-infected cell because the exposure of the cell to immune surveillance, particularly by CTLs, might be delayed or diminished. Impairment of CTL surveillance may similarly allow HTLV-1-transformed leukemic cells to survive and proliferate (Furukawa *et al.*, 2001; Nomura *et al.*, 2004). It will be important to identify the mechanisms responsible, and to quantify the degree and the rate of this suppression of proviral expression.

Why do a minority of HTLV-1-infected people develop HAM/TSP, while the majority remain healthy asymptomatic carriers?

No unique sequence variant of HTLV-1 has been associated with HAM/TSP (Daenke *et al.*, 1990; Kinoshita *et al.*, 1991; Komurian *et al.*, 1991; Slattery *et al.*, 1999). There is evidence from southern Japan (Furukawa *et al.*, 2000) of a significantly greater risk of HAM/TSP in people infected with the cosmopolitan subtype A of HTLV-1 than in those with cosmopolitan subtype B. However, this effect of HTLV-1 genotype is small, so the factors that determine the different outcomes of HTLV-1 infection must lie chiefly in the host. Specifically, it was likely that polymorphisms in genes that influence the immune response account for much of the variation in outcome, that is, the risk of HAM/TSP in HTLV-1-infected individuals and the proviral load.

The protective effects of *HLA-A*02* and *HLA-Cw*08* in HTLV-1 infection in southern Japan (Jeffery *et al.*, 1999, 2000; Vine *et al.*, 2002) have been described above, where it was pointed out that the likely mechanism of this protection is efficient lysis of HTLV-1-expressing cells by HLA-A2-restricted and Cw08-restricted CTLs. In the same cohort, the host genotype was determined (Vine *et al.*, 2002; Kodama *et al.*, 2004; Sabouri *et al.*, 2004) at over 70 single-nucleotide polymorphisms (SNPs) in over 50 loci outside HLA class 1. Of these, polymorphisms at four loci (*TNFA*, *IL-15*, *SDF-1* and *IL-10*) had statistically significant independent effects on the proviral load or the risk of HAM/TSP, or both. The results are shown in Table 1, in which the contribution of each significant factor to the risk of HAM/TSP was quantified by standard multivariate techniques.

The effect of the protective alleles *HLA-A*02* and *-Cw*08* in Kagoshima is exerted mainly through a reduction in proviral load. However, there is an additional statistically significant protective effect of each of these two alleles that remains even after the proviral load has been taken into account (Bangham, 2003). The mechanism of this additional protective effect associated with HLA class 1 alleles is unknown.

Table 1 Genetic polymorphisms that influence the outcome of HTLV-1 infection in southern Japan

Locus	*Function*	*Allele*	*Effect on HTLV-I infection*	*Odds ratio of HAM/TSP*	*Reference*
HLA-A	Presentation of peptides to cytotoxic T lymphocytes	*HLA-A*02*	Decreases odds of HAM/TSP; reduces proviral load	0.53	Jeffery *et al.* (1999)
HLA-B		*HLA-B*54*	Increases proviral load	2.51[a]	Jeffery *et al.* (2000)
HLA-C		*HLA-Cw*08*	Decreases odds of HAM/TSP; reduces proviral load	0.41	Jeffery *et al.* (2000)
HLA-DR	Presentation of peptides to helper T lymphocytes	*HLA-DRB1*0101*	Increases odds of HAM/TSP	1.72	Jeffery *et al.* (1999)
TNF-α	Proinflammatory cytokine produced by CD4+, CD8+, NK and ?? T cells	*TNFA −863A*	Increases odds of HAM/TSP in individuals with a high proviral load	—[b]	Vine *et al.* (2002)
SDF-1β	Chemoattractant for activated and resting T cells	*SDF-1β +801A*	Decreases odds of HAM/TSP; no effect on proviral load	0.45 (heterozygote) 0.18 (homozygote)	Vine *et al.* (2002)
IL-15	Immunoregulatory cytokine, maintains NK cells and CTL memory	*IL-15 +191C*	Decreases proviral load	0.69[a]	Vine *et al.* (2002)
IL-10	Inhibits production of antiviral TH1, proinflammatory cytokines	*IL-10 −592A*	Decreases odds of HAM/TSP; decreases proviral load in both ACs and patients with HAM/TSP	0.50[a]	Sabouri *et al.* (2004)
MMP9	Proteolysis of collagen type IV in extracellular matrix	*MMP-9* promoter long (CA) repeats	Increase odds of HAM/TSP	2.15[c]	Kodama *et al.* (2004)

[a]The odds ratios for *HLA-B*54*, *IL-15 +191C* and *IL-10 −592A* are calculated in single-factor analyses, not from logistic regression, since the effect of each allele appears to be exerted through an effect on the proviral load of HTLV-1. [b]A simple odds ratio cannot be calculated for the TNF-α allele, because its effect is exerted through a strong interaction with the proviral load. [c]Odds ratio of HAM/TSP conferred by possession of *either* $(CA)_{23}$ *or* $(CA)_{24}$

In the same study, a multivariate regression was used to identify the significant correlates of proviral load. These results (Vine *et al.*, 2002) showed that, among all the loci tested, the only alleles that exerted a significant influence on the proviral load in healthy carriers were *HLA-A*02* and *-Cw*08*, that is, the two protective class 1 HLA alleles. However, within the patients with HAM/TSP, the HLA class 1 genotype was not a significant predictor of proviral load. This apparent difference in the control of proviral load between patients with HAM/TSP and healthy carriers suggested either that the CTL response was ineffective in HAM/TSP or that other factors override the influence of CTLs on the proviral load in patients with the disease.

These data (Table 1) give an estimate of the relative risk of HAM/TSP, measured as the odds ratio, in a comparison between HTLV-1-infected individuals in Kagoshima. The data do not give a measure of the influence of host genotype on the absolute risk of HAM/TSP. Further, the study was designed to answer the question: what is the influence of host genetic polymorphism on the course of HTLV-1 infection? The question whether host genetic polymorphism also influences the risk of acquiring HTLV-1 infection was not addressed in this study, because exposure to HTLV-1 infection occurs by different routes and at different ages; the intensity of exposure by any of these routes is difficult to measure with precision. Owing to these known sources of variation in the intensity of exposure to HTLV-1 infection, it is likely that a large population sample would be required to detect a true effect of host genetics on the risk of acquisition of HTLV-1. However, Plancoulaine *et al.* (2000) have obtained evidence for such an effect at the level of genetic linkage analysis: it is hoped that the genes responsible can be mapped and identified, to allow a deeper understanding of this interesting and potentially important effect.

Rafatpanah *et al.* (2004) have found a suggestive increase in the frequency of a single-nucleotide polymorphism at nt +418 in the perforin gene in patients with HAM/TSP, when compared with uninfected controls. However, there was no significant difference between HAM/TSP patients and asymptomatic carriers of HTLV-1 in the frequency of this polymorphism either at the allele or the genotype level. However, perforin plays a central part in CTL-mediated lysis of virus-infected cells, and Vine *et al.* (2004) found upregulation of perforin gene expression in subjects with a low proviral load, compared with those with a high load (in two of three experiments; see Figure 1). Therefore, it is possible that the *+418C* allele of perforin is associated with more efficient CTL-mediated lysis of HTLV-1-infected cells. Here, the term 'CTL efficiency' refers to the per-CD8$^+$ cell lytic efficiency as described above (Figure 2a; Asquith *et al.*, 2005).

HLA-DR1

The first host gene that was found to influence HTLV-1 infection was HLA-DRB1*0101 (HLA-DR1). Usuku *et al.* (1990) found that possession of HLA-DR1 was associated with a higher prevalence of HAM/TSP in Japan; this effect was confirmed in subsequent studies (Nishimura *et al.*, 1991; Sonoda *et al.*, 1996; Kitze *et al.*, 1998). There is recent evidence of a similar susceptibility effect of HLA-DR1 in an HTLV-1-infected population in northern Iran (Sabouri *et al.*, 2005). Although the effect of HLA-DR1 is somewhat weaker than that of the other polymorphic loci in Kagoshima, in which HLA-DR1 is not a significant independent predictor of the risk of HAM/TSP or proviral load after taking account of the other significant predictors, the reproducibility of the effect at the population level suggests that it is real. However, it is not clear which of several possible mechanisms is responsible for the effect of HLA-DR1 on HTLV-1 infection. This contrasts with the effects of the class 1 HLA genes, where the likely mechanism is straightforward to identify.

Conclusions on the genetic influence on the immune response to HTLV-1

The immunogenetic results obtained to date have corroborated other lines of evidence that variation in the efficiency of HTLV-1-specific CTLs determines variation in the outcome of HTLV-1 infection. The results have also served to identify factors not directly related to the CTL response, which suggest additional mechanisms that influence the outcome of HTLV-1 infection. However, there remains a quantitative disparity between the functional data on the importance of the CD8$^+$ lymphocyte response (Asquith *et al.*, 2005; see above) and the immunogenetic data. That is, the functional data suggest that the variation in the CTL lytic rate accounts for between 30 and 50% of the observed variation in proviral load, whereas the best available genetic model (Vine *et al.*, 2002) accounts for only 5–10% of the observed variation in proviral load. We suggest that other host genetic polymorphisms, still unidentified, are likely to account for the remaining difference between individuals in the proviral load: these polymorphisms may act by determining the efficacy (lytic rate) of HTLV-1-specific CTLs. There are indications that the relative importance of the respective mechanisms of immune surveillance against HTLV-1 might differ quantitatively between populations (Hisada *et al.*, 2004; Sabouri *et al.*, 2005). As noted above, such differences could be due to variation between the populations in the frequency of certain genetic polymorphisms, particularly HLA alleles, or in the prevalent genotype of HTLV-1. However, it seems unlikely that the fundamental mechanisms will differ qualitatively between populations.

Natural killer (NK) cell response to HTLV-1

The NK cell response to HTLV-1, as in other infections, has been less studied than the antibody and T-cell responses, partly because of the lack of a single surface marker that identifies NK cells in the human, and because of the existence of various lymphocyte subsets – such as 'NKT' cells – that are intermediate in function

and surface marker expression between T cells and 'classical' NK cells.

Evidence was obtained in Japan (Fujihara *et al.*, 1991; Yu *et al.*, 1991) that patients with HAM/TSP had both lower frequency and a lower activity of NK cells (especially the $CD3^+$ $CD16^+$ subset) than did asymptomatic HTLV-1 carriers, although the results were not normalized with respect to the proviral load.

An important mechanism of induction of NK cell-mediated killing is recognition by the NK cell of a complex of the nonpolymorphic MHC molecule HLA-E bound to a peptide derived from HLA-A, -B, or -C molecules (Braud *et al.*, 1998). Using synthetic tetramers of HLA-E^+ peptide to identify NK cells, Saito *et al.* (2003) found a low frequency of HLA-E tetramer-binding cells in patients with HAM/TSP. Interestingly, as in the earlier studies (Fujihara *et al.*, 1991; Yu *et al.*, 1991), this reduction in frequency was particularly notable in the $CD3^+$ cells, whereas there was no significant difference in the frequency of HLA-E tetramer-binding $CD3^-$ cells between patients with HAM/TSP and asymptomatic carriers.

The study by Saito *et al.* (2003) and the earlier work by Yu *et al.* (1991) and Fujihara *et al.* (1991) suggested that the activity of the NK or NK-like cell response was associated with the presence or absence of HAM/TSP; the NK frequency and activity did not appear to correlate with proviral load. However, the recent data on lymphocyte gene expression (Vine *et al.*, 2004) indicated that high levels of expression of certain genes that encode proteins involved in NK cell-mediated lysis were associated with a low proviral load of HTLV-1. These genes, which include *NKG2D*, *NKG2B* and *NKG2C* and *NKP46*, were highly expressed on $CD8^+$ cells. In particular, *NKG2D* was consistently the gene that was most differentially expressed between high-load and low-load subjects. The NKG2D protein serves as a costimulatory molecule on $CD8^+$ CTLs (Groh *et al.*, 2001) and as a primary recognition receptor on NK cells (Cosman *et al.*, 2001; Pende *et al.*, 2001; Sutherland *et al.*, 2002). It appears that the cytolytic lymphocyte population that reduces HTLV-1 proviral load contains both 'classical' $CD8^+$ CTLs and NK or NK-related cells.

$CD4^+$ (helper) T-cell response to HTLV-1

Research on the T-cell response to HTLV-1 has focused mainly on the CTL response, because these CTLs are abundant and chronically activated. Indeed, the evidence reviewed above favours the interpretation that CTLs play a major part in controlling the outcome of HTLV-1 infection. The $CD4^+$ (helper) T-cell response has been more difficult to study, because HTLV-1 Tax protein activates both IFNγ transcription and T-cell proliferation, which are the basis of the two most widely used assays of antigen-specific $CD4^+$ T-cell responses. However, Goon *et al.* (2002, 2003, 2004a, b) have shown that a short (6–7 h) ELISpot assay can detect antigen-induced cytokine production before the spontaneously expressed Tax protein results in host gene transactivation or T-cell activation. Using this technique, Goon *et al.* have made three chief observations on the HTLV-1-specific $CD4^+$ T-cell response. First, the response is dominated by IFNγ-producing cells, that is, the potentially pro-inflammatory (Th1) cells (Goon *et al.*, 2002). Second, the frequency of HTLV-1-specific IFNγ producing $CD4^+$ T cells was between 10 and 25 times greater in HAM/TSP patients than in asymptomatic carriers with a similar proviral load (Goon *et al.*, 2003, 2004a). This difference in T-cell frequency between patients with HAM/TSP and asymptomatic carriers is considerably greater than the two- to four-fold difference observed in the frequency of CTLs between these respective subject groups at a given proviral load. Whatever the cellular dynamics that result in such a high frequency of HTLV-1-specific $CD4^+$ cells, it seems probable that these cells contribute to the inflammatory tissue damage seen in HAM/TSP, especially since $CD4^+$ T cells predominate in active (early) inflammatory lesions in this disease (Usuku *et al.*, 1990; Iwasaki *et al.*, 1992). Finally, Goon *et al.* (2004b) used the IFNγ ELISpot assay to identify the immunodominance hierarchy of HTLV-1 antigens in both the $CD4^+$ and $CD8^+$ T-cell response. The results are shown in Table 2, with the typical range of frequencies of cells of each specificity. The HTLV-1 antigen most commonly recognized by $CD4^+$ T cells is the Env protein, in contrast with the immunodominance of Tax in the $CD8^+$ T-cell response. This result is not unusual: glycoproteins frequently predominate as target antigens in the $CD4^+$ T-cell response to viruses. These results also confirmed the observation of Pique *et al.* (2000) that a detectable $CD8^+$ T-cell response is mounted against the regulatory HTLV-1 proteins Tof and Rof.

Table 2 Immunodominance hierarchies in the helper ($CD4^+$) and cytotoxic (CTL, $CD8^+$) T-cell response to HTLV-1

$CD4^+$ (N = 14)		*$CD8^+$ (N = 17)*	
Antigen	*Percentage of responders*	*Antigen*	*Percentage of responders*
Env	71	Tax	94
Gag	71	Pol	71
Tax	64	Env	65
Pol	43	Gag	59
Pro	36	Rof	35
Rex	36	Tof	29
Rof	36	Pro	18
Tof	7	Rex	6

IFNγ ELIspot data taken from Goon *et al.* (2004a, b)

Pathogenesis of HAM/TSP and other HTLV-1-associated inflammatory diseases

It is widely assumed that the immune response causes the inflammatory tissue damage that is seen in diseases such as HAM/TSP, because the diseases are usually accompanied by high titres of HTLV-1 antibody (Ishihara *et al.*, 1994; Nagai *et al.*, 1998) and high frequencies of activated T cells (Jacobson *et al.*, 1990, 1992; Elovaara *et al.*, 1993; Greten *et al.*, 1998; Jacobson, 2002). Also, the tissue damage in the central

nervous system is associated with dense infiltrates of mononuclear cells, largely T cells (Iwasaki *et al.*, 1992; Iwasaki, 1993). But how might the immune system actually cause this tissue damage? Broadly, we can identify three possibilities. First, HTLV-1 might itself infect neurons or other resident cells in the central nervous system, and these infected cells could then be attacked by the HTLV-1-specific immune response. This possibility appears to be excluded by the observation that there is little or no HTLV-1 infection of resident cells in the central nervous system in HAM/TSP (Lehky *et al.*, 1995; Matsuoka *et al.*, 1998). Similarly, in HTLV-1-associated polymyositis, the provirus is present in invading $CD4^+$ T cells, not in macrophages or myocytes (Higuchi *et al.*, 1996).

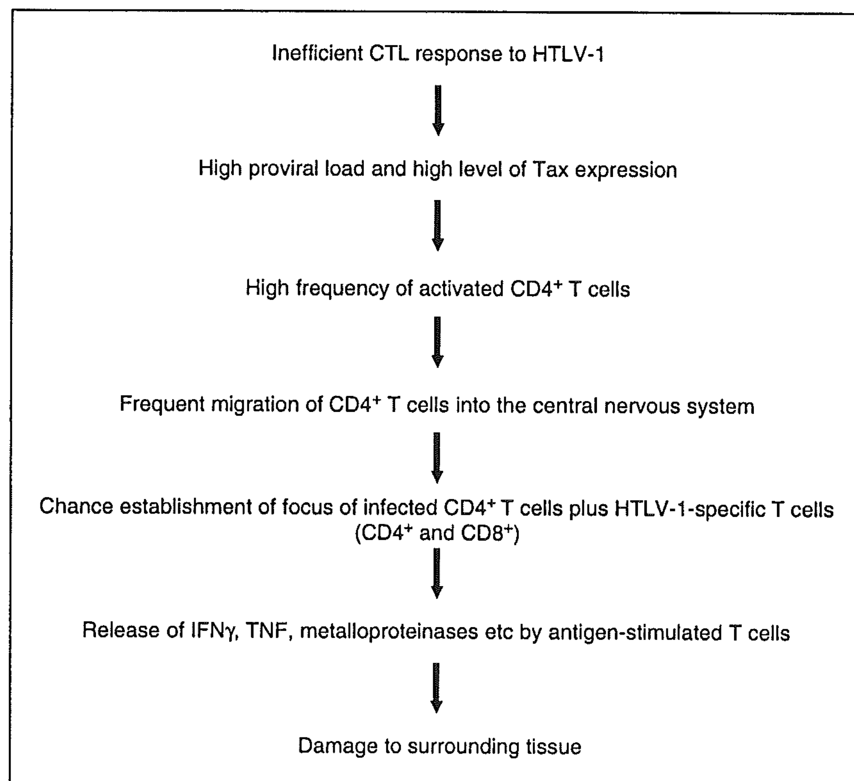
Second, antibodies or T cells that recognize certain HTLV-1 epitopes might crossreact with certain host proteins, a phenomenon known as antigen mimicry. Levin *et al.* (2002) obtained some intriguing evidence for antigen mimicry in HTLV-1 infection: antibodies that recognize Tax protein can crossreact with a host nuclear riboprotein hnRNP-A1. It is plausible that such autoreactive antibodies might contribute to the tissue damage in an established lesion in HAM/TSP. However, it is unlikely that this mechanism explains the onset or the distribution of the inflammatory lesions, because the host protein hnRNP-A1 is not confined to the central nervous system but is widely expressed, and is not normally accessible to antibody attack.

Finally, the cell damage in the CNS in HAM/TSP might be caused by inflammatory substances released by lymphocytes during the course of an immune response to HTLV-1-infected $CD4^+$ T cells that have invaded the central nervous system. We suggest that this process, known as 'bystander' or 'collateral' damage, is the most likely pathogenetic mechanism in HAM/TSP (Ijichi *et al.*, 1993; Daenke and Bangham, 1994). A simple hypothesis of the chain of causes that results in HAM/TSP can then be suggested: see box 1. Formal tests of the mechanisms of pathogenesis of HAM/TSP are difficult to devise, because of the paucity of animal models of this disease. The prospects of studying the role of the immune response to HTLV-1 in an animal model are perhaps best in the squirrel monkey (Kazanji *et al.*, 1997) or the pig-tailed macaque (McGinn *et al.*, 2004), but at present there are few appropriate reagents available for experiments in these animals.

Lymphocyte dynamics in HTLV-1 infection

It is now clear that HTLV-1 causes a persistent and highly dynamic infection, with rapid turnover of certain lymphocyte populations. Owing to the dynamic and interconnected nature of the processes involved, including proviral transcription, antigenic stimulation, lymphocyte proliferation and CTL-mediated killing, a full understanding cannot be reached without the use of mathematics (Nowak and Bangham, 1996; Wodarz and

Box 1 Hypothetical chain of causation in HTLV-1-associated inflammatory diseases



Bangham, 2000; Asquith *et al.*, 2002; Asquith and Bangham, 2003). Even after the molecular details of viral replication and cell–cell interactions are well understood, in order to explain the outcome of infection at the host level, it is necessary to consider the fate of populations of cells, not of individual cells. That is, it is helpful to consider the fate of an individual cell as a balance of probabilities. For example, a $CD4^+$ T cell in a lymph node that carries the HTLV-1 provirus may be more likely to transcribe HTLV-1 if the cell is stimulated, for example, by antigens or cytokines. Once the cell starts to express Tax protein there is a certain probability (say, p1) that the cell will be stimulated to undergo mitosis, a probability p2 that the cell will be killed by a CTL, and a probability p3 that the cell, activated by Tax protein, crosses the blood vessel endothelium and enters a tissue such as the central nervous system. Not all of these processes are, of course, mutually exclusive. An important challenge in HTLV-1 infection, as in other persistent viral infections, is to identify the critical processes and parameterize them – that is, to estimate the magnitudes of these probabilities or processes – and to identify critical points where these processes can be interrupted.

Conclusions

Variation in the genetically determined efficiency with which an individual's CTLs kill HTLV-1-infected cells appears to be the main cause of variation between hosts in the proviral load of HTLV-1. This efficiency can be measured *ex vivo* as the rate of lysis of Tax-expressing cells by autologous CTLs. Since the proviral load is strongly correlated with the risk of HAM/TSP, this conclusion implies that CTL lytic efficiency also determines the risk of inflammatory diseases associated with HTLV-1. However, CTL efficiency does not account for all the observed variation in the outcome of HTLV-1 infection; further work is needed to identify the additional factors that result in a higher proviral load in HAM/TSP patients than in asymptomatic carrers at a given level of CTL efficiency (lytic rate).

Patients with HAM/TSP have a 10- to 100-fold greater frequency of HTLV-1-specific $CD4^+$ T cells than do asymptomatic carriers; this high frequency, which might play a causative role in HAM/TSP, needs explaining. More work is also required to quantify the contribution of NK, NK-related and $\gamma\delta$ T-cell receptor + T cells to the control of HTLV-1 infection.

HTLV-1 infection has proved to be a very useful model to understand certain points of principle in persistent viral infections, because both the infected cells and the immune effector cells are accessible and present in high and stable frequencies in the blood, and because the degree of immunosuppression caused by HTLV-1 is small compared with that caused by HIV.

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Relapsing cervical cord lesions on MRI in patients with HTLV-I–associated myelopathy

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Human T lymphotropic virus type I (HTLV-I) is associated with adult T cell leukemia (ATL) and a chronic progressive disease of the CNS termed HTLV-I–associated myelopathy (HAM)/tropical spastic paraparesis (TSP).[1,2] The most common MRI findings of HAM/TSP are spinal cord atrophy predominantly involving the thoracic cord levels. In this article, we report two patients with HAM/TSP, who developed transient cervical cord lesions on MRI during the course of the illness.

Case reports. Patient 1 is 39-year-old woman who developed progressive gait and urinary disturbance when she was 22 years old. At age 35, she was diagnosed with HAM/TSP. MRI taken at age 37 showed spinal cord atrophy predominantly in the thoracic region (see figure E-1 on the *Neurology* Web site at www.neurology.org). Despite various treatments, her symptoms gradually worsened. At age 39, she noticed numbness and pain in the right upper limbs. On neurologic examination, muscle strength in the lower limbs was moderately decreased. Deep tendon reflexes were mildly exaggerated in the upper limbs and highly exaggerated in the lower limbs. Babinski signs were positive bilaterally. Superficial and deep sensations were disturbed below T10 level. She had urinary frequency and residual urine (170 mL). Anti-HTLV-I antibody was positive both in serum (×32,768) and in CSF (×512). HTLV-I provirus load was 55 copies/10^4 peripheral blood mononuclear cells. CSF showed increased protein content (53 mg/dL), IgG level of 12.5 mg/dL, and cell count of 7/mm^3. Myelin basic protein level was normal, and there were no oligoclonal bands detected in the CSF. Western blotting of CSF for anti-HTLV-I was positive for p19, p24, p28, p53, and env. Cervical MRI demonstrated swelling of the spinal cord at C5 to C6 levels with gadopentetate dimeglumine enhancement (figure). T2-weighted image showed high intensity signals at the same levels on sagittal section. Axial T2-weighted image at the C5 level showed high intensity signals in the lateral columns bilaterally. She was treated with IV high-dose methylpredonisolone followed by oral prednisolone treatment; cervical MRI gradually decreased with improvement of symptoms in the upper limb.

Patient 2 is a 72-year-old woman who developed progressive gait and pollakiuria at age 59 years and was subsequently diagnosed with HAM/TSP. Despite various treatments, her symptoms gradually worsened, and she became unable to walk. Since age 72, she noticed muscle weakness in the upper limbs. On neurologic examination, muscle strength in the lower limbs was moderately decreased. Deep tendon reflexes were mildly exaggerated in the upper limbs and highly exaggerated in the lower limbs. Babinski signs were positive bilaterally. She had difficulty in urination and residual urine (250 mL). Anti-HTLV-I antibody was positive both in serum (×131,072) and in CSF (×512). CSF showed increased protein content (53 mg/dL) and cell count (1/mm^3). Cervical MRI demonstrated swelling of the entire cervical spinal cord region (see figure E-2, A and B). T2-weighted image showed high intensity signals at the same level on sagittal section. Axial T2-weighted image at the C5 level showed high intensity signals in the lateral columns bilaterally. She was treated with IV high-dose methylprednisolone followed by oral prednisolone treatment; cervical MRI gradually decreased with improvement of muscle weakness in the upper limbs (figure E-2, C).

Discussion. The characteristic finding in these two patients is the appearance of abnormal lesions in the cervical cord on MRI during the long-standing course of the illness. Disappearance of high intensity lesions in the cervical cord after corticosteroid treatment suggests that inflammation or edematous changes may be the cause.

Recently, several cases of HAM/TSP with MRI abnormalities in the spinal cord have been reported.[3-5] These changes consisted of spinal cord swelling with high intensity signals and contrast enhancement from the cervical to the thoracic levels. Recently, we reported four patients who developed slowly progressive myelopathy with abnormal MRI lesions at the cervical cord levels; we proposed that these four cases may belong to a variant form of HAM/TSP, predominantly involving the cervical cord levels.[6] In the two patients presented here, MRI abnormalities at the cervical cord level were associated with worsening of symptoms in the upper limbs, suggesting that these MRI abnormalities also indicate active inflammatory changes. Neuropathologic analysis of HAM/TSP demonstrated diffuse parenchymal infiltration involving the entire spinal cord, including the cervical cord levels.[7] Therefore, abnormal MRI findings at the cervical cord also indicate active inflammatory changes in the current patients.

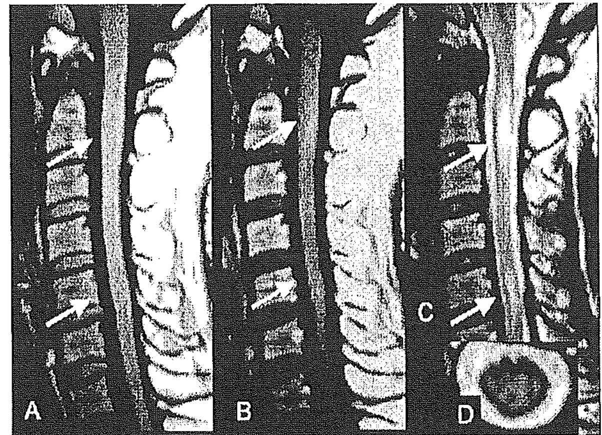

Figure. Cervical MRI showed focal swelling (A, arrows) with slight gadolinium enhancement (B, arrows) on T1-weighted imaging. T2-weighted image showed high intensity signals at the same level on sagittal section (C, arrows), which was located at the center of the spinal cord (D, C2 level).

Additional material related to this article can be found on the *Neurology* Web site. Go to www.neurology.org and scroll down the Table of Contents for the January 24 issue to find the title link for this article.

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HTLV-I viral escape and host genetic changes in the development of adult T Cell leukemia

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In the pathogenesis of adult T cell leukemia (ATL), an oncogenetic role of the human T cell lymphotropic virus type I (HTLV-I) Tax protein, viral escape from the host immune system, and host genetic changes have been proposed as contributory factors. We examined the premature stop codons in *tax* gene as one of the mutations that may lead to escape of HTLV-I from the cytotoxic T lymphocyte (CTL) response in HTLV-I carriers, to test whether a putative CTL escape mutant can emerge in the early stage of ATL development and whether HTLV-I infected cells with such a mutation can proliferate subsequently. We also examined deletion of cyclin-dependent kinase inhibitor 4 (*INK4*) genes and mutation of *p53* gene in combination with changes in the HTLV-I genome in acute type ATL to test whether host genetic changes promoted the malignant transformation of ATL cells that carry putative CTL escape mutations. The premature stop codon in *tax* gene existed in many non-ATL HTLV-I carriers as a minor population but not in the commonest HTLV-I sequence of the individual. This minor population with a premature stop codon did not expand subsequently in 3 asymptomatic carriers tested. There were cases who had a mutation or deletion in HTLV-I who also have either deletion of *INK4* genes or mutation in *p53* gene. Our findings suggest that CTL escape mutation can occur at an early stage of ATL development, and that certain host genetic changes favor the development of the aggressive form of ATL.
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Key words: viral escape; INK4; p53; ATL

Adult T cell leukemia (ATL) is a T cell malignancy with clonal proliferation of human T cell leukemia virus type I (HTLV-I) infected cells.[1,2] HTLV-I is also an etiologic agent for HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP).[3,4] ATL is subdivided into 4 types (smoldering, chronic, lymphoma and acute).[5] ATL has a long incubation period and smoldering and chronic type ATL sometimes transform into a more aggressive acute/lymphoma type of ATL, suggesting a multistep leukemogenesis model[6] for the development of ATL.

HTLV-I Tax protein is a key regulator for immortalization, transformation and oncogenesis of the HTLV-I infected lymphocytes through its interaction with many cellular proteins. For example, Tax binds to CBP/p300 and determines the accessibility of CBP/p300 to protein complexes on specific DNA elements,[7] resulting in Tax mediated trans-activation of viral genes[8] and growth factors,[9] or trans-repression of p18,[10] DNA polymerase β and bax genes.[11] Tax also modifies the cell cycle through binding $p16^{INK4A}$,[12] hDLG[13] and MAD1[14] and contributes to the development of ATL.

Tax also plays a role as an immunodominant target antigen for the cytotoxic T lymphocyte response (CTL)[15,16] to HTLV-I and Tax-expressing cells will be rejected by the host immune response. It is possible therefore that immortalized cells that elicit weaker CTL responses are selected during the development of ATL. We have reported previously several mutations and deletions in the *tax* gene in ATL that can escape from the host immune system.[17] A premature stop codon in the *tax* gene (substitution at nt. position 7464 from G–A) that was observed frequently in ATL, is one such escape mutation, because the resulting truncated Tax protein loses its transactivational activity[18] and expression of HTLV-I related proteins is diminished. This premature stop codon was also observed in the chronic type of ATL as a consensus sequence of the patient,[17] suggesting that Tax is dispensable at least in some chronic ATL cases.

These findings suggest that viral escape from CTL recognition is one of the important steps for the development of ATL; however host genetic changes such as cyclin-dependent kinase inhibitor genes and *p53* gene have also been observed in ATL.

The signaling pathway governed by G1 cyclins, cyclin-dependent kinases (CDK), pRb and E2F plays a major regulatory role during G1 to S transition in the cell cycle.[19,20] The complex formed by CDK4 and D-type cyclins controls the passage of cells through G1 phase, and the function of CDK4/CDK6 complexes is inhibited by a number of inhibitor of CDK4 (INK4), *i.e.*, $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$.[21–25] The human $p16^{INK4A}$ and $p15^{INK4B}$ genes are situated within 30kb on chromosome 9p21.[26] $p18^{INK4C}$ and $p19^{INK4D}$ proteins also inhibit the activities of D-type CDK.[23–25] Among these INK4, however, $p16^{INK4A}$ is most impaired frequently in tumor cells.[27] There is another tumor suppressor gene named $p14^{ARF}$,[28] encoded in an alternative reading frame (ARF) of the $p16^{INK4A}$ gene. The *p53* gene is another tumor suppressor gene.[29] Mutations of the *p53* gene have been found in several malignancies including ATL.[30]

We wondered when a premature stop codon in the tax gene, a putative CTL escape mutant, emerged during the development of ATL, and whether HTLV-I with this stop codon would subsequently proliferate. To this end, we examined the occurrence of a premature stop codon in the tax gene in 219 asymptomatic carriers and 143 HAM/TSP patients. We also examined the proportion of HTLV-I infected cells with this stop codon in 3 asymptomatic carriers at different time points to test whether such HTLV-I infected cells continuously proliferate in asymptomatic HTLV-I carriers without ATL. We also examined the deletion of cyclin-dependent kinase (CDK) 4 inhibitor genes ($p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, $p19^{INK4D}$) in 23 acute ATL patients and mutations of *p53* gene in 22 ATL patients to investigate whether additional host genetic changes favor the development of the aggressive form of ATL.

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The authors have no commercial or other associations that might pose a conflict of interest.

Informed consent was obtained from all HTLV-I carriers and patients. This research was approved by the institutional review boards of the author's institutions, and human experimentation guidelines of the US Development of Health and Human Services and those of the author's institutions were followed in the conduct of clinical research.

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Material and methods

Study population

Two hundred and nineteen HTLV-I seropositive asymptomatic blood donors (AC) and 143 cases of HAM/TSP whose *tax* gene had not been sequenced in our previous study[17] were examined for the stop codon in the *tax* gene. Twenty-three cases of acute type ATL whose tax genes were sequenced in the previous study[17] were tested for the deletion of cyclin-dependent kinase inhibitor 4 genes and 22 of these cases were tested for a mutation in the *p53* gene. All cases were of Japanese ethnic origin and resided in Kagoshima prefecture (Japan). The diagnosis and clinical subtyping of ATL were made according to Shimoyama's criteria.[5] The diagnosis of HAM/TSP was made according to WHO diagnostic criteria.[31]

Proviral load measurement

The HTLV-I provirus load in peripheral blood mononuclear cells (PBMC) was measured in HAM/TSP patients and HC as described.[32] A quantitative PCR reaction was carried out using the ABI PRISM 7700 sequence detector (Perkin-Elmer Applied Biosystems, Tokyo, Japan). The amount of HTLV-I proviral DNA was calculated as follows: copy number of HTLV-I (*tax*) per 10^4 PBMC = [copy number of *tax*/(copy number of β-actin/2)] × 10^4. The lower limit of detection was 1 copy/10^4 PBMC.

Statistical analysis

The Mann-Whitney *U*-test was used for statistical analysis of HTLV-I provirus load and the variables were treated as continuous.

Restriction fragment length polymorphism analysis of the HTLV-I tax gene

Substitution at nt. position 7464 from G–A on the *tax* gene created a premature stop codon[17] and also created a Bln I restriction site (CCTGGG–CCTAGG). This stop codon was observed frequently in ATL in the commonest sequence of the individual ATL patients. Restriction fragment length polymorphism (RFLP) analysis using Bln I was done on 219 AC and in 143 HAM/TSP cases. Nested polymerase chain reaction (PCR) was carried out on the extracted DNA to amplify proviral DNA and the amplified product was digested with Bln I. One hundred nanograms of DNA were amplified by 35 cycles of PCR using expand high fidelity PCR system (Boehringer Mannheim, Tokyo, Japan) and 1 μM primers (PXO1+: 5′-TCGAAACAGCCCTGCAGATA-3′ [7257–7276] and PXO2−: 5′-TGAGCTTATGATTTGTCTTCA-3′ [8447–8467]). After the first PCR reaction, 1 μl aliquots of the amplified products were subjected to further 20 cycles of the second PCR using internal primers (PXI1+: 5′-ATACAAAGTTAACCATGCTT-3′ [7274–7293] and PXI1−: 5′-GGGTTCCATGTATCCATTTC-3′ [7644–7663]). Each PCR cycle consisted of denaturation at 94°C for 60 sec, annealing at 58°C for 75 sec, extension at 72°C for 90 sec and extension of the final cycle at 72°C for 10 min. Two μl of the nested PCR product was digested with 5 U of Bln I (Takara, Tokyo, Japan) in 10 μl volume at 37°C for 18 hr and was then electrophoresed on 1% agarose gel.

Proportion of HTLV-I infected cells with stop codon in the tax gene in asymptomatic carriers at different time points

RFLP analysis showed that there are AC and HAM/TSP patients that have a stop codon in the *tax* gene as a minor subpopulation of HTLV-I infected cells. To test whether such HTLV-I infected cells with a premature stop codon in the *tax* gene that can escape from the host immune response to HTLV-I can expand subsequently as a major population, we carried out RFLP analysis at different time points in 3 asymptomatic carriers. Case 1 was examined with samples taken on June 25 1999 and June 26 2000. Case 2 was examined with samples taken on Jan 14 2000 and Jan 26 2001. Case 3 was examined with samples taken on May 26 2000, Jan 25 2002 and Oct. 22 2004. RFLP analysis suggested that in Case1, the proportion of HTLV-I infected cells with the premature stop codon decreased after 1 year. In this case, to quantify the ratio of HTLV-I infected cells with the premature stop codon to HTLV-I infected cells without this premature stop codon, the nested PCR product was cloned into pCR-Blunt II-TOPO vector (Zero Blunt TOPO PCR cloning kit: Invitrogen), transformed into competent *E. coli* cells and spread on LB plates containing 50 μg/ml kanamycin. Colonies from the plate were cultured overnight in LB medium containing 50 μg/ml kanamycin, and plasmids containing subcloned tax genes were extracted. Purified plasmids containing the subcloned tax gene were digested with Eco RI and Bln I and then electrophoresed on 1 % agarose gel. When the subcloned *tax* gene was cleaved by Bln I, the subclone was judged as having the stop codon, and if uncleaved, the subclone was judged as not having the stop codon. The proportion of HTLV-I cells that carry the stop codon was then calculated.

Southern blot analysis of p16^{INK4A}, p15^{INK4B}, p18^{INK4C} *and* p19^{INK4D} *and HTLV-I*

Southern blot analysis of *p16^{INK4A}*, *p15^{INK4B}*, *p18^{INK4C}* and *p19^{INK4D}* was carried out in 23 cases with acute type ATL. Southern blot analysis of HTLV-I was also carried out. High molecular weight DNA was extracted by a standard method using phenol extraction. In Southern blot analysis for cyclin-dependent kinase inhibitor genes (*p16^{INK4A}*, *p15^{INK4B}*, *p18^{INK4C}*, *p19^{INK4D}*), 10μg of genomic DNA was digested with Hind III, separated on a 1% agarose gel, and transferred to a nylon membrane. Probes used in hybridization were a EcoRI–XhoI fragment of *p16^{INK4A}* cDNA, EcoRI–XhoI fragment of *p15^{INK4B}* cDNA, BamHI–BamHI fragment of *p18^{INK4C}* cDNA and EcoRI–EcoRI fragment of *p19^{INK4D}* cDNA. All of these probes were provided from Dr. Hirai (Banyu Tsukuba Research Institute). The same filters were rehybridized successively with the respective probes. Nylon membranes were also hybridized with β-globin probe. Probe DNA fragments were labeled with α-^{32}P-dCTP by random priming. Blots were hybridized at 65°C for 12 hr in a mixture containing 4× SSC (1× SSC; 0.15 M NaCl, 0.015 M sodium citrate) and 50μg of sonicated and denatured salmon sperm DNA and then washed in 0.1% sodium dodecylsulfate (SDS) and 1× SSC at 65°C for 30 min, and autoradiographed, then exposed to a imaging plate and analyzed by a laser image analyzer (MAC-BAS-1000). Southern blot analysis of HTLV-I was also done in our previous study[17] with 10μg of genomic DNA digested with Pst I and hybridized with total sequence of HTLV-I as a probe. The same filters were rehybridized with a ^{32}P-labeled HTLV-1 long terminal repeat (LTR) probe.

Sequence of p53 *gene*

The sequence of *p53* was examined in 22 ATL cases. Three *p53* fragments were amplified using nested PCR: (*i*) 371 bp encompassing the entire exon 4; (*ii*) 499 bp encompassing the entire exons 5 and 6; and (*iii*) 692 bp encompassing the entire exons 7 and 8. The primers used for PCR encompassing exon 4 were sense 5′-AACGTTCTGGTAAGGACAAGGG-3′ (p53_41) and antisense 5′-AAGGGTGAAGAGGAATCCCAAA-3′ (p53_42) for the first PCR and sense 5′-AGGACCTGGTCCTCTGACTG-3′ (p53_43) and antisense 5′-ATACGGCCAGGCATTGAAGT-3′ (p53_44) for the second PCR. The primers used for PCR encompassing exons 5 and 6 were sense 5′-TAGTGGGTTGCAGGAGGTGCTT-3′ (p53_51) and antisense 5′-GCAGGAGAAAGCCCCCCTACTG-3′ (p53_62) for the first PCR and sense 5′-TATCTGTTCACTTGTGCCCT-3′ (p53_53) and antisense 5′-GGCCACTGACAACCACCCTT-3′ (p53_64) for the second PCR. The primers used for PCR encompassing exons 7 and 8 were sense 5′-GACAGAGCGAGATTCCATCTCA-3′ (p53_71) and antisense 5′-GCTGGTGTTGTTGGGCAGTGCT-3′ (p53_82) for the first PCR and sense 5′-AGGTCTCCCCAA- GGCGCACTGG-3′ (p53_73) and antisense 5′-GGCATAACTGCACCCTTGGTCT-3′ (p53_84) for the second PCR. One hundred nanogram DNA was amplified by 35 cycles for the first PCR using the Expand

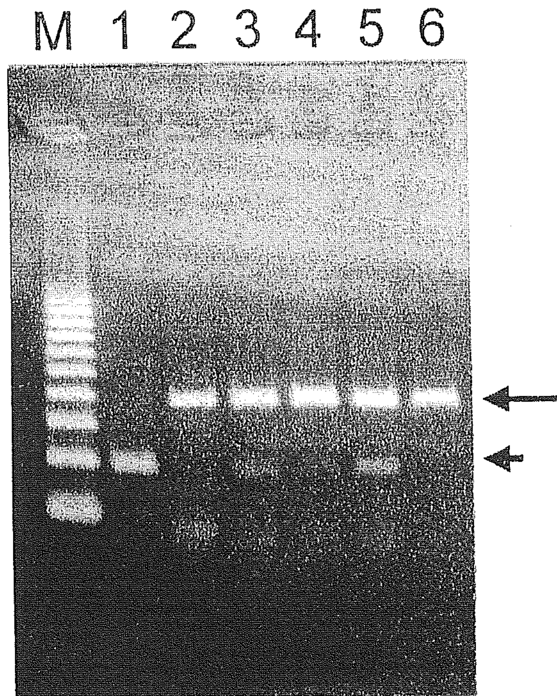

FIGURE 1 – RFLP analysis of *tax* gene. Nested PCR products of *tax* was digested by restriction enzyme Bln I. PCR product in ATL case with a premature stop codon at nt. 7464 in HTLV-I genome was completely cut by Bln I (*lane 1*), whereas in ATL case without the stop codon at nt. 7464 was not cut by Bln I (*lane 2*). PCR products in HAM case (*lane 3*) and in AC case (*lane 5*) was partially cut by Bln I, suggesting existence of a minor population of HTLV-I infected cells with the premature stop codon at nt. 7464 in HTLV-I *tax* genome, whereas in other HAM case (*lane 4*) and in AC case (*lane 6*), PCR products were not cut by Bln I. Long arrow indicates the nested PCR product and short arrow indicates the band cut by Bln I. M, 100 base marker.

high-fidelity PCR system (Boehringer Mannheim, Tokyo, Japan) and 1 μM of each primers. After the first PCR , 1 μl of aliquots of the amplified products were subjected to an additional 20 cycles of the second PCR using internal primers. Each PCR cycle consisted of denaturation at 95°C for 60 sec, annealing at 60°C for 75 sec, extension at 72°C for 120 sec and extension of the final cycle at 72°C for 10 min. Amplified DNA products were purified using QIA quick purification kit (Qiagen, Tokyo, Japan) and 0.1μg of PCR products were sequenced using dye terminator DNA sequencing kit (Applied Biosystems, Tokyo, Japan) with 3.2 pmol of each primers (p53_43 and p53_44 for exon 4, p53_53 and p53_64 for exons 5 and 6, p53_73 and p53_84 for exons 7 and 8) in an automatic sequencer (377 DNA Sequencer, Applied Biosystems).

Results

Premature stop codon in the tax *gene in AC and in HAM/TSP patients*

In 219 asymptomatic carriers and in 143 HAM/TSP patients, there was no case that had a premature stop codon in the *tax* gene in the commonest sequence of the individual. There are cases, however, who had HTLV-I infected cells with this premature stop codon in the *tax* gene as a minor population of the HTLV-I infected cells. Figure 1 shows representative results. In the ATL case with a premature stop codon in the HTLV-I *tax* gene, all of the nested PCR product was cut by Bln I (Fig. 1, *lane 1*). Under the same experimental conditions, there were no AC or HAM/TSP patients whose nested PCR products were completely cut by Bln I, but there was partial cleavage in some cases (Fig. 1., *lanes 3,5*).

TABLE I – HTLV-I PROVIRUS LOAD IN AC AND IN HAM/TSP IN ASSOCIATION WITH OR WITHOUT THE PREMATURE STOP CODON IN TAX GENE AS MINOR POPULATION OF HTLV-I INFECTED CELLS[1]

	AC (n = 219)		HAM (n = 143)	
	+	–	+	–
n	79	140	78	65
Median	166*	34*	523	420

[1]HTLV-I copy number per 10^{-4} PBMC was represented. n = number of subjects. + Subjects with the premature stop codon in tax gene as minor population of HTLV-I infected cells detected by RFLP analysis – Subjects without premature stop codon in tax gene; HAM, patients with HAM; AC, asymptomatic carriers. *p-value < 0.001 by Mann-Whitney U-test.

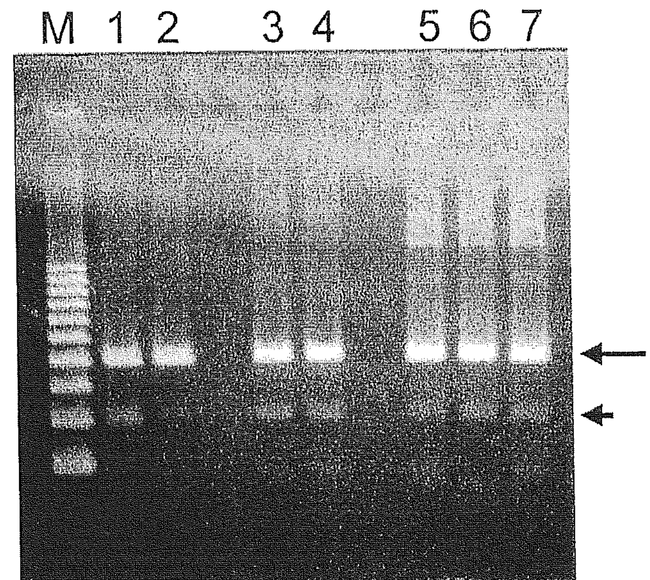

FIGURE 2 – RFLP analysis of *tax* gene at different time points. RFLP analysis of nested PCR product of *tax* gene digested by Bln I was done in 3 asymptomatic carriers at different time points. Case 1 (*lane 1* at June 25 1999 and *lane 2* at June 26 2000). Case 2 (*lane 3* at Jan 14 2000 and *lane 4* at Jan 26 2001). Case 3 (*lane 5* at May 26 2000, *lane 6* at Jan 25 2002 and *lane 7* at Oct. 22 2004). Long arrow indicates the nested PCR product and short arrow indicates the band cut by Bln I. M, 100 base marker.

There were 79 cases of 219 AC (36.1%) and 78 cases of 143 HAM/TSP patients (54.5%) that had HTLV-I infected cells with the premature stop codon in the *tax* gene as a minor population of the individuals (Table I).

The median provirus load in AC who had the premature stop codon in the *tax* gene as a minor population of HTLV-I infected cells was 166 and the median provirus load in AC who did not have this premature stop codon in the HTLV-I infected cells was 34.5, and this difference was significant ($p < 0.001$). The median provirus load in HAM patients who had the premature stop codon in *tax* gene as a minor population of HTLV-I infected cells was 523 and the median provirus load in HAM patients who did not have this premature stop codon in HTLV-I infected cells was 420, and this difference was not significant ($p = 0.305$) (Table I).

Proportion of HTLV-I infected cells with a stop codon in the tax gene as a minor population in asymptomatic carriers at different time points

In 3 asymptomatic carriers having the premature stop codon in the *tax* gene as a minor population of HTLV-I infected cells, we examined whether this minor population expanded subsequently.