Chronic graft-versus-host disease-like autoimmune disorders spontaneously occurred in rats with neonatal thymus atrophy

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We earlier reported that the human T cell leukemia virus type-1 *pX* gene transduced into rat thymic epithelial cells had an impact on biology of the cells. We report here that FW-pX rats born by mating of F344 transgenic rats expressing the *pX* gene without tissue specificity with nontransgenic Wistar rats developed disorders, including atrophy of the thymus, lymphocytopenia, and inflammatory cell infiltration into multiple organs, similar to events in chronic graft-*vs.*-host disease (GVHD). Vanishment of thymic epithelial cells especially in the cortex and marked depletion of CD4 CD8 double-positive thymocytes were evident in the neonatal thymus in these rats. The relative abundance of CD8 compared to CD4 T cells may be related to dominant infiltration of CD8 T cells into the affected organs. Additionally, adoptive transfer of FW-pX splenocytes could induce lymphocytic infiltration into sublethally irradiated wild-type syngeneic recipients. Analysis of the expression level of the Foxp3 gene in peripheral blood mononuclear cells revealed that the numbers of immunoregulatory T cells were less in FW-pX rats than in wild-type rats. The collective evidence suggested that the FW-pX rats spontaneously developed chronic GVHD-like autoimmune diseases, following abortive differentiation of T cells in the thymus in early days of the newborn. This rat model may shed light on the pathogenesis of chronic GVHD and also other systemic autoimmune diseases, the etiology of which is unknown.

Key words: Thymus · Autoimmune disease · Animal model · Chronic GVHD

Introduction

Human T cell leukemia virus type 1 (HTLV-1) is the etiological agent of adult T cell leukemia/lymphoma [1, 2] and other inflammatory diseases including HTLV-1-associated myelopathy/tropical spastic paraparesis [3, 4] and HTLV-1 uveitis [5]. Since Tax (protein product of the *pX* gene of HTLV-1) modulates expression and function of host molecules such as cytokines, cytokine receptors, growth factors, transcription factors, and cell cycle-related proteins, it is considered that the *pX* gene plays major pathogenetic roles in HTLV-1-associated diseases [6, 7].

To investigate the roles of Tax *in vivo*, we established transgenic rat models carrying the *pX* gene [8–10]. Among them, F344 rats expressing the *pX* gene under control of the rat lymphocyte-specific protein tyrosine kinase p56lck proximal promoter (lck-pX rats) fre-

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Abbreviations: **HTLV-1:** Human T cell leukemia virus type 1 · **DP:** Double-positive **SP:** Single-positive · **HSCT:** Hematopoietic stem cell transplantation · **ssDNA:** Single-stranded DNA

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quently developed epithelial thymoma which originated in the medulla [10]. Although we expected that the *pX* gene was exclusively expressed in lymphoid cells, the transgene was actually expressed in systemic organs including thymic epithelial cells in these rats. These findings suggested that the *pX* gene strongly affected the biology of thymic epithelial cells. To determine whether the phenomenon depended on host genetic backgrounds, we mated F344 lck-pX rats with other strains. We found that epithelial thymoma occurred at a high frequency only when lck-pX rats were mated with syngeneic F344 rats, indicating that host genetic factors are critical for tumorigenicity by the *pX* gene. This interesting theme is now under investigation.

In the present study, we focused on a unique phenotype other than epithelial thymoma seen in F1 rats which were born after mating F344 lck-pX with nontransgenic Wistar rats (FW-pX rats), including atrophy of the thymus, lower body weight than of age-matched controls (FW-wt rats), and dermatitis with hair loss. Lymphocytopenia with unrecognizable formation of follicles in LN and the spleen, and inflammatory cell infiltration into multiple organs were also noted in these rats. We considered that the disorders which occurred in FW-pX rats were similar to events in patients with chronic graft-*vs.*-host disease (GVHD) who had undergone hematopoietic stem cell transplantation (HSCT).

Results

Development of phenotype different from epithelial thymoma in FW-pX rats

F344 transgenic rats expressing the HTLV-1 *pX* gene in systemic organs (lck-pX rats) developed epithelial thymoma, suggesting that the *pX* gene strongly affected the biology of thymic epithelial cells [10]. To examine whether the phenomenon depended on host genetic backgrounds, mating of F344 lck-pX rats with other strains was done. Since epithelial thymoma occurred more frequently in male than in female lck-pX rats (reason currently unknown), we used male lck-pX rats for mating in the present study. Male rats in F1 generation from mating lck-pX rats with syngeneic wild-type F344 rats developed epithelial thymoma at a high frequency. On the other hand, male FW-pX rats obtained by mating of male lck-pX rats with female Wistar rats frequently developed disorders other than epithelial thymoma from early days after birth. In these rats, dermatitis with hair loss, lower body weight than of age-matched controls, and atrophy of the thymus were observed (Fig. 1). Out of 29 male FW-pX rats, 26 (90%) showed a similar phenotype, while most females did not. Incidence of the disease was highest in male FW-pX rats, though frequency of the disease in other F1 strains was up to 20% (data not shown).

Fig. 1. Representative photographs of F1 rats (male, 5 weeks of age). The upper and lower photographs show the FW-wt [(male nontransgenic F344) × (female Wistar) F1] and FW-pX [(male F344 lck-pX) × (female Wistar) F1] rat, respectively. FW-pX rats were physically smaller than age-matched FW-wt rats and manifested dermatitis with hair loss (left). In FW-pX rats, atrophy of the thymus was observed (right). Arrows indicate the thymus.

Structural abnormality of the thymus and apoptosis of thymic epithelial cells and CD4 CD8 double-positive thymocytes in neonatal FW-pX rats

To determine how the thymus atrophied in FW-pX rats, neonatal thymus was examined chronologically. Demarcation between the cortex and medulla was missing due to marked depletion of thymocytes in 10-day-old FW-pX thymus (Fig. 2A). In 2-day-old FW-pX thymus, thymocytes were maintained relatively. Thymic epithelial cells positively stained with anti-cytokeratin Ab were diminished in the FW-pX thymus predominantly in the cortex compared to the medulla, while these cells spread like a mesh in 2-, 5-, and 10-day-old control FW-wt rats (Fig. 2B). In the 5-day-old FW-pX thymus, some nuclei of epithelial cells labeled with anti-cytokeratin Ab were positively stained with anti-single-stranded DNA (anti-ssDNA) Ab (Fig. 2C). These were also positive by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL, data not shown), suggesting that the loss of thymic epithelial cells was caused by apoptosis. Contrarily, in the age-matched FW-wt thymus, most ssDNA-positive cells were cytokeratin-negative thymocytes (Fig. 2C).

The rate of apoptotic thymocytes in the FW-pX thymus was at a significantly high level at 5 (32.1±5.7%) and 10 days of age (13.0±2.1%) compared with findings in the FW-wt thymus (less than 5% at 2, 5, and 10 days of age) (Fig. 3A). In line with these findings, the numbers of viable thymocytes in the FW-pX

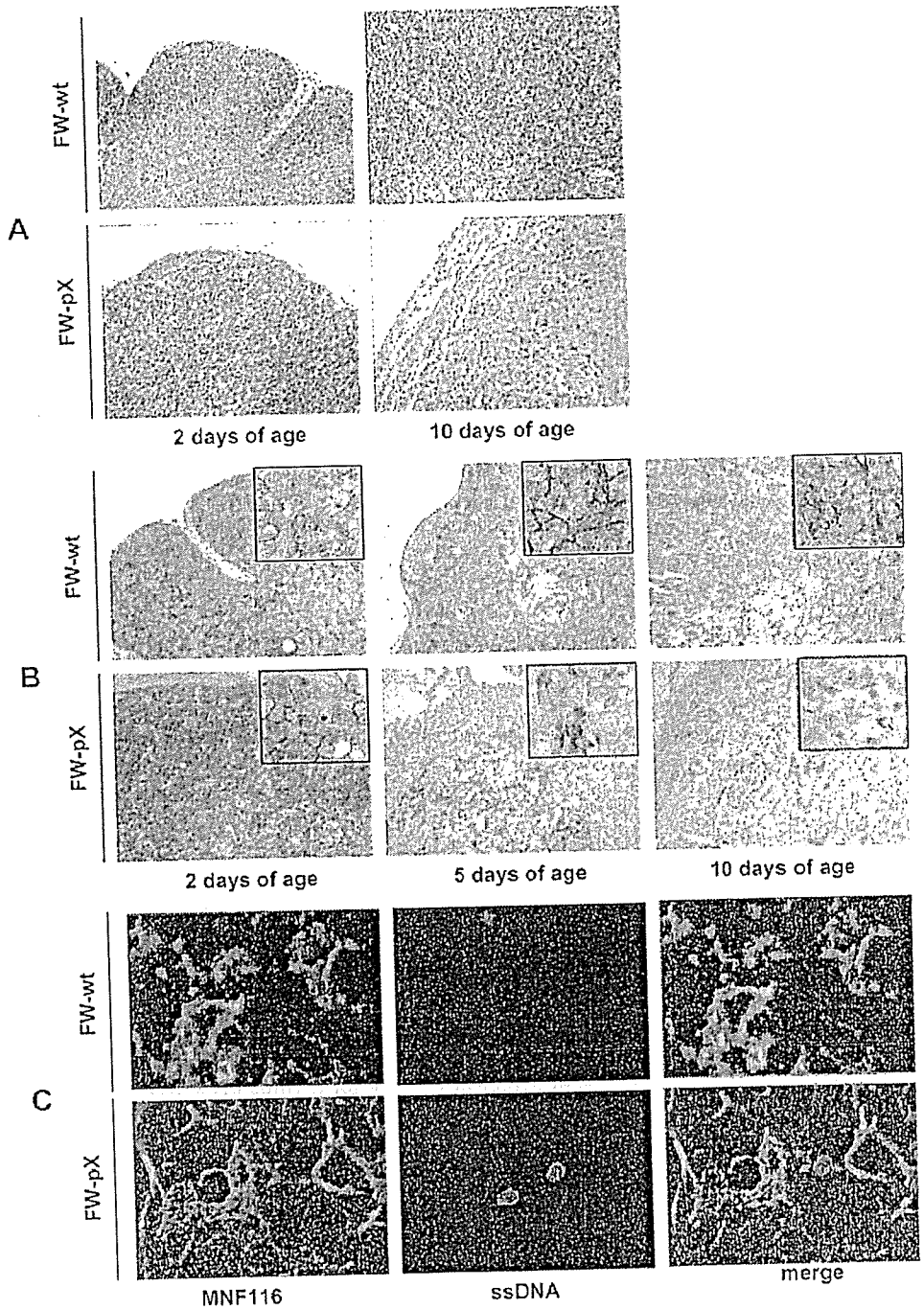

Fig. 2. Structural alteration of the thymus in neonatal FW-pX rats. Photographs show representative findings. (A) H&E staining (original magnification: ×100). (B) Immunostaining for cytokeratin (AE1+AE3) to label thymic epithelial cells (original magnification: ×100). Inset: high-power view of the cortex (×400). (C) Immunofluorescence double staining for cytokeratin (MNF116, green) and ssDNA (A4506, red), using the thymus from 5-day-old rats. The merged image shows that some nuclei of thymic epithelial cells were stained for anti-ssDNA Ab, indicating that thymic epithelial cells are dying by apoptosis in 5-day-old FW-pX rats (lower panels, original magnification: ×400). On the other hand, cells positive for ssDNA do not merge to thymic epithelial cells, indicating apoptosis of thymocytes in FW-wt rats (upper panels, original magnification: ×400).

thymus (5 days of age: $55.4\pm21.8\times10^5$, 10 days of age: $37.6\pm8.4\times10^5$) were significantly less than those of the FW-wt thymus (5 days of age: $365.7\pm67.8\times10^5$, 10 days of age: $1{,}461.8\pm202.7\times10^5$) (Fig. 3B). The rate of CD4 CD8 double-positive (DP) cells in the FW-pX thymus (5 days of age: 7.76%, 10 days of age: 2.77%) was

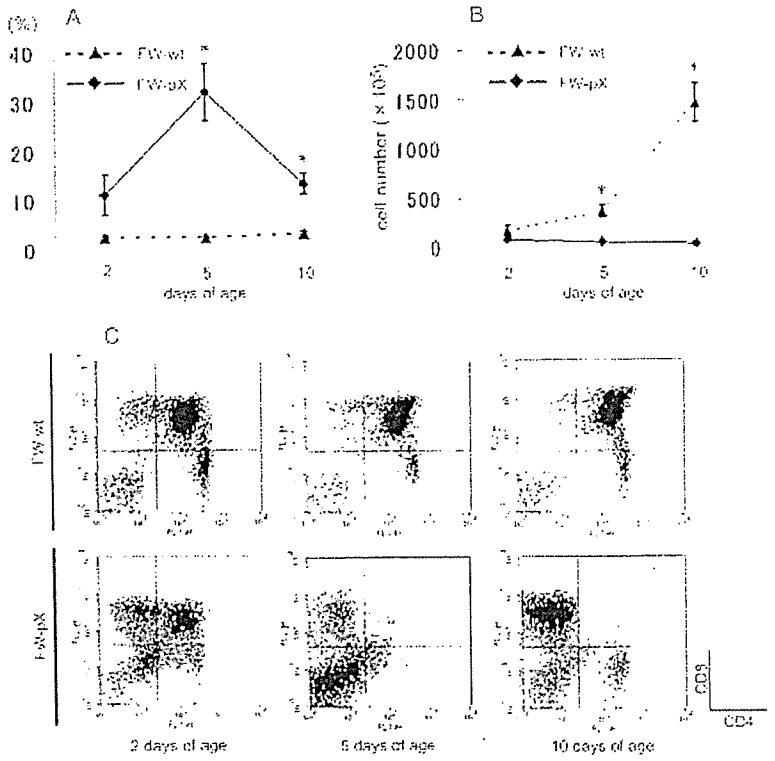

Fig. 3. Analyses of the thymus. (A) Thymocytes were stained with Annexin-V-FLUOS Staining Kit to detect apoptosis, and then analyzed using FACScan. (B) Total number of thymocytes. Viable cell number was counted after Trypan blue staining. (C) Thymocytes from FW-wt (top) and FW-pX rats (bottom) were stained with FITC-conjugated anti-CD4 (OX-35) and PE-conjugated anti-CD8 (OX-8) Ab. In both groups at each time-point, at least three rats were examined. In (A, B), data are represented as mean ± SD (*$p<0.05$). Each panel in (C) shows the representative profile of reproduced results.

significantly low compared with findings in the FW-wt thymus (5 days of age: 80.26%, 10 days of age: 83.87%), suggesting that the most affected cells in the neonatal FW-pX thymus were CD4 CD8 DP thymocytes (Fig. 3C). In addition, CD8 single-positive (SP) cells were noted at a higher ratio than CD4 SP cells in the FW-pX thymus at 2, 5, and 10 days of age.

Alteration of populations of peripheral blood mononuclear cells and abnormality of peripheral lymphoid organs in FW-pX rats

The rate of peripheral T cells reactive with anti-αβ TCR Ab was significantly low in FW-pX rats (4 weeks of age: 25.9±9.2%, 7 weeks of age: 15.0±6.7%) compared with findings in FW-wt rats (4 weeks of age: 49.2±1.0%, 7 weeks of age: 47.7±1.1%) (Fig. 4A). Contrarily, the rate of peripheral myeloid cells reactive with anti-CD11b/c Ab was significantly high in the FW-pX rats (4 weeks of age: 41.4±2.3%, 7 weeks of age: 68.9±11.0%) compared with findings in the FW-wt rats (4 weeks of age: 20.0±0.5%, 7 weeks of age: 20.3±3.2%) (Fig. 4B). The majority (87.2%) of peripheral $CD3^+$ cells were CD8 T cells, while a smaller population (9.8%) was composed of CD4 T cells (Fig. 4C). Expressions of CD25 (predominantly on CD4 T cells), MHC class II, and CD44 were increased on CD4 and CD8 T cells from FW-pX rats (Fig. 4D). On the other hand, expression of CD62L was decreased on CD4 and CD8 T cells from FW-pX rats (data not shown). These findings suggested that peripheral T cells were activated in FW-pX rats.

The absolute numbers of peripheral T cells were significantly decreased with marked depletion of CD4 T cells in FW-pX rats (Table 1). The CD4/CD8 ratio was converse between FW-pX and FW-wt rats, though the numbers of CD8 T cells were also less in FW-pX rats than in FW-wt rats. These findings suggested that CD8 T cells were positively selected or expanded among the small number of T cells in FW-pX rats. Alternatively, dominant defect in the generation of CD4 compared to CD8 T cells may be evident in FW-pX rats. In any case, these findings indicated that T cell differentiation was impaired in FW-pX rats. In contrast to this, higher numbers of peripheral myeloid cells were evident in FW-pX rats than in FW-wt rats. In LN and the spleen in the FW-pX rats, follicular structures were missing due to marked depletion of lymphocytes (Fig. 5).

Inflammatory cell infiltration into multiple organs in FW-pX rats

In FW-pX rats, inflammatory cell infiltration was evident in multiple organs including the skin, liver, salivary glands, and heart (Fig. 6A–D). The major cells infiltrating these organs were mononuclear. In the liver and salivary glands, the epithelium of bile and exocrine ducts, respectively, was affected by infiltrating cells.

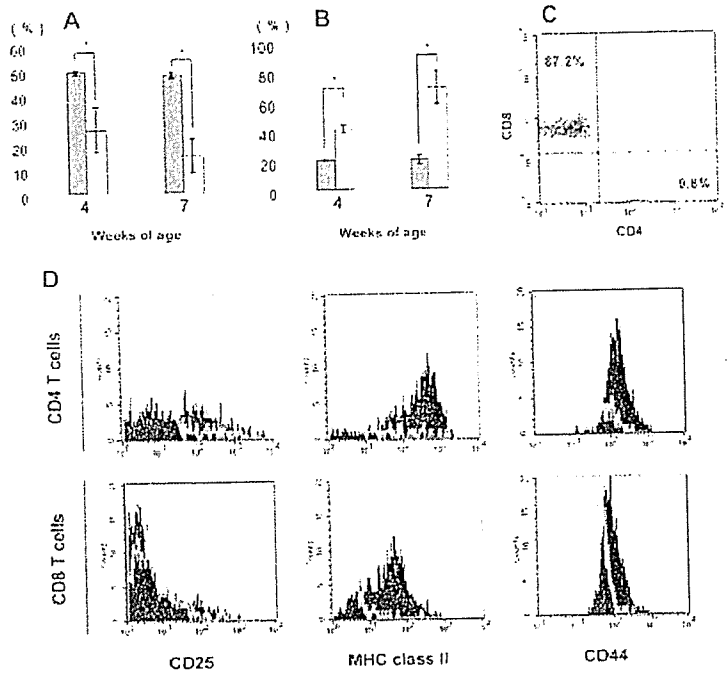

Fig. 4. Population of T and myeloid cells in peripheral blood. (A, B) PBMC were stained with FITC-conjugated anti-αβ TCR (R73) and PE-conjugated anti-CD11b/c (OX-42) Ab, and then analyzed using FACScan. The rate of T cells reactive with R73 (A) and myeloid cells reactive with OX-42 (B) are shown. In both FW-wt (gray bars) and FW-pX rats (white bars) at each time-point, at least three rats were examined. Data are represented as mean ± SD (*$p<0.05$). (C) PBMC were stained with FITC-conjugated anti-CD3 (G4.18), PE-conjugated anti-CD4 (OX-35), and PerCP-conjugated anti-CD8 (OX-8) Ab, and then analyzed using FACScan. Profile was obtained by gating $CD3^+$ cells. (D) PBMC were stained with FITC or PE-conjugated anti-CD4 (OX-35), PerCP-conjugated anti-CD8 (OX-8), and PE or FITC-conjugated Ab for CD25 (OX-9), MHC class II (MRC OX-6), or CD44 (OX-49), and then analyzed using FACScan. CD4 and CD8 SP cells were gated to obtain histograms, respectively. Black and gray histograms represent data from FW-pX and FW-wt rats, respectively. Representative profiles of reproduced results are shown.

Immunohistochemistry revealed that T cells and macrophages dominantly infiltrated into the affected sites (Fig. 6E, F), while B cells and granulocytes were observed to be small in number (data not shown). Approximately a quarter of all the infiltrating $CD3^+$ cells were CD4 T cells, and others CD8 T cells (Fig. 6G, H). In systemic organs, the heart, liver, kidneys, salivary glands, skin, pancreas, and skeletal muscle were susceptible to the inflammatory cell infiltration (Table 2).

Autoimmune response was involved in the development of inflammatory tissue damage in FW-pX rats

To examine the association of affected organs with the transgene, expression level of the *pX* gene was quantified by real-time reverse transcription (RT)-PCR. The *pX* transgene was expressed in all samples examined (data not shown). Among them, the expression was relatively high in the lung and low in the heart, suggesting no significant correlation between the affected organs and expression level of the transgene.

Adoptive transfers of FW-pX splenocytes into sublethally irradiated FW-wt rats were done to determine whether the inflammatory response in FW-pX rats was caused by autoimmune mechanisms. Five days after transplantation, infiltration of mononuclear cells was evident in the liver and heart in recipients (three of five, and one of five cases, respectively) of transferred splenocytes from FW-pX but not FW-wt rats (Fig. 7A, B). Histological aspects were similar to findings in FW-pX rats. Infiltrating cells were mainly composed of T cells and macrophages (Fig. 7C, D).

A possible implication of defect of immunoregulatory T cells in the development of GVHD-like autoimmune diseases in FW-pX rats

Recent studies show that peripheral $CD25^+CD4^+$ immunoregulatory T (T-reg) cells are engaged in inhibiting proliferation of autoreactive T cells, and in the maintenance of immunological self tolerance [11]. It is also shown that Foxp3 is the master gene of T-reg cells in the normal thymus [12, 13]. To assess the contribution of defect of T-reg cells to the development of GVHD-

Table 1. The number of T and myeloid cells in peripheral blood (/μl)[a)]

	T cells	CD4 T cells	CD8 T cells	Myeloid cells
FW-wt	2,319.9±120.3	1,307.6±121.8	704.9±83.9	941.5±70.2
FW-pX	624.7±301.1*	162.6±36.7*	470.4±64.4*	3,709.1±739.7*

a) In both groups, three rats (6 weeks old) were examined. Data are represented as mean ± SD.
*Significant difference ($p<0.05$) between FW-wt and FW-pX rats.

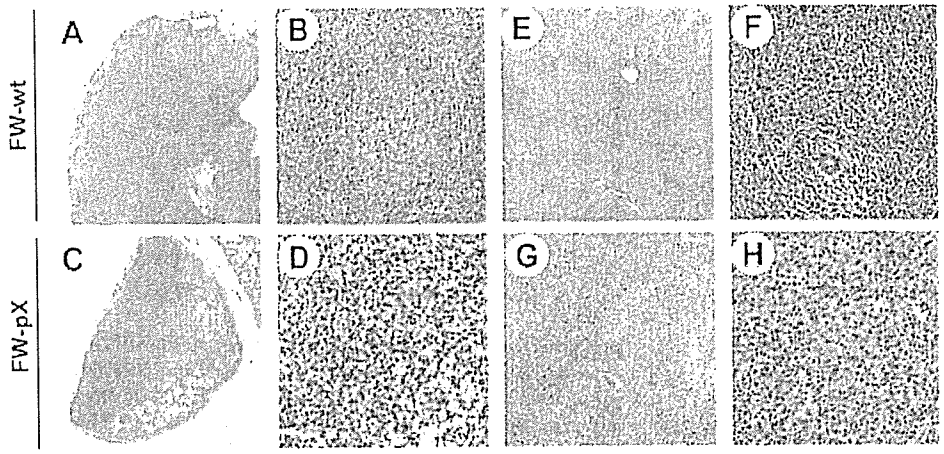

Fig. 5. Structural alteration of peripheral lymphoid organs of FW-pX rats (6 weeks of age). The upper and lower panels show representative findings in FW-wt and FW-pX rats, respectively. Tissue sections of LN (A–D) and the spleen (E–H) were stained with H&E [original magnification: ×20 (A, C, E, G), ×100 (B, D, F, H)]. In both groups, at least three rats were examined.

like autoimmune diseases in FW-pX rats, we compared the expression levels of the Foxp3 gene in PBMC between FW-pX and FW-wt rats, using the quantitative real-time RT-PCR method. When the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used for standardization, the expression level of the Foxp3 gene was significantly lower in FW-pX rats than in FW-wt rats (Fig. 8). On the other hand, the expression levels appeared to be equivalent between FW-pX and FW-wt rats when the gene for CD3 δ chain was used as a reference. Since the total numbers of PBMC were comparable between FW-pX and FW-wt rats (data not shown), these findings suggested that the numbers of peripheral T-reg cells were less in FW-pX rats than in FW-wt rats, though the proportional alteration in T cells was not evident. The defect of T-reg cells may be associated with the development of GVHD-like autoimmune diseases in FW-pX rats.

Discussion

Chronic GVHD usually appears several months after HSCT and is characterized by weight loss, atrophy of the thymus, reduction in number and function of peripheral T cells, structural alteration of LN and the spleen, and lymphocytic infiltration into systemic organs [14–18]. The susceptible sites of inflammation include the epithelium of bile ducts and salivary glands. Since GVHD occurs after not only allogeneic but also

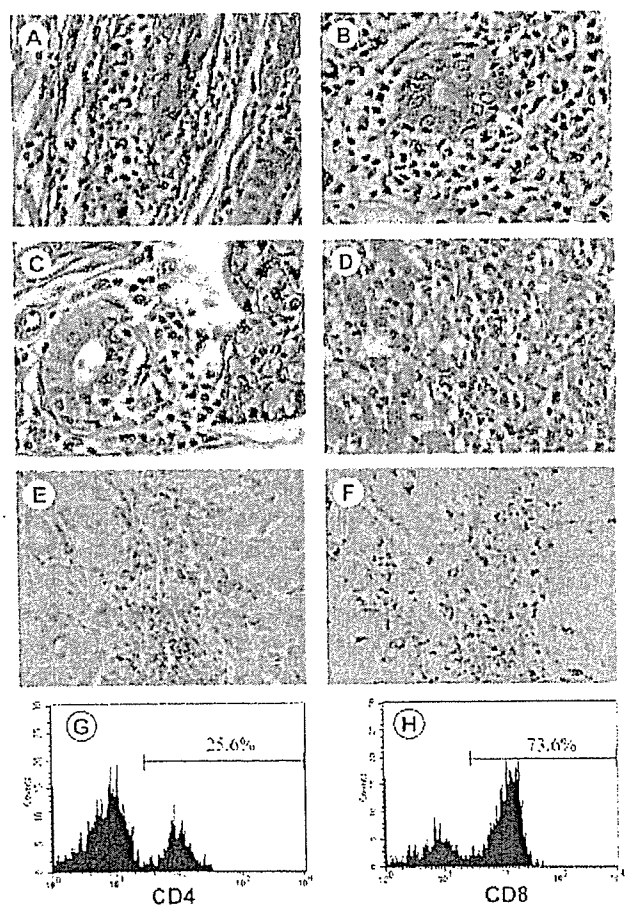

Fig. 6. Inflammatory cell infiltration in systemic organs of FW-pX rats (6 weeks of age). (A–D) Tissue sections from systemic organs were stained with H&E. Photographs show representative findings in the skin (A), liver (B), salivary glands (C), and heart (D). Arrows indicate the epithelium of bile duct (B) and salivary glands (C) affected by lymphocytic infiltration. Tissue sections of the liver were stained with anti-CD3 (CL020AP) (E) and CD68 (ED-1) (F) Ab (original magnification: ×400). Infiltrating cells were eluted from the liver by collagenase digestion, stained with FITC-conjugated anti-CD3 (G4.18) and PE-conjugated anti-CD4 (OX-35), or FITC-conjugated G4.18 and PE-conjugated anti-CD8 (OX-8) Ab, and then analyzed using FACScan. Histograms for CD4 (G) and CD8 (H) were obtained by gating $CD3^+$ cells. Representative data of reproduced results are shown.

Table 2. The incidence of inflammatory cell infiltration in systemic organs in male FW-pX rats

Organs	Incidence (%)
Heart	26/29 (90)
Liver	24/29 (83)
Kidney	22/29 (76)
Salivary gland	16/23 (70)
Skin	18/29 (62)
Pancreas	18/29 (62)
Skeletal muscle	16/29 (55)
Lung	5/29 (17)

syngeneic or autologous transplantation [19], and production of autoantibodies is sometimes accompanied with the disease [17], autoimmune mechanisms seem to be involved in the pathogenesis. It is considered that putative disruption of the thymus by conditioning regimens and/or acute GVHD may induce abortive negative selection of autoreactive T cells [17, 18, 20, 21]; however, details remain unclear. To better understand

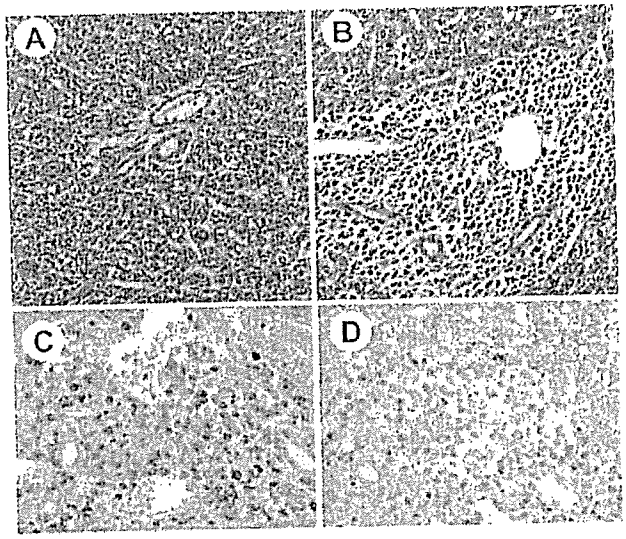

Fig. 7. Adoptive transfer experiments. Splenocytes isolated from 4-week-old FW-wt (A) and FW-pX rats (B–D), respectively, were stimulated by PMA (20 ng/ml) and ionomycin (2 μg/ml) for 24 h, and then injected intravenously into FW-wt rats (1×10^8/rat) which had been given sublethal total-body irradiation (9 Gy) (B–D). Five days later, recipient rats were killed for pathological examinations. Splenocytes from one donor were injected into one recipient. Experiments were repeated independently, and five rats were examined in each experimental group. Tissue sections of the liver from recipients were stained with H&E (A, B) and with anti-CD3 (CL020AP) (C) and CD68 (ED-1) (D) Ab [original magnification: ×200 (A, B), ×400 (C, D)]. Photographs show representative findings of reproduced results.

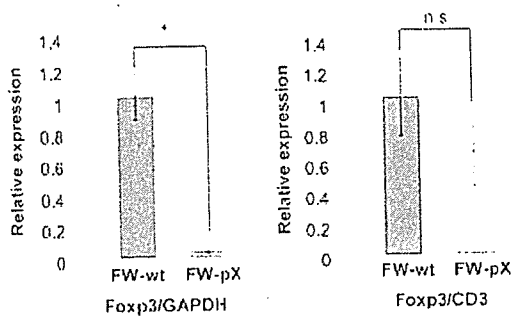

Fig. 8. Comparative analyses of mRNA expression of the Foxp3 gene in FW-pX and FW-wt rats. PBMC were separated from FW-pX and FW-wt rats (6 weeks old, $n=3$), respectively. Total RNA extracted from the cells was reverse-transcribed, and then the random-primed cDNA served as a template. Quantitative real-time PCR monitored by the SYBR Green I dye was carried out using the ABI PRISM 7700 Sequence Detector System. Amounts of the specific mRNA were quantified at the point where the system detected the uptake in exponential phase of PCR accumulation, and were standardized by the GAPDH gene or the gene for CD3 δ chain. The expression level in PBMC from FW-wt rats was set as 1.0, and the relative expression was calculated. Data are represented as mean ± SD in experiments done in triplicate; $^*p<0.05$, n.s.: not significant.

the pathophysiology of chronic GVHD, development of highly satisfactory animal models is needed.

In the present study, we found that FW-pX rats (F1 generation of F344 lck-pX rats and nontransgenic Wistar rats) showed atrophy of the thymus, lymphocytopenia, structural alteration of lymphoid tissues, and inflammatory cell infiltration into multiple organs. These disorders resembled chronic GVHD in patients given HSCT. Adoptive transfer of FW-pX splenocytes could induce lymphocytic infiltration into sublethally irradiated wild-type syngeneic recipients, clearly indicating that autoimmune mechanisms are involved in the pathogenesis of the disorders in FW-pX rats. These findings suggested that FW-pX rats spontaneously developed chronic GVHD-like autoimmune diseases. Among the strain combinations of mating, incidence of the disease was highest in male FW-pX rats (90%), though 0–20% of other F1 strains of lck-pX rats showed a similar phenotype (data not shown). The significance of strain combination or sex dependency of the disease will be considered in our ongoing works.

We previously reported that transgenic rats established in WKAH strains which expressed the HTLV-1 *pX* gene without tissue specificity developed systemic autoimmune diseases [9]. Although atrophy of the thymus was sometimes observed, there was no significant association with the development of most diseases in these rats (env-pX rats). Moreover, lymphocytopenia, which is a characteristic of GVHD, is never seen in env-pX rats. Therefore, regardless of the common transgene, it is considered that FW-pX and

env-pX rats develop autoimmune diseases via diverse pathogenesis.

In the FW-pX thymus, atrophy occurred by increased apoptosis of thymic epithelial cells and CD4 CD8 DP thymocytes from early days after birth. Thymic epithelial cells are essential for positive and negative selections of T precursors, and CD4 CD8 DP thymocytes are strongly influenced by cortical epithelial cells in the differentiation process [22]. According to this dogma, it is considered that severe depletion of CD4 CD8 DP thymocytes may be mediated by early diminution of thymic cortical epithelial cells in FW-pX rats. Tax (protein product of the HTLV-1 *pX* gene) plays paradoxical roles in proliferation and apoptosis depending on host cell types and conditions [6, 23]. Putative genetic factors in (F344 x Wistar) F1 rats may be associated with apoptosis of thymic cortical epithelial cells expressing the *pX* gene. Interestingly, CD8 SP cells were noted at a higher ratio than CD4 SP cells in FW-pX thymocytes, suggesting that abortive differentiation of T cells occurred in neonatal days. Nuclear factor (NF)-κB plays an important role in the differentiation into CD8 SP cells from CD4 CD8 DP thymocytes [24]. Since Tax activates NF-κB [25], the relative dominancy of CD8 SP cells compared to CD4 SP cells may be mediated, at least in part, by the *pX* transgene in thymocytes.

However, framework of the thymus appeared to be essential for impairment of T cell differentiation in FW-pX rats, because lethally irradiated nontransgenic FW-wt rats reconstituted with FW-pX BM cells never showed a similar alteration of profile of thymocytes or developed GVHD-like diseases (unpublished results). The altered profile of thymocytes corresponded with numerical dominancy of CD8 T cells compared to CD4 T cells in the peripheral blood and also in the sites of inflammation in FW-pX rats. Analyses for phenotype of peripheral CD4 and CD8 T cells showed that these cells were activated in FW-pX rats. Further investigations are needed to determine the causal association of the *pX* transgene with vanishing of thymic epithelial cells especially in the cortex, depletion of CD4 CD8 DP thymocytes, and generation and activation of autoreactive T cells.

Neonatal thymectomy, leading to lack of peripheral T-reg cells, generally results in organ-specific but not systemic autoimmune diseases in mouse models [26, 27]. Teshima et al. [28] reported that BM chimeras defective in expression of MHC class II on thymic APC led to impaired differentiation of T cells and resulted in development of systemic autoimmune diseases. Thymectomy of recipients prior to BM transplantation prevented the diseases in their model, suggesting that framework of the thymus was essential for generation of autoreactive T cells. The collective evidence suggests that systemic autoimmune diseases may occur when abortive differentiation of T cells in the thymus induces both active generation of autoreactive T cells and lack of peripheral T-reg cells. Our FW-pX rats, in which both impaired T cell differentiation associated with atrophy of the thymus and inflammatory cell infiltration into multiple organs related to autoimmune response are evident, may be useful for understanding the mechanisms of generation of autoreactive T cells and loss of peripheral T-reg cells.

Although several models of chronic GVHD have been reported [29–31] in which animals were given irradiation and lymphoid cell transplantation, influences of diverse factors including irradiation and acute GVHD complicated the pathogenesis in these models. FW-pX rats spontaneously developed chronic GVHD-like systemic autoimmune diseases, following abortive differentiation of T cells in the thymus at early ages in the newborn. This rat model may shed light on the pathogenesis of chronic GVHD and also other systemic autoimmune diseases whose etiology is unknown.

Materials and methods

Rats

Inbred F344 and closed colony Wistar rats were purchased from SLC (Shizuoka, Japan) and Charles River (Kanagawa, Japan), respectively. F344 lck-pX rats (line 38) [10] were maintained at the Institute of Animal Experimentation of Hokkaido University Graduate School of Medicine. All experiments using rats were done according to the Guide for the Care and Use of Laboratory Animals in Hokkaido University Graduate School of Medicine (http://www.hokudai.ac.jp/animal/houki/ hokudaisisin.html).

Mating

FW-pX rats were obtained by mating of male lck-pX rats with female Wistar rats. Since epithelial thymoma occurred more frequently in male than in female lck-pX rats (reason currently unknown), we used male lck-pX rats for mating in the present study. All FW-pX rats were screened for the *pX* gene by genomic PCR as described [10]. FW-wt rats were obtained by mating of nontransgenic male F344 rats with female Wistar rats, and served as controls. Eight-week-old rats were used for mating.

Monoclonal antibodies

Murine mAb used were anti-rat CD3 (CL020AP for immunohistochemistry; Cedarlane, Fornby, Canada; and G4.18 for FCM; PharMingen, San Diego, CA), CD4 (OX-35; PharMingen), CD8 (OX-8; PharMingen), CD11b/c (OX-42; PharMingen), CD25 (OX-39, PharMingen), CD44 (OX-49, PharMingen), CD62L (HRL1; PharMingen), CD68 (ED-1; Serotec, Oxford, UK), MHC class II (MRC OX-6, Serotec), αβ TCR (R73; PharMingen), and anti-human cytokeratin (AE1+AE3 and MNF116; DAKO, Glostrup, Denmark). We have previously

shown that AE1+AE3 and MNF116 were cross-reactive with rat epithelial cells [32]. Mouse IgG1 and IgG2 (CBL600P and CBL601P, respectively; Chemicon International, Temecula, CA) served as isotype controls.

Histopathology and immunohistochemistry

Tissue samples were fixed in 10% phosphate-buffered formaldehyde and embedded in paraffin blocks. Each 4-μm section was stained with hematoxylin and eosin (H&E). For immunohistochemistry, an avidin-biotin immunoperoxidase kit (DAKO) was used. After immunostaining, tissue sections were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany).

Immunofluorescence staining

To determine the co-localization of staining for cytokeratin and ssDNA, tissue sections were incubated with MNF116 (mouse monoclonal IgG1) and rabbit anti-ssDNA antisera (A4506; DAKO) followed by incubation with Alexa Fluor 488-goat anti-mouse IgG (Molecular Probes, Leiden, Netherlands) and Alexa Fluor 594-goat anti-rabbit IgG (Molecular Probes). After being washed with PBS, the sections were mounted in Fluorescent Mounting Medium (DAKO). Immunofluorescence was detected using a confocal microscope (1×70; OLYMPUS, Tokyo, Japan). TUNEL was done using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI).

Flow cytometry

Expression of cell surface molecules was analyzed by FCM. To detect apoptotic cells, thymocytes were stained using Annexin-V-FLUOS Staining Kit (Roche, Mannheim, Germany). FACScan (Becton Dickinson, Franklin Lakes, NJ) was used for these purposes.

Quantitative real-time RT-PCR

Total RNA was isolated from various organs of FW-pX rats, using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). Complementary DNA was synthesized from total RNA, using a random primer set and murine Moloney leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative real-time RT-PCR was done using the ABI PRISM 7000 Sequence Detection System (Applied Biosystem, Foster City, CA) with SYBR Green I as a double-stranded DNA-specific binding dye, and the reaction was continuously monitored based on the fluorescence levels, according to Wittwer et al. [33]. Each cDNA was amplified, using QuantiTect SYBR Green Master Mix (QIAGEN) containing 0.3 μM of the specific primers for the *pX* gene (sense: 5′-GTCTTCTTTTCGGATACCCAGTCTA-3′; antisense: 5′-AAG-GAGGGGAGTCGAGGGATAAGGA-3′) in a total volume of 10 μl, according to the following PCR conditions: 50°C for 2 min, 95°C for 15 min, followed by 40 cycles of 95°C for 30 s and 60°C for 30 s. The relative expression of the *pX* mRNA was analyzed by the ΔΔCT method [34]. GAPDH was used as a control for the amount of RNA (sense: 5′-ATGGGAGTTGCTGTTGAAGT-CA-3′; antisense: 5′-CCGAGGGCCCACTAAAGG-3′). Each reaction was done in triplicate.

Adoptive transfer

Mononuclear cells were isolated from the BM and spleen of 4-week-old FW-pX rats using Histopaque-1083 (Sigma-Aldrich, St. Louis, MO). For BM cell transfer experiments, isolated cells from the BM (1×10^7) were injected intravenously into FW-pX rats which had been lethally irradiated (12 Gy). Two months after transplantation, recipients were killed for examinations. For spleen cell transfer experiments, isolated splenocytes were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. For activation of lymphocytes *in vitro*, 20 ng/ml PMA and 2 μg/ml ionomycin were added to the culture medium, as described [35]. Twenty-four hours later, cells were harvested, washed twice, and injected intravenously into FW-wt rats (1×10^8/rat) with sublethal irradiation (9 Gy). Five days later, recipient rats were killed for pathological examinations. As negative controls, similarly irradiated FW-wt rats given splenic mononuclear cells from FW-wt rats with same treatments were used.

Comparison of the expression levels of the Foxp3 gene

Total RNA was extracted from PBMC of FW-pX and FW-wt rats (6 weeks old), respectively. In both groups, three rats were used for the experiments. Complementary DNA was synthesized, and then quantitative real-time RT-PCR was performed, using the specific primers for the Foxp3 gene (sense: 5′-GAGCCAGCTCTACTCTGCAC-3′, antisense: 5′-CCTCGAA-GACCTTCTCACAA-3′), the gene for CD3 δ chain (sense: 5′-cgaatgtgccagaactgtgt-3′, antisense: 5′-agtgtcaacagccccagaaa-3′), and the GAPDH gene. Each reaction was done in triplicate.

Statistical analysis

Data are represented as mean ± standard deviation (SD). Statistical significance between any two groups was determined by two-tailed Student's *t*-test. *p* values less than 0.05 were considered to be significant.

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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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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 ($\sim$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,

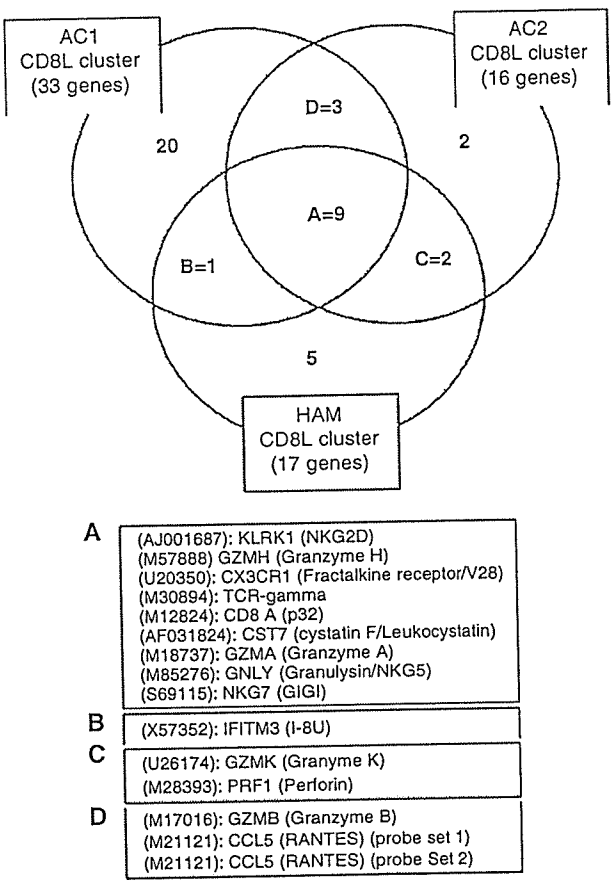


Figure 1 Venn diagram showing the number of genes that occurred in each CD8L cluster and the number and identity of genes that occurred in two or more CD8L clusters. In the three experiments, 27, 56 and 53% of genes present in the respective CD8L cluster occurred in this cluster in all three experiments: 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

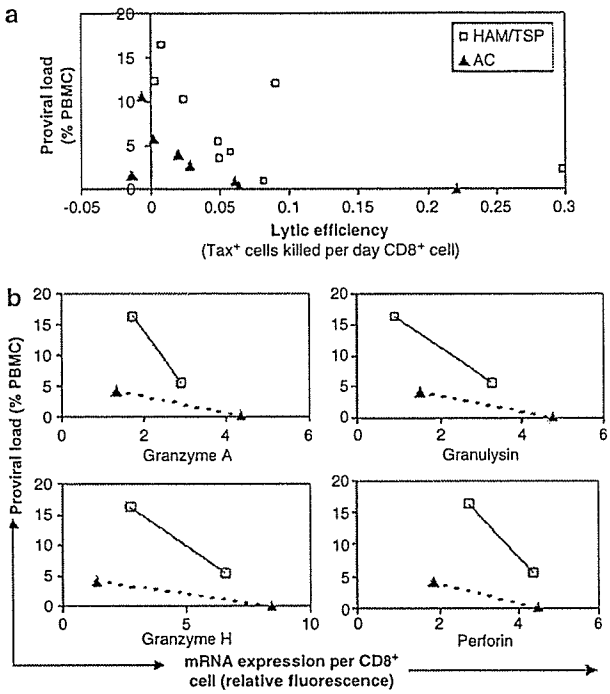


Figure 2 (a) A high rate of destruction of HTLV-1 Tax-expressing cells by $CD8^+$ cell-mediated lysis shows a statistically significant negative correlation with a low proviral load of HTLV-1 *in vivo* in both asymptomatic HTLV-1 carriers (AC) and patients with HAM/TSP. These results suggest that there 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 I 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 I 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 (^2H) 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).

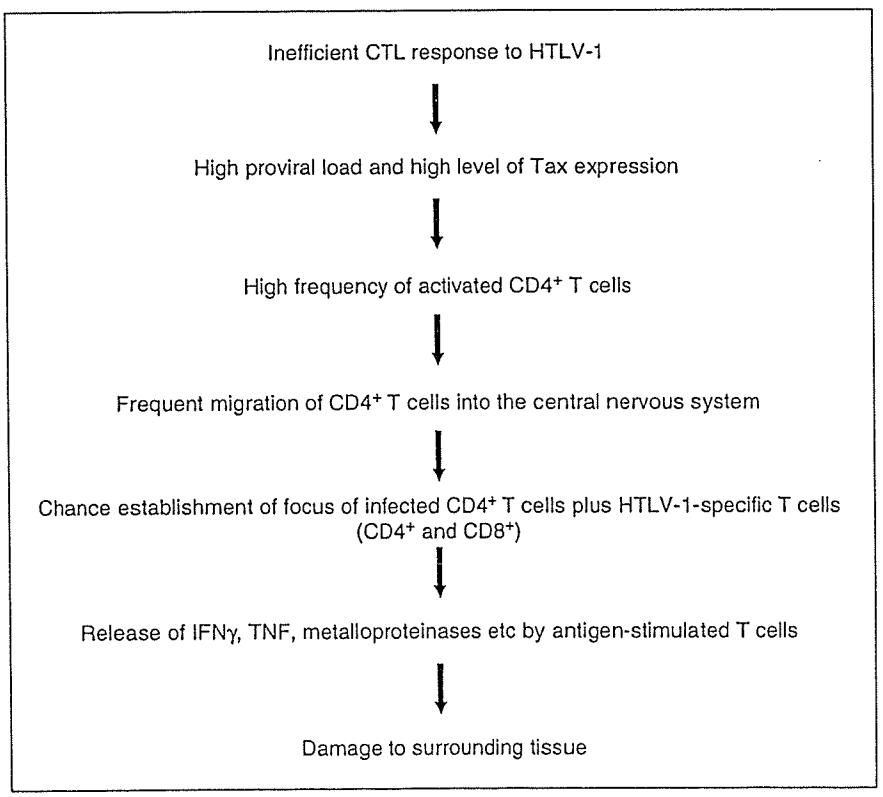
Second, antibodies or T cells that recognize certain HTLV-1 epitopes might crossreact with certain host proteins, a phenomenon known as antigen mimicry. Levin *et al.* (2002) obtained some intriguing evidence for antigen mimicry in HTLV-1 infection: antibodies that recognize Tax protein can crossreact 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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