TABLE 3. Cytotoxic T Lymphocytes, Human T-Lymphotrophic Virus-1–Infected Cells and Apoptotic Cells in the CNS

Patient ID	Tax-Specific CTLs in CD8-Positive Cells	Env-Positive Cells in CD4-Positive Cells	Caspase-3–Positive Cells in CD4-Positive Cells	Caspase-3–Positive Cells in Env-Positive CD4-Positive Cells	Caspase-3–Positive Cells in Env-Negative CD4-Positive Cells
8624	22.1% (62/280)*	60.3% (44/73)	26.0% (19/73)	36.4% (16/44)	10.3% (3/29)
6315	31.1% (96/309)	82.4% (103/125)	12.0% (15/125)	12.6% (13/103)	9.1% (2/22)
6664	N/A†	N/A	N/A	N/A	N/A

*The numbers of positive cells/total cells are indicated within parentheses.
†Not applicable; cell infiltration was not significant for evaluation.
CTL, cytotoxic T lymphocytes.

were not positive for active caspase-3 (Fig. 7H). Neurons identified by their size as well as several particles within the neuronal body induced strong autofluorescence but were not positive for active caspase-3 (data not shown).

DISCUSSION

One of the most striking features of the cellular immune responses in patients with HAM/TSP is the highly increased numbers of HTLV-1–specific CTLs in PBMCs and CSF (14, 15); however, little is known about CTLs in the CNS. The fixation of human CNS samples with a very low concentration of PFA made it possible to visualize antigen-specific CTLs using tetramers or a pentamer in the CNS. Strikingly, their frequency reached more than 20% in CD8-positive cells that had migrated to the CNS. In a flow cytometric study in our cohort, we detected HTLV-1 Tax11-19–specific and HTLV-1 Tax301-309–specific CTLs in PBMCs from patients with HAM/TSP at 2.25% (0.0%–18.7%) (26) and 4.34% (0.2%–17.6%) (unpublished data), respectively. The present data are consistent with another report in which the frequency of HTLV-1 Tax–specific CTLs was higher in CSF than in PBMCs (18). Although the frequency of the CTLs differs by the case, it may be attributed to the difference in the phase of the disease or the duration of the illness.

Granzyme B and perforin, both known as cytotoxic molecules of CTLs, were detected along with IFN-γ in the parenchyma near the vessels in the CNS. Some CTLs contained granzyme B. Human T-lymphotropic virus type-1–specific CTLs were in contact with HTLV-1–infected cells, and apoptotic cells were frequently noted near the CD8-positive cells. These results strongly suggest that the infiltrating CTLs function as effector cells in the CNS. Interestingly, a considerable number of oligodendrocytes underwent apoptosis in the affected lesions. Meanwhile, the oligodendrocytes neither increase the expression levels of HLA-ABC nor express HTLV-1 proteins. The HTLV-1–positive cells were only infiltrating CD4-positive T cells. These results suggest that HTLV-1–infected CD4-positive T cells, but not oligodendrocytes, are the main targets of HTLV-1–specific CTLs in the CNS, and that an interaction between these infected CD4-positive cells and CTLs may cause bystander damage in oligodendrocytes that is associated with demyelination. Similarly, in a previous study on an animal model of neurotropic mouse coronavirus infection, activated CD8-positive T cells specific to neither the virus nor CNS antigens caused demyelination (27). Mechanisms of demyelination in other viral infections in the CNS, even those exhibiting dense infiltration of activated CTLs such as measles or lymphocytic choriomeningitis virus encephalitis, are unclear. Whereas a CD8-positive CTL has been considered to be beneficial for the host infected by a certain virus by diminishing virus-infected cells, recent studies clearly show that strong CTL responses to a pathogen sometimes induce an immunopathology that is harmful to the host. For example, in a mouse model of CNS lymphocytic choriomeningitis virus infection, the depletion of CD8-positive T cells rescues the animal from a fatal condition (28). In another report, highly activated CD8-positive T cells in the brain were correlated with early CNS dysfunction in simian immunodeficiency virus infection (29). Similarly, markedly increased HTLV-1–specific CTLs in the CNS may induce the development of HAM/TSP.

Previous studies reported that the HTLV-1 antigen is hardly detected in PBMCs (30, 31), despite a high proviral

FIGURE 3. Frequency of human T-lymphotropic virus type-1 (HTLV-1)-1 Tax–specific cytotoxic T lymphocytes (CTLs) in CD8-positive lymphocytes. Double staining with anti-CD8 monoclonal antibody (mAb) (red) and HLA-A*0201/Tax11-19 or HLA-A*2402/Tax301-309 tetramer (green) was performed with confocal laser scanning microscopy (CLSM). DAPI (blue) was used for counterstaining the nuclei. The 3 colors were captured sequentially using CLSM. Merged images are shown in the right-hand column. **(A–C)** Spinal cords from the 3 patients with HAM/TSP were stained with the tetramers. CTLs stained with the tetramer were exclusively positive for CD8. The arrowheads indicate CTLs. **(A)** Double staining with HLA-A*0201/Tax11-19 tetramer and anti-CD8 mAb (Patient 8624). HTLV-1–specific CTLs are observed in the parenchyma and comprise 29% of CD8-positive cells in the field. **(B)** Double staining with HLA-A*2402/Tax301-309 tetramer and anti-CD8 mAb (Patient 6315). The CTLs comprise 25% of CD8-positive cells in the field. **(C)** Double staining with HLA-A*2402/Tax301-309 tetramer and anti-CD8 mAb (Patient 6664). CTLs are visible in the perivascular area and comprise 11% (1 of 9) of CD8-positive cells in the field. **(D)** Double staining with HLA-A*2402/HIV Gag tetramer (tetramer control) and CD8 in Patient 6315. No cells are stained with the tetramer. **(E)** To corroborate the staining with the tetramer, an HLA pentamer was used for double staining (Patient 8624). Similarly, fluorescence of the HLA-A*02/Tax11-19 pentamer (red in left-hand column) is exclusively colocalized with that of CD8 (green in middle column), as shown by yellow in the merged image (right-hand column) in the thickened meninges. The percentage of Tax pentamer–positive cells in CD8-positive cells is 31% (5 of 16) of the CD8-positive cells in the field. White bars indicate 20 μm.

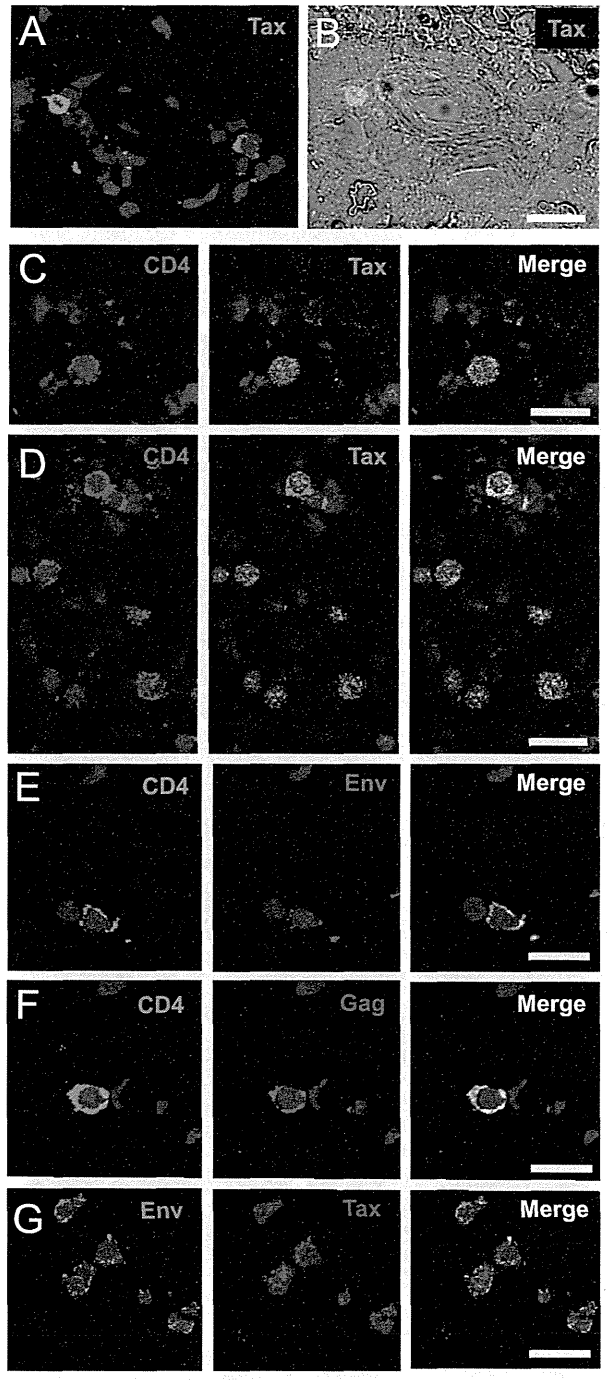

FIGURE 4. Detection of CD4-positive human T-lymphotropic virus type-1 (HTLV-1)–infected cells in CNS parenchyma. **(A, B)** Cells expressing HTLV-1 Tax protein were found near the vessels of the spinal cord of Patient 6315. **(C, D)** Double staining for CD4 (purple) and HTLV-1 Tax (green) revealed that visualized HTLV-1 Tax showed a patchy staining pattern in the nuclei in parenchyma **(C)** and in meninges **(D)**. **(E)** Double staining for CD4 (green) and HTLV-1 Env protein (red) revealed that HTLV-1 Env is costained with CD4 on the cell surface. **(F)** Double staining for CD4 (green) and HTLV-1 Gag protein (red) revealed that HTLV-1 Gag is detected in the cytoplasm of CD4-positive cells. **(G)** Double staining for HTLV-1 Env (green) and HTLV-1 Tax (red) revealed that both proteins were detected separately in the same cells. The nuclei were counterstained with DAPI. White bars indicate 20 μm.

load (12). We also failed to detect any HTLV-1 proteins by the immunohistochemical or flow cytometric study in PBMCs from 20 HAM/TSP patients, even though the proteins became detectable after short-term culturing (Figure, Supplemental Digital Content 1, part C, http://links.lww.com/NEN/A676). Although the evidence of both vigorous persistent CTLs immune responses and increased IgM antibody specific for HTLV-1 in the peripheral blood of HAM/TSP patients have suggested that

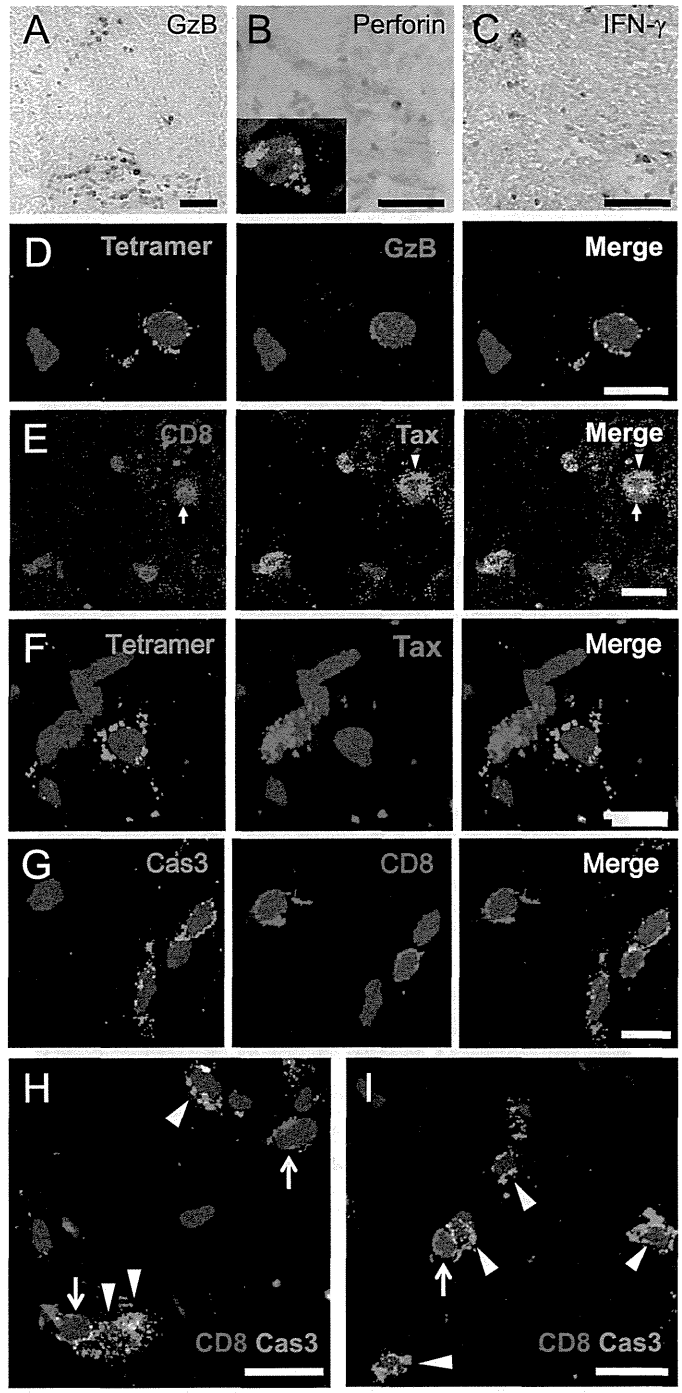

FIGURE 5. Cytotoxic T-lymphocyte (CTL) molecules in the CNS. **(A–C)** Immunohistochemistry shows granzyme B (GzB)–positive cells **(A)**, perforin-positive cells **(B)**, and interferon-γ (IFN-γ)–positive cells **(C)** in the perivascular area of the spinal cord of Patient 8624. Black bars indicate 100 μm. **(D)** Double staining with HLA-A*2402/Tax301-309-tetramer (green) and anti–granzyme B monoclonal antibody (mAb) (red) reveals a GzB-positive HTLV-1–specific CTL in the parenchyma of the spinal cord (Patient 6315). **(E)** A cell expressing HTLV-1 Tax protein is in contact with a CD8-positive cell in the spinal cord of Patient 6315. **(F)** An HTLV-1 Tax–specific CTL is next to the cell expressing Tax protein in the spinal cord of Patient 8614. **(G–I)** Double staining for active caspase-3 (Cas3) (green) and CD8 (red, arrows in **[H]** and **[I]**) reveals CD8-positive cells in contact with active caspase-3–positive cells in the spinal cord of Patient 8624. Nuclei were counterstained with DAPI. White bars indicate 10 μm.

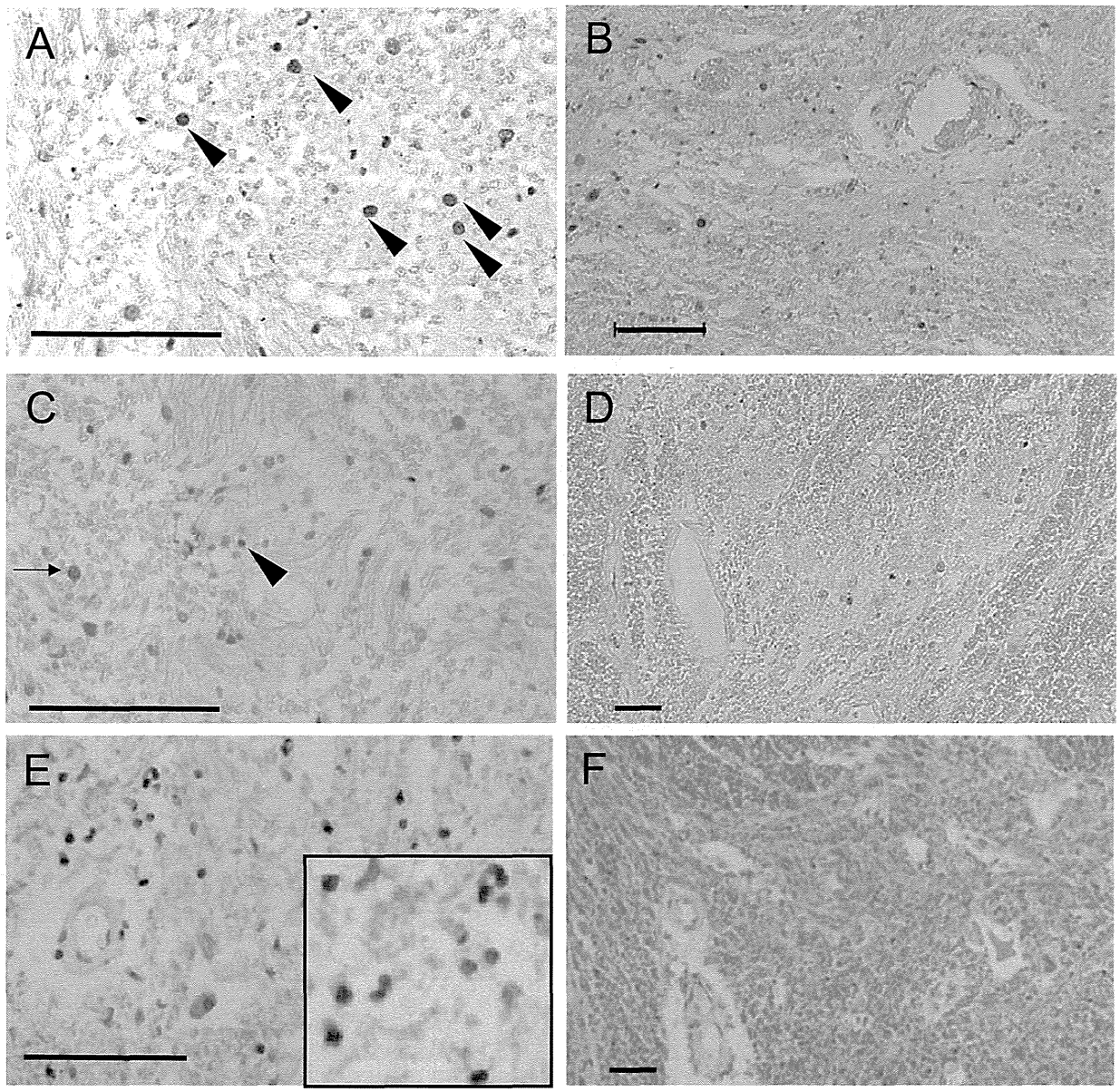

FIGURE 6. Detection of apoptotic cells in the CNS. **(A)** Some small cells are apoptotic (DAB; brown, arrowheads) detected by anti–active caspase-3 antibody (Ab). **(B–D)** TdT-mediated dUTP nick end labeling assay. **(B)** A number of apoptotic cells (DAB; brown) are detected in the spinal cord of a patient with HTLV-1–associated myelopathy/tropical spastic paraparesis (Patient 8624). **(C)** Some infiltrating small cells around a small vessel (arrowhead) and some relatively large cells in the parenchyma (arrow) are apoptotic. **(D)** The apoptotic cells are barely detectable in the control spinal cord from an HTLV-1–seronegative patient with hepatoma. **(E, F)** Anti-single-stranded DNA antibody staining. **(E)** Numerous apoptotic cells (AEC; red) are detected in the spinal cord (Patient 8624). A higher magnification picture in the inset shows apoptotic cells. **(F)** Apoptotic cells are barely detectable in the control patient spinal cord. Scale bar = 100 μm.

HTLV-1–infected cells could express viral antigens anywhere in the body of the infected individuals (14, 32), the expression of HTLV-1 proteins in vivo has remained elusive so far.

In this study, we succeeded in detecting HTLV-1 proteins in the CD4-positive T cells infiltrating the CNS. This is consistent with our previous reports in which HTLV-1–infected cells were determined to be CD4-positive lymphocytes in the CNS by in situ hybridization for HTLV-1 mRNA and in situ polymerase chain reaction for HTLV-1 DNA (33, 34). The infiltrating HTLV-1–infected CD4-positive cells may easily express the viral antigens in the CNS, which in turn facilitates the accumulation of HTLV-1–specific CTLs.

Human T-lymphotropic virus type-1 infection causes several organ-specific inflammatory diseases including HAM/TSP (2, 3). Previous reports demonstrating that HTLV-1 proviral loads are high in affected organs such as the muscles, lungs, and CNS suggest that HTLV-1–infected cells accumulate in the organs (13, 35). The pathogenesis model in which both

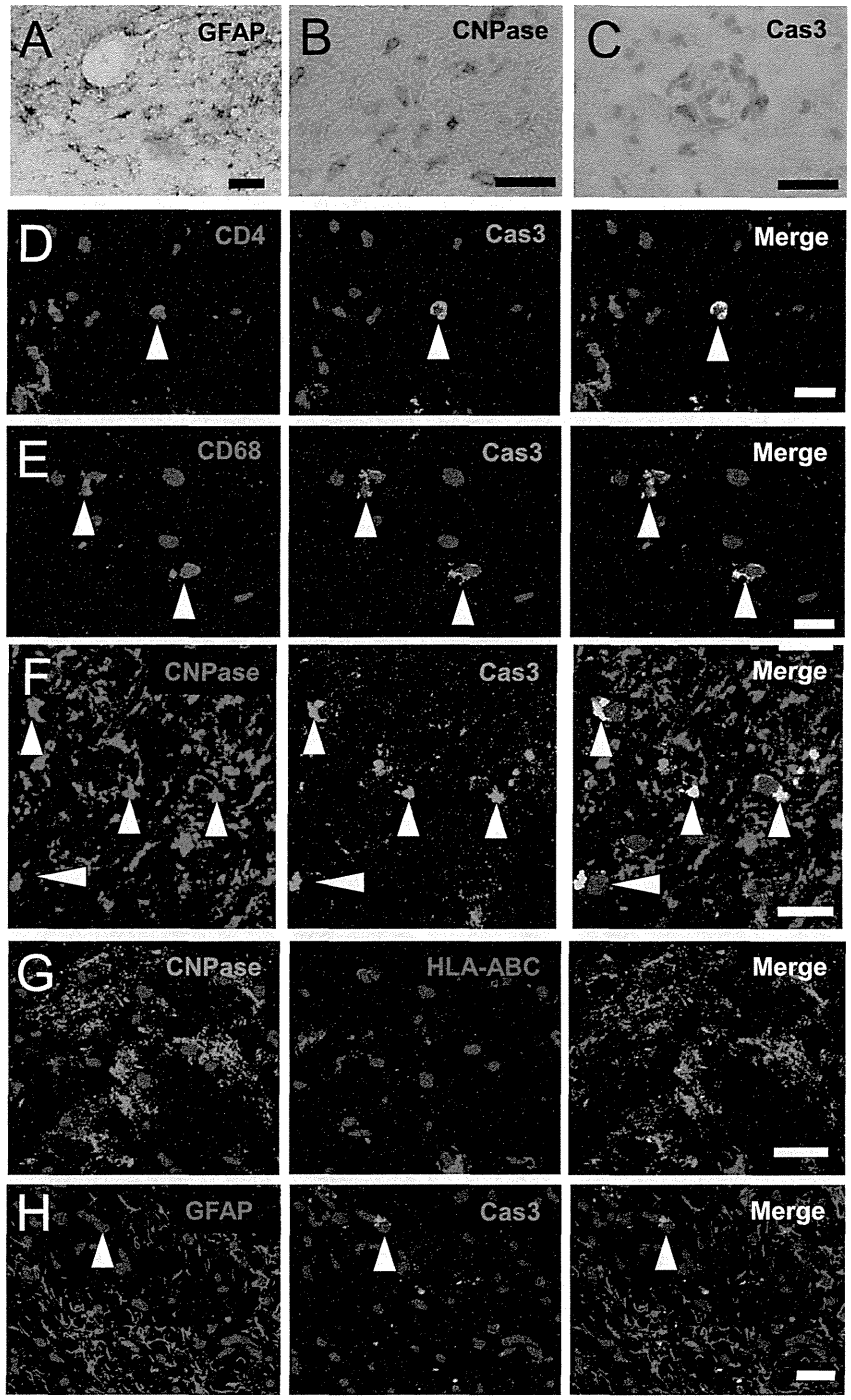

FIGURE 7. Cell identification of apoptotic cells. **(A–C)** Astrocytes **(A)**, oligodendrocytes **(B)**, and apoptotic cells **(C)** were stained with anti–glial fibrillary acidic protein (GFAP) antibody (Ab), anti–2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase) monoclonal antibody (mAb), or anti–active caspase-3 Ab, respectively, in the spinal cord of Patient 8624. Nuclei were counterstained with hematoxylin. **(D–F, H)** Double staining revealed that a CD4-positive cell (red, **D**), a CD68-positive cell (red, **E**), and some oligodendrocytes (red, **F**), but no astrocytes (red, **H**), were apoptotic (green) (arrowheads) in the spinal cord of Patient 8624. **(G)** Double staining with anti-CNPase mAb (green) and anti–HLA-ABC mAb (red) revealed that no oligodendrocyte expresses HLA-ABC. There is no double-positive signal (yellow) in the merged image. White bars indicate 20 μm. Cas3, active caspase-3.

HTLV-1–infected CD4-positive T cells and the virus-specific CD8-positive CTLs infiltrate the organs from the peripheral blood followed by bystander tissue damage may explain why HTLV-1 infection can cause several chronic inflammatory diseases in various organs. Further studies are needed to determine whether the similar immunopathologic model can be applied to HTLV-1–associated inflammatory diseases in other organs.

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REVIEW

Neuroimmunological aspects of human T cell leukemia virus type 1-associated myelopathy/tropical spastic paraparesis

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Abstract Human T cell leukemia virus type 1 (HTLV-1) is a human retrovirus etiologically associated with adult T cell leukemia/lymphoma and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Only approximately 0.25–4 % of infected individuals develop HAM/TSP; the majority of infected individuals remain lifelong asymptomatic carriers. Recent data suggest that immunological aspects of host–virus interactions might play an important role in the development and pathogenesis of HAM/TSP. This review outlines and discusses the current understanding, ongoing developments, and future perspectives of HAM/TSP research.

Keywords HTLV-1 · HAM/TSP · Host immune response

Introduction

Human T cell leukemia virus type 1 (HTLV-1) is a replication-competent human retrovirus associated with two distinct types of disease: a malignancy of mature $CD4^+$ T cells called adult T cell leukemia/lymphoma (ATL) (Hinuma et al. 1981; Poiesz et al. 1980; Yoshida et al. 1984) and a chronic inflammatory central nervous system disease HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al. 1985; Osame et al. 1986). Like human immunodeficiency virus (HIV), HTLV-1 is never eliminated from the host despite vigorous cellular and humoral immune responses. However, in contrast to HIV infection, few with HTLV-1 develop disease; only approximately 2–3 % of infected persons develop ATL (Tajima 1990), another 0.25–4 % develop HAM/TSP (Hisada et al. 2004; Kramer et al. 1995; Nakagawa et al. 1995; Osame et al. 1990), and the majority of infected individuals remain lifelong asymptomatic carriers (ACs). Therefore, evaluation of the individual risk of developing disease in ACs would certainly be of considerable importance, especially in HTLV-1 endemic areas.

The viral, host, and environmental risk factors as well as the host immune response against HTLV-1 infection appear to regulate the development of HTLV-1-associated diseases (Bangham and Osame 2005). In particular, a strong immune response, especially the cytotoxic T lymphocyte (CTL) response, to HTLV-1 is seen in patients with HAM/TSP and suggested to be strongly associated with the pathogenesis of HTLV-1-associated diseases (Matsuura et al. 2010; Saito et al. 2012). For more than two decades, the investigation of HTLV-1-mediated immunopathogenesis has focused on Tax, an HTLV-1-encoded viral oncoprotein, because Tax activates many cellular genes by binding to groups of transcription factors and co-activators and is necessary and sufficient for cellular transformation. However, recent reports have identified that another regulatory protein, HTLV-1 basic leucine zipper factor (HBZ), also has a critical role in the development of ATL and HAM/TSP (Matsuoka and Jeang 2011). This review summarizes past and recent studies of HAM/TSP, attempting to answer the following fundamental questions: Why do some HTLV-1-infected people develop disease whereas the vast majority remain healthy? How does HTLV-1 persist in the individual host despite a strong host immune response? How is the inflammatory lesion in HAM/TSP initiated and maintained?

History and epidemiology of HTLV-1

HTLV-1 belongs to the *Deltaretrovirus* genus of the *Orthoretrovirinae* subfamily and infects 10–20 million people worldwide (de The and Bomford 1993; Proietti et al. 2005; Uchiyama 1997). HTLV-1 can be transmitted through sexual contact (Roucoux et al. 2005), intravenous drug use (Proietti et al. 2005), and breastfeeding from mother to child (Hino

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et al. 1985; Kinoshita et al. 1987). At present, the infection is endemic in southwest Japan, the Caribbean, Sub-Saharan Africa, and South America, with smaller foci in Southeast Asia, South Africa, and northeast Iran (Verdonck et al. 2007). HTLV-1 was initially isolated in 1980 from two T cell lymphoblastoid cell lines and the blood of a patient originally thought to have a cutaneous T cell lymphoma (Poiesz et al. 1980). It was the first retrovirus ever associated with cancer in a human. Three years before the isolation of HTLV-1, Takatsuki et al. reported ATL, a rare form of leukemia endemic to southwest Japan, as a distinct clinical entity (Uchiyama et al. 1977). In 1981, Hinuma et al. clearly demonstrated that ATL was caused by a new human retrovirus, originally termed ATLV (Hinuma et al. 1981; Miyoshi et al. 1981). Since then, ATLV and HTLV have been shown to be identical, and a single name, HTLV-1, has been adopted. In the mid-1980s, epidemiological data linked HTLV-1 infection to a chronic progressive neurological disease, which was termed tropical spastic paraparesis in the Caribbean (Gessain et al. 1985) and HTLV-1-associated myelopathy in Japan (Osame et al. 1986). HTLV-1-positive TSP and HAM were subsequently found to be clinically and pathologically identical, and the disease was given a single designation as HAM/TSP (Hollsberg and Hafler 1993). To date, more than 3,000 cases of HAM/TSP have been reported in HTLV-1 endemic areas. Sporadic cases have also been described in non-endemic areas such as the USA and Europe, mainly in immigrants from an HTLV-1 endemic area (Araujo and Silva 2006). HTLV-1 can cause other chronic inflammatory diseases such as uveitis (Mochizuki et al. 1992), arthropathy (Nishioka et al. 1989), pulmonary lymphocytic alveolitis (Maruyama et al. 1988; Sugimoto et al. 1989; Sugimoto et al. 1987), polymyositis (Higuchi et al. 1992; Morgan et al. 1989), Sjögren syndrome (Terada et al. 1994), and infective dermatitis (LaGrenade et al. 1990), although there is no clear evidence for an etiological role of HTLV-1 in these diseases.

Clinical and pathological features of HAM/TSP

HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities (Nakagawa et al. 1996). The period from initial HTLV-1 infection to the onset of HAM/TSP is assumed to range from months to decades, a shorter time than that for the onset of ATL (Nakagawa et al. 1995; Olindo et al. 2006). HAM/TSP occurs both in vertically infected individuals and in those who become infected later in life, that is, through sexual contact almost exclusively from male to female, intravenous drug use, contaminated blood transfusions, etc. The mean age at onset is 43.8 years, and like autoimmune diseases, the frequency of cases of HAM/TSP is greater in women than in men (the male-to-female ratio of occurrence is 1:2.3) (Nakagawa et al. 1995). In addition to HTLV-1 antibody (Ab) positivity both in serum and cerebrospinal fluid (CSF), the presence of atypical lymphocytes (the so-called "flower cells") in peripheral blood and CSF, a moderate pleocytosis, and raised protein content in CSF is observed in patients with HAM/TSP (Araujo and Silva 2006). Oligoclonal immunoglobulin bands in the CSF; raised concentrations of inflammatory markers such as neopterin, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and interferon (IFN)-γ; and increased intrathecal Ab synthesis specific for HTLV-1 antigens have also been described (Jacobson 2002). Clinical progression of HAM/TSP is associated with an increase in the proviral load (PVL) in individual patients, and a high ratio of PVL in CSF cells/peripheral blood mononuclear cells (PBMCs) is also significantly associated with clinically progressive disease (Takenouchi et al. 2003). Thus, a pro-inflammatory environment associated with increased numbers of HTLV-1-infected cells is a characteristic immunological profile of HAM/TSP.

Pathological analysis of HAM/TSP autopsy materials showed the loss of myelin and axons in the lateral, anterior, and posterior columns of the spinal cord. These lesions are associated with perivascular and parenchymal lymphocytic infiltration with the presence of foamy macrophages, reactive astrocytosis, and fibrillary gliosis, predominantly at the thoracic level (Iwasaki 1990; Izumo et al. 2000; Yoshioka et al. 1993), suggesting that the immune response against HTLV-1 causes the inflammatory spinal cord damage seen in patients with HAM/TSP (Bangham 2000). In patients with active chronic lesions in the spinal cord, perivascular inflammatory infiltration with similar composition of cell subsets was also seen in the brain (Aye et al. 2000). The peripheral nerve pathology of patients with HAM/TSP with sensory disturbance showed varying degrees of demyelination, remyelination, axonal degeneration, regeneration, and perineural fibrosis (Bhigjee et al. 1993; Kiwaki et al. 2003).

Treatment of HAM/TSP

To date, no generally agreed standard treatment regimen has been established for HAM/TSP, and no treatment has proven to be consistently effective on a long-term basis. Therefore, clinical practice for treatment of patients with HAM/TSP is based on case series and open, nonrandomized, uncontrolled studies. Mild to moderate beneficial effects have been reported for a number of agents in open-label studies including corticosteroids (Nakagawa et al. 1996), danazol (Harrington et al. 1991), pentoxifylline (Shirabe et al. 1997), immunosuppressants such as ciclosporin A (Martin et al. 2012), high-dose intravenous gamma globulin (Kuroda et al. 1991), plasmapheresis (Matsuo et al. 1988), antibiotics (erythromycin and fosfomycin), and vitamin C (Nakagawa et al. 1996). It should be noteworthy that oral prednisolone was effective in 81.7 %

of 131 patients in a large-scale case series study (Nakagawa et al. 1996). However, the complications of corticosteroids limit their use, particularly in post-menopausal women, who are at higher risk for developing HAM/TSP. Multicenter double-blind randomized placebo-controlled trials for the IFN-α treatment indicate that IFN-α is an effective therapy with an acceptable side effects profile (Izumo et al. 1996), although the benefit of long-term IFN-α therapy has not been well studied. In regard to oral antiviral drugs zidovudine plus lamivudine, no evidence of significant benefit yet exists from randomized placebo-controlled trials (Taylor et al. 2006). Recently, oral administration of histone deacetylase inhibitor valproic acid (VPA) has been conducted as a single-center, open-label trial (Olindo et al. 2011). Although administration of VPA induced a transient increase of HTLV-1 expression to expose virus-positive cells to the host immune response, clinical measures and PVL were stable overall. It has also been reported that the antibiotic minocycline significantly inhibited spontaneous lymphocyte proliferation and degranulation/IFN-γ expression in $CD8^+$ T cells of patients with HAM/TSP, suggesting its potential for treatment (Enose-Akahata et al. 2012). Overall, more clinical trials with adequate power are needed in the future.

Risk factors for developing HAM/TSP

It is well-known that HAM/TSP occurs in only a minority of HTLV-1-infected individuals. A previous population association study in HTLV-1 endemic southwest Japan revealed that one of the major risk factors is the HTLV-1 PVL, because the PVL is significantly higher in patients with HAM/TSP than in ACs (Nagai et al. 1998). A higher PVL in patients with HAM/TSP than in ACs was also observed in other endemic areas such as the Caribbean (Manns et al. 1999), South America (Adaui et al. 2006), and the Middle East (Sabouri et al. 2005). In southwest Japan, it was suggested that genetic factors such as the human leukocyte antigen (HLA) genotype are related to the high PVL in patients with HAM/TSP and genetic relatives. Namely, possession of the HLA class I genes HLA-A*02 and Cw*08 was associated with a statistically significant reduction in both HTLV-1 PVL and the risk of HAM/TSP, whereas possession of HLA class I HLA-B*5401 and class II HLA-DRB1*0101 predispose to HAM/TSP in the same population (Jeffery et al. 2000; Jeffery et al. 1999). Because the function of class I HLA proteins is to present antigenic peptides to CTL, these results imply that individuals with HLA-A*02 or HLA-Cw*08 mount a particularly efficient CTL response against HTLV-1, which may be an important determinant of PVL and the risk of HAM/TSP. In accordance with this observation, it has been reported that CTL spontaneously kill autologous HTLV-1-infected cells ex vivo (Hanon et al. 2000), granzymes and perforin are more highly expressed in individuals with a low PVL (Vine et al. 2004), and the lytic efficiency of the CD8+ T cell response (i.e., the fraction of autologous HTLV-1-expressing cells eliminated per CD8+ T cell per day) was inversely correlated with both PVL and the rate of spontaneous proviral expression (Kattan et al. 2009). Furthermore, the major histocompatibility complex (MHC) class I tetramer analysis of lymphocytes isolated from the CSF of patients with HAM/TSP showed even higher frequencies of HTLV-1 Tax11-19-specific, HLA-A*02-restricted CD8+ lymphocytes compared with PBMCs (Nagai et al. 2001b). These findings indicate that an increased proliferation or migration of HTLV-1-infected and/or HTLV-1-specific lymphocytes to the central nervous system might be closely associated with the pathogenesis of HAM/TSP (Hayashi et al. 2008a), and the CTLs against HTLV-1 reduce both PVL and the risk of HAM/TSP. Recently, using a combination of computational and experimental approaches, MacNamara et al. reported that a CTL response against HBZ restricted by protective HLA alleles such as HLA-A*02 or Cw*08, but not a response to the immunodominant protein Tax, also determines the outcome of HTLV-1 infection (Macnamara et al. 2010).

Meanwhile, analysis of non-HLA host genetic factors by candidate gene approaches revealed that non-HLA gene polymorphisms also affect the risk of developing HAM/TSP. Namely, the TNF-α promoter −863 A allele (Vine et al. 2002) and the longer CA repeat alleles of matrix metalloproteinase 9 promoter (Kodama et al. 2004) predisposed to HAM/TSP, whereas IL-10 −592 A (Sabouri et al. 2004), stromal-derived factor 1 +801A (Vine et al. 2002), and IL-15 +191 C alleles (Vine et al. 2002) conferred protection against HAM/TSP. The polymorphisms in the matrix metalloproteinase 9 and IL-10 promoters were each associated with differences in the HTLV-1 Tax-mediated transcriptional activity of the respective gene (Kodama et al. 2004; Sabouri et al. 2004). However, the contributions of these non-HLA genes to the pathogenesis of HAM/TSP are largely unknown and these data have not yet been reproduced in different populations. Further candidate gene studies together with genome-wide association studies in different ethnic populations in a larger sample size may provide evidence for the association of non-HLA genes with the pathogenesis of HAM/TSP.

It has been reported that the lifetime risk of developing HAM/TSP differs among ethnic groups, ranging between 0.25 and 4 %. The annual incidence of HAM/TSP is higher among Jamaican subjects than among Japanese subjects (20 versus 3 cases/100,000 population), with a two to three times greater risk for women in both populations (Hisada et al. 2004; Kramer et al. 1995; Nakagawa et al. 1995; Osame et al. 1990). Although most studies of HTLV-1 genotype have reported no association between variants of HTLV-1 and the risk of HAM/TSP, Furukawa et al. reported the association between HTLV-1 Tax gene variation and the risk of HAM/TSP (Furukawa et al. 2000). Tax subgroup A, which belongs to cosmopolitan

subtype A, was more frequently observed in patients with HAM/TSP, and this association was independent of the protective effect of HLA-A*02. Interestingly, HLA-A*02 appeared to give protection against only one of the two prevalent sequence variants of HTLV-1, Tax subgroup B, which belongs to cosmopolitan subtype B, but not against Tax subgroup A in the Japanese population (Furukawa et al. 2000). Jamaican subjects, who had a higher annual incidence of HAM/TSP, also have cosmopolitan subtype A, whereas approximately 80 % of Japanese subjects, who had a lower annual incidence of HAM/TSP, have cosmopolitan subtype B. Interestingly, HLA-A*02 did not appear to provide protection against HAM/TSP development with cosmopolitan subtype A in a population in Iran (Sabouri et al. 2005).

To test whether the genomic integration site determines the abundance and the pathogenic potential of an HTLV-1-positive T cell clone, Gillet et al. recently reported the results of high-throughput mapping and quantification of HTLV-1 proviral integration in the host genome (Gillet et al. 2011). They mapped >91,000 unique insertion sites (UISs) of the provirus in primary PBMCs from 61 HTLV-1-infected individuals and showed that a typical HTLV-1-infected host carries between 500 and 5,000 UISs in 10 μg of PBMC genomic DNA. They calculated an oligoclonality index to quantify the clonality of HTLV-1-infected cells in vivo and found that the oligoclonality index did not distinguish between ACs and patients with HAM/TSP and that there was no correlation between the oligoclonality index and HTLV-1 PVL in either ACs or patients with HAM/TSP. These results indicate that the higher PVL observed in patients with HAM/TSP was attributable to a larger number of UISs but not, as previously thought, to a difference in clonality. They also obtained evidence that the abundance of established HTLV-1 clones is determined by genomic features of the host DNA flanking the provirus. Namely, HTLV-1 clonal expansion in vivo is favored by a proviral integration site near a region of host chromatin undergoing active transcription or same-sense transcriptional orientation of the provirus. In contrast, negative selection of infected clones, probably by CTLs during chronic infection, favors establishment of proviruses integrated in transcriptionally silenced DNA, and this selection is more efficient in ACs than in HAM/TSP, indicating the selection of HTLV-1-infected T cell clones with low pathogenic potential. More recent reports indicate that circulating HTLV-1-positive cells each contain a single integrated proviral copy (Cook et al. 2012), and cells expressing HTLV-1 Tax protein (i.e., viral protein expression) were significantly more frequent in clones of low abundance in vivo, whereas certain transcription start sites immediately upstream of the viral integration site were associated with virus latency (i.e., no viral protein expression). In particular, Tax-expressing, more "pathogenic" clones were efficiently controlled by the immune response, especially CTLs, whereas non-Tax-expressing "invisible" infected clones were associated with mitotic clonal expansion in vivo (Melamed et al. 2013).

The innate immune response in HAM/TSP

Type I IFN is a key innate immune cytokine produced by cells in response to viral infection. The type I IFN response protects cells against invading viruses by inducing the expression of IFN-stimulated genes, which execute the antiviral effects of IFN (Samuel 2001). The IFN-stimulated genes then generate soluble factors including cytokines that activate adaptive immunity or directly inhibit the virus itself (Liu et al. 2011). In PBMCs of HTLV-1-infected individuals, the level of HTLV-1 messenger RNA is very low and viral protein is not detectable, but these molecules are rapidly expressed after a short time in culture in vitro (Hanon et al. 2000). However, the mechanisms of this phenomenon are largely unknown. Recently, it has been reported that HTLV-1 expression in HTLV-1-infected T cells is suppressed by stromal cells (i.e., epithelial cells and fibroblasts) in culture through type I IFNs (Kinpara et al. 2009). Namely, HTLV-1 Gag protein expression was suppressed when contacted with stromal cells and restored when separated from the stromal cells. Although neutralizing antibodies against human IFN-α/β receptor only partly abrogated this phenomenon, the results indicate that the innate immune system suppresses HTLV-1 expression in vitro and in vivo, at least through type I IFN. More recently, it has been reported that IFN-stimulated genes were overexpressed in circulating leukocytes and the expression correlated with the clinical severity of HAM/TSP (Tattermusch et al. 2012).

Previous reports indicated that patients with HAM/TSP had both a lower frequency and a lower activity of natural killer (NK) cells (especially the CD3+ CD16+ subset) than ACs, although the results were not normalized with respect to the PVL (Yu et al. 1991). Because an important mechanism of induction of NK cell-mediated killing is recognition by the NK cell of a complex of the non-polymorphic MHC molecule HLA-E bound to a peptide derived from the signal sequence of some other MHC class I molecules, the synthetic tetramers of HLA-E with the HLA-G signal sequence peptide were used to identify NK cells in patients with HAM/TSP (Saito et al. 2003). The results clearly showed a lower frequency of HLA-E tetramer-binding cells in patients with HAM/TSP than in ACs; as in the earlier studies (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 ACs (Saito et al. 2003). Recent reports also suggest that the frequency of invariant natural killer T (NKT) cells in the peripheral blood of patients with HAM/TSP is significantly decreased when compared with that in healthy subjects and/or ACs (Azakami et al. 2009; Ndhlovu et al. 2009). These findings indicated that the activity of the NK or NKT cell response was associated with the presence or absence of HAM/TSP. Interestingly, a previous uncontrolled preliminary trial of viable *Lactobacillus casei* strain Shirota-containing

fermented milk in patients with HAM/TSP resulted in a significant increase in NK cell activity with improvements in clinical symptoms (Matsuzaki et al. 2005). Thus, circulating NK and NKT cells might also play an important role in the disease progression and pathogenesis of HAM/TSP.

The acquired immune response in HAM/TSP

It has been reported that patients with HAM/TSP generally have higher anti-HTLV-1 Ab titers than ACs with a similar PVL (Ishihara et al. 1994; Kira et al. 1992; Nagasato et al. 1991), suggesting the existence of an augmented humoral immune response to HTLV-1. Interestingly, although Ab responses to the immunodominant epitopes of the HTLV-1 Envelope (Env) proteins were similar in all three clinical groups of HTLV-1 infection (HAM/TSP, ATL, and ACs), reactivity to four Tax immunodominant epitopes was higher in patients with HAM/TSP (71–93 %) than in patients with ATL (4–31 %) or ACs (27–37 %) (Lal et al. 1994). A recent report indicates that the Ab response against HBZ was associated with reduced CD4+ T cell activation in patients with HAM/TSP, and HBZ-specific Ab inhibited spontaneous in vitro lymphocyte proliferation in the PBMCs of patients with HAM/TSP (Enose-Akahata et al. 2013). Among these anti–HTLV-1 antibodies, anti-Env Ab is particularly important because some anti-Env Abs have neutralizing activity against HTLV-1. Antisera raised against recombinant HTLV-1 Env polypeptides (Kiyokawa et al. 1984; Nakamura et al. 1987), vaccinia virus containing the HTLV-1 env gene (Hakoda et al. 1995; Shida et al. 1987), immunization with neutralizing epitope peptides (Tanaka et al. 1994), and passive transfer of human immunoglobulin G that has neutralizing activity (Murata et al. 1996; Tanaka et al. 1993) were all shown to neutralize HTLV-1 infectivity. In HTLV-1 infection, the roles of HTLV-1 neutralizing Ab in vivo are still largely unknown. It will be interesting to examine whether HTLV-1 neutralizing Ab titers correlate with disease status and PVL in infected individuals. Because the mutation rate of HTLV-1 provirus is significantly lower than that of HIV-1, passive immunization with human monoclonal Ab may be a beneficial and effective method to prevent HTLV-1 infection.

Antiviral CD4+ T cell responses are of central importance in driving B cell and CD8+ T cell responses in vivo. The most common HTLV-1 antigen recognized by CD4+ T cells is the Env protein (Goon et al. 2004b; Kitze et al. 1998), in contrast to the immunodominance of Tax in the CD8+ T cell response (Goon et al. 2004a; Jacobson et al. 1990; Kannagi et al. 1991). At a similar PVL, patients with HAM/TSP had a significantly increased frequency of virus-specific CD4+ T cells compared with ACs (Goon et al. 2004b; Nose et al. 2007). The antiviral T-helper (Th) 1 phenotype is also dominant among HTLV-1-specific CD4+ T cells in both ACs and patients with HAM/TSP (Goon et al. 2002), and there is a higher frequency of IFN-γ, TNF-α, and IL-2 production by CD4+ T cells in patients with HAM/TSP compared with ACs of a similar PVL (Goon et al. 2002; Goon et al. 2003). A role for CD4+ T cells in initiating and causing HAM/TSP is also consistent with the immunogenetic observations that the possession of HLA-DRB1*0101, which restricts the immunodominant epitope of HTLV-1 Env gp21, was associated with susceptibility to HAM/TSP in independent HTLV-1-infected populations in southern Japan (Jeffery et al. 1999, 2000) and northeast Iran (Sabouri et al. 2005). Accordingly, a synthetic tetramer of DRB1*0101 and the immunodominant HTLV-1 Env380-394 peptide was used to analyze Env-specific CD4+ T cells directly ex vivo (Nose et al. 2007). The results showed that the frequency of tetramer+ CD4+ T cells was significantly higher in patients with HAM/TSP than in ACs with a similar PVL. Furthermore, direct ex vivo analysis of tetramer+ CD4+ T cells from two unrelated DRB1*0101-positive patients with HAM/TSP indicated that certain T cell receptor Vβs were utilized and antigen-specific amino acid motifs were identified in complementarity determining region 3 from both patients. These results suggest that the observed increase in virus-specific CD4+ T cells in patients with HAM/TSP, which may contribute to CD4+ T cell-mediated antiviral immune responses and to an increased risk of HAM/TSP, was not simply due to the rapidly growing HTLV-1-infected CD4+ T cells but was the result of in vivo selection by specific MHC–peptide complexes, as observed in freshly isolated HLA-A*0201/Tax11-19 tetramer+ CD8+ T cells (Saito et al. 2001) and muscle-infiltrating cells from patients with HAM/TSP and HTLV-1-infected patients with polymyositis (Saito et al. 2002).

Previous reports indicated that HTLV-1-specific CD8+ CTLs are typically abundant, chronically activated, and mainly targeted to the viral transactivator protein Tax (Bangham 2000). Further, as already mentioned, the median PVL in PBMCs of patients with HAM/TSP was more than 10 times higher than that in ACs, and a high PVL was also associated with an increased risk of progression to disease (Nagai et al. 1998). Furthermore, HLA-A*02 and HLA-Cw*08 genes were independently and significantly associated with a lower PVL and a lower risk of HAM/TSP (Jeffery et al. 2000; Jeffery et al. 1999), and CD8+ T cells efficiently kill autologous Tax-expressing lymphocytes in fresh PBMCs in HTLV-1-infected individuals (Hanon et al. 2000). These data have raised the hypothesis that the class I-restricted CD8+ CTL response plays a critical part in limiting HTLV-1 replication in vivo and that genetically determined differences in the efficiency of the CTL response to HTLV-1 account for the risk of developing HAM/TSP. The analysis of gene expression profiles using microarrays in circulating CD4+ and CD8+ lymphocytes indicated that granzymes and perforin are more highly expressed in individuals with a low PVL (Vine et al. 2004), suggesting that a strong CTL response is associated

with a low PVL and a low risk of HAM/TSP. In accordance with this observation, the lytic capacity of HTLV-1-specific CTLs in patients with HAM/TSP and ACs quantified by a CD107a mobilization assay showed significantly lower CD107a staining in HTLV-1-specific CTLs in patients with HAM/TSP than in ACs (Sabouri et al. 2008); this suggests that patients with HAM/TSP have a high frequency of HTLV-1-specific CD8+ T cells with poor lytic capacity, whereas ACs have a lower frequency of cells with high lytic capacity. Moreover, it has been reported that the high CTL avidity, which is closely associated with the lytic efficiency of CTLs, correlates with low PVL and proviral gene expression (Kattan et al. 2009), indicating that the efficient control of HTLV-1 in vivo depends on the quality of CTLs, which determines the position of virus–host equilibrium and also the outcome of persistent HTLV-1 infection. More recently, MacNamara et al. (Macnamara et al. 2010) showed that HLA class 1 alleles, which strongly bind oligopeptides from the HBZ protein, enable the host to have a more effective immune response against HTLV-1; therefore, such individuals have a lower PVL and are more likely to be asymptomatic. Another recent report showed the presence of HBZ-specific CD4+ and CD8+ cells in vivo in patients with HAM/TSP and in ACs and a significant association between the HBZ-specific CD8+ cell response and asymptomatic HTLV-1 infection (Hilburn et al. 2011). These findings provide strong evidence to support the hypothesis of the crucial role of CTLs and confirm the importance of HBZ for persistent infection. However, because the frequency of HTLV-1-specific CD8+ T cells was significantly elevated in patients with HAM/TSP compared with ACs (Greten et al. 1998; Nagai et al. 2001a), and these cells have the potential to produce proinflammatory cytokines (Kubota et al. 1998), there is debate on the role of HTLV-1-specific CD8+ T cells, namely, whether these cells contribute to the inflammatory and demyelinating processes of HAM/TSP or whether the dominant effect of such cells in vivo is protective against disease, although a protective role and a pathogenic role of CTLs are not mutually exclusive. Indeed, there are other examples of viral infections in which the virus-specific CTLs exert both beneficial (antiviral) and detrimental (inflammatory) effects, such as lymphocytic choriomeningitis virus infection in the mouse (Klenerman and Zinkernagel 1997). It is difficult to separate cause and effect in analyzing the association between T cell attributes and the efficiency of viral control in a persistent infection at equilibrium.

Regulatory T cells (Tregs) are important mediators of peripheral immune tolerance and play an important role in chronic viral infections. HTLV-1 preferentially and persistently infects CD4+ CD25+ lymphocytes in vivo (Yamano et al. 2005), which contain the majority of the Foxp3+ Tregs (Sakaguchi et al. 2006). In patients with HAM/TSP, the percentage of Foxp3+ Tregs in CD4+ CD25+ cells is lower than that in ACs and uninfected healthy controls (Oh et al. 2006; Yamano et al. 2005), whereas the percentage of Foxp3+ cells in the CD4+ population tends to be higher in patients with HAM/TSP than in ACs (Best et al. 2009; Hayashi et al. 2008b; Toulza et al. 2008). Because CD25 is induced by HTLV-1 Tax oncoprotein (Inoue et al. 1986), the proportion of Foxp3+ cells falls in the CD4+ CD25+ population, which contains both Tregs and activated non-Tregs, in HTLV-1-infected individuals, especially patients with HAM/TSP. Therefore, it is inappropriate to use CD25 as a marker of Tregs in HTLV-1 infection, and the best current working definition of Treg phenotype is CD4+ Foxp3+. The high frequency of CD4+ Foxp3+ T cells in HTLV-1-infected individuals is maintained by CCL22 produced by HTLV-1-infected PBMCs (Toulza et al. 2010). The frequency of HTLV-1-negative Foxp3+ CD4+ cells positively correlated with the HTLV-1 PVL (Hayashi et al. 2008a; Toulza et al. 2008), and the CTL activity negatively correlated with the frequency of HTLV-1-negative Foxp3+ CD4+ cells (Toulza et al. 2008). These results suggest that an increase in HTLV-1-negative FoxP3+ CD4+ Tregs is one of the chief determinants of the efficiency of T cell-mediated immune control of HTLV-1. If such Tregs reduce CTL activity, which in turn increases the HTLV-1 PVL, this activity increases the risk of developing HAM/TSP.

Dendritic cells and the other reservoirs of HTLV-1

Dendritic cells (DCs) are antigen-presenting cells that play a critical role in the regulation of the adaptive immune response. In HTLV-1 infection, it has been shown that the DCs from patients with HAM/TSP were infected with HTLV-1 (Macatonia et al. 1992), and the development of HAM/TSP is associated with rapid maturation of DCs (Ali et al. 1993). In vitro culture of lymphocytes from HTLV-1-infected individuals results in "spontaneous lymphocyte proliferation" (SLP), which is the in vitro proliferation of PBMCs without any exogenous stimuli such as antigen or mitogen. In patients with HAM/TSP, the levels of SLP reflect the severity of the disease (Ijichi et al. 1989; Itoyama et al. 1988). Interestingly, depletion of DCs from the PBMCs of patients with HAM/TSP abolished SLP, whereas supplementing DCs restores proliferation (Macatonia et al. 1992); supplementing B cells or macrophages had no effect. A DC-dependent mechanism of SLP was further supported by data showing that antibodies to MHC class II, CD86, and CD58 can block SLP (Makino et al. 1999). Recently, Jones et al. demonstrated that human-derived myeloid and plasmacytoid DCs are susceptible to infection with cell-free HTLV-1 and that HTLV-1-infected DCs can rapidly transfer virus to autologous primary CD4+ T cells (Jones et al. 2008). Furthermore, it was recently demonstrated that transmission of HTLV-1 from DCs to T cells was mediated primarily by DC-SIGN (Jain et al. 2009), and the DCs are the major cell type responsible for the generation and maintenance of Tax-specific CD8+ T cells both in vitro and in vivo (Manuel et al. 2009).

These findings suggest that the interaction of DCs with HTLV-1 is also crucial for the pathogenesis of HAM/TSP. Moreover, using transgenic mouse models that permit conditional transient depletion of CD11c+ DCs, and a chimeric HTLV-1 that carries the envelope gene from Moloney murine leukemia virus, Rahman et al. demonstrated the critical role of DCs in their ability to mount both innate and adaptive immune responses during early cell-free HTLV-1 infection (Rahman et al. 2011, 2010). Because HTLV-1 can impair the differentiation of monocytes into DCs (Nascimento et al. 2011), the interaction of DCs with HTLV-1 plays a central part in the persistence and pathogenesis of HTLV-1.

Conclusions

During the three decades since the discovery of HTLV-1, advances in research have successfully helped us to understand the clinical features of HTLV-1-associated diseases and the virological properties of HTLV-1, although the precise mechanism of disease pathophysiology is still incompletely understood and treatment is still unsatisfactory. Accumulating evidence suggests that the virus–host immunological interactions play a pivotal role in the pathogenesis of HAM/TSP. A genetically determined, less efficient CTL response against HTLV-1 may cause higher PVL and antigen expression in infected individuals, which lead to the activation and expansion of antigen-specific T cell responses, subsequent induction of large amounts of proinflammatory cytokines and chemokines, and progression of the development of HAM/TSP. Future studies should be conducted to identify the precise mechanism of disease development to allow effective treatment and prevention of disease. This will require the development of a humanized small animal model that could be exploited as a tool for screening and evaluation of HTLV-1-associated diseases.

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Elimination of Human T Cell Leukemia Virus Type-1-Infected Cells by Neutralizing and Antibody-Dependent Cellular Cytotoxicity-Inducing Antibodies Against Human T Cell Leukemia Virus Type-1 Envelope gp46

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Abstract

Human T cell leukemia virus type-1 (HTLV-1) is prevalent worldwide with foci of high prevalence. However, to date no effective vaccine or drug against HTLV-1 infection has been developed. In efforts to define the role of antibodies in the control of HTLV-1 infection, we capitalized on the use of our previously defined anti-gp46 neutralizing monoclonal antibody (mAb) (clone LAT-27) and high titers of human anti-HTLV-1 IgG purified from HAM/TSP patients (HAM-IgG). LAT-27 and HAM-IgG completely blocked syncytium formation and T cell immortalization mediated by HTLV-1 *in vitro*. The addition of these antibodies to cultures of $CD8^+$ T cell-depleted peripheral blood mononuclear cells (PBMCs) from HAM/TSP patients at the initiation of culture not only decreased the numbers of Tax-expressing cells and the production of HTLV-1 p24 but also inhibited the spontaneous immortalization of T cells. Coculture of *in vitro*-HTLV-1-immortalized T cell lines with autologous PBMCs in the presence of LAT-27 or HAM-IgG, but not an $F(ab')_2$ fragment of LAT-27 or non-neutralizing anti-gp46 mAbs, resulted in depletion of HTLV-1-infected cells. A 24-h ^{51}Cr release assay showed the presence of significant antibody-dependent cellular cytotoxicity (ADCC) activity in LAT-27 and HAM-IgG, but not $F(ab')_2$ of LAT-27, resulting in the depletion of HTLV-1-infected T cells by autologous PBMCs. The depletion of natural killer (NK) cells from the effector PBMCs reduced this ADCC activity. Altogether, the present data demonstrate that the neutralizing and ADCC-inducing activities of anti-HTLV-1 antibodies are capable of reducing infection and eliminating HTLV-1-infected cells in the presence of autologous PBMCs.

Introduction

HUMAN T CELL LEUKEMIA VIRUS type-1 (HTLV-1) is the first human retrovirus that was etiologically associated with adult T cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP).[1–4] HTLV-1 is prevalent worldwide with foci of high prevalence in southwest Japan, the Caribbean islands, South America, and a part of Central Africa. The total number of HTLV-1 carriers is currently estimated to be 10–20 million.[5] The majority of HTLV-1 carriers remain asymptomatic throughout their lives, and approximately 5% of HTLV-1-infected individuals will develop either ATL or HAM/TSP after prolonged latency periods.

HTLV-1 is transmitted through contact with bodily fluids containing infected cells most often from mother to child through breast milk or via blood transfusion. It has been previously established that HTLV-1 efficiently spreads from cell to cell via the formation of virological synapses.[6] More recently, however, the formation of extracellular HTLV-1 viral particles similar to the formation of bacterial films has also been shown to be effective in viral transmission.[7] HTLV-1-antigen-expressing cells are difficult to detect at least in fresh peripheral blood mononuclear cells (PBMCs) from HTLV-1-infected individuals.[8] However, when these PBMCs are isolated from the blood and cultured *in vitro*, some T cells begin to produce HTLV-1 antigen[9,10] followed by spontaneous immortalization of the cells in media containing interleukin-2 (IL-2).[11]

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Although it has been suggested that HTLV-1 can stay dormant in infected cells and become resistant to immune effector mechanisms by ratcheting down its antigen production,[12] the continued presence of strong $CD8^+$ cytotoxic T lymphocyte (CTL) responses[13] and readily detectable levels of antibodies specific for HTLV-1 antigens in HTLV-1-infected people[14] indicates that persistent production of HTLV-1 must occur *in vivo* to maintain such effector mechanisms. Escape from immune effector mechanisms by spontaneous mutation of key residues is unlikely, due to the high degree of genomic stability that is characteristic of the HTLV-1 genome.[15] It has been suggested that HTLV-1-infected cells expressing HTLV-1 antigens occur at a low enough frequency that they are constantly being eliminated by HTLV-1-specific CTL *in vivo*[10] without leading to immune exhaustion. Besides CTL and virus neutralizing antibodies, there has been renewed interest in the potential role of antibody-dependent cellular cytotoxicity (ADCC) as an effector mechanism against a number of viral infections. This view has been highlighted by the recent demonstration of the potential role of ADCC in the only known partially successful human RV144 trial of a vaccine against human immunodeficiency virus type-1 (HIV-1).[16] The ADCC activity against HTLV-1 was first reported by Miyakoshi *et al.* in 1984[17] followed by a number of other reports.[18–21]

So far, several lines of evidence show that the HTLV-1 envelope gp46 antigen serves as a major target of ADCC.[22–24] Antibodies against gp46 antigen are commonly detected in the sera of HTLV-1-infected individuals.[25–27] However, the precise role of ADCC effector mechanism(s) in controlling HTLV-1 infection has been lacking. A possible involvement of anti-HTLV-1 antibodies in the suppression of spontaneous HTLV-1 antigen expression by HTLV-1-infected cells was first reported by Tochikura *et al.*[28] These investigators showed that serum IgG from HTLV-1-infected donors interfered with HTLV-1 antigen expression by *in vitro*-cultured PBMCs from both ATL patients and healthy HTLV-1 carriers. However, the precise mechanism by which this was mediated remained unclear.

In efforts to define the role of antibodies with neutralizing and ADCC-inducing activities in the control of HTLV-1 infection, we capitalized on the use of our previously defined rat anti-gp46 neutralizing monoclonal antibody (mAb) (LAT-27)[29] and pooled human anti-HTLV-1 IgG purified from HAM/TSP patients (HAM-IgG). Studies were conducted to evaluate the potential of these antibodies to block HTLV-1 infection and eliminate HTLV-1-infected cells from autologous T cell cultures that had previously been infected with HTLV-1 *in vivo* or *in vitro*. Results of these studies show that monoclonal LAT-27 and the polyclonal HAM-IgG are not only capable of mediating neutralization and ADCC, but are also highly effective in the elimination of HTLV-1-infected cells in the presence of fresh autologous PBMCs while preventing *de novo* infection with HTLV-1.

Materials and Methods

Reagents

The medium used throughout was RPMI 1640 medium (Sigma-Aldrich. Inc., St. Louis, MO) supplemented with 10% fetal calf serum (FCS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (hereinafter called RPMI medium). Anti-human CD3 (clone OKT-3) and anti-CD28 (clone 28.2) mAbs were purchased from the American Type Culture Collection (Rockville, MD) and Biolegend (San Diego, CA), respectively.

The rat and mouse mAbs utilized in the studies reported herein were produced and characterized by our laboratory previously.[29–34] These antibodies were rat IgG2b anti-gp46 (clones LAT-27 and LAT-25), rat IgG2a anti-gp46 (clone LAT-12), rat IgG2b anti-HCV (clone Mo-8), rat IgG2a anti HTLV-1 p24 (clone WAG-24), mouse IgG1 anti-HTLV-1 gp46 (clone MET-3), mouse IgG3 anti-HTLV-1 Tax (clone Lt-4), mouse IgG1 anti-p24 (clone NOR-1), and mouse IgG1 anti-HIV-1 p24 (clone 2C2). These in-house mAbs were purified from the ascites fluids of groups of CB.17-SCID mice carrying the appropriate hybridoma cell line. The ascites fluid was subjected to ammonium sulfate precipitation followed by gel filtration using Superdex G-200 (GE Healthcare, Tokyo, Japan). Aliquots of these mAbs were labeled with either fluorescein isothiocyanate (FITC), Alexafluor 488, Alexafluor 647, HRP (Dojindo, Kumamoto, Japan), or Cy-5 (GE Healthcare) according to the manufacturer's instructions. The FITC- or phycoerythrin (PE)-labeled mouse mAbs against human CD3, CD4, CD8, CD14, CD16, CD19, or CD56 and unlabeled mouse anti-CD16 and anti-CD32 mAbs were purchased from Abcam.

For cell depletion, magnetic beads labeled with anti-CD4, CD8, CD14, CD16, CD19, and antimouse IgG (Dynal) and those labeled with anti-CD56 mAb (LifeTec) were used according to the manufacturer's recommendations. Mitomycin-C (MMC) was commercially purchased from Kyowa Kirin (Tokyo, Japan) and used at 50 μg/ml in RPMI medium. A purified $F(ab')_2$ fragment of LAT-27 IgG generated by enzymatic digestion of LAT-27 IgG was purchased from IBL Inc. (Gunma, Japan). Human IgG was purified from pooled plasma from three normal donors (normal IgG) and three HAM patients (HAM-IgG) using protein-G affinity purification kits (GE Healthcare).

The protocols for the use of human PBMCs and animals were approved by the Human IRB and the Institutional Animal Care and Use Committee (IACUC) on clinical and animal research of the University of the Ryukyus prior to initiation of the present study.

Cell cultures

PBMCs were isolated from heparinized blood by standard density gradient centrifugation using Lympholyte (Cedarlane, Burlington, Canada). Some PBMCs were cryopreserved using a cell freezing media (Cell reservoir, Nakarai Tesque Inc., Kyoto, Japan). The method to activate PBMCs with anti-CD3 and CD28 mAbs has been described previously.[34] The HTLV-1-producing T cell lines utilized included MT-2, HUT102, IL-2-dependent $CD4^-CD8^+$ ILT-M1 cells derived from an HAM/TSP patient, $CD4^+CD8^-$ ILT-H2 cells, ATL-3 cells derived from ATL patients, and a number of other T cell lines derived from normal PBMCs following *in vitro* immortalization by cocultivation with MMC-treated ILT-M1 cells. These cell lines were maintained in culture using RPMI medium containing 20 U/ml IL-2.

The syncytium inhibition assay was performed using an assay that involved the coculture of ILT-M1 and Jurkat cells.[35] A suspension of ILT-M1 cells in a volume of 25 μl containing 5×10^4 cells in 20 U/ml IL-2 media was mixed with 50 μl of serially diluted antibody to be tested in a flat-bottom 96-well microtiter plate for 5 min followed by the addition of 5×10^4 Jurkat cells in a volume of 25 μl of medium. After coculture for 18 ~ 24 h at 37°C in a 5% CO_2 humidified incubator, syncytium formation was microscopically observed using an inverted microscope and the minimum concentration of antibody that showed complete blocking of syncytium formation was determined. In some experiments, gp46 antigen that had been affinity purified from the culture supernatants of MT-2 cells using our anti-gp46 mAb (MET-3) antibody-coupled Sepharose 4B column (GE Healthcare) was used as a target antigen to serve as a specificity control to block the syncytia neutralization of antibodies.[36]

The HTLV-1-immortalization inhibition assay was performed according to the method described previously with a slight modification.[29] Briefly, PBMCs from HTLV-1-negative healthy donors were activated with immobilized OKT-3 together with soluble anti-CD28 mAb overnight, and these cells (5×10^4 cells) were cocultured with an equal number of MMC-treated ILT-M1 cells in wells of round-bottom 96-well microtiter plates (BD) in 0.2 ml media containing 20 U/ml IL-2 at 37°C in a humidified 5% CO_2 incubator in the presence or absence of the test antibodies. The medium was replaced with fresh IL-2-containing media with or without antibody every 3–5 days. Aliquots of the cocultured cells were monitored every week for intracellular expression of Tax antigen, and the culture supernatants were monitored for the production of p24.

The assay for inhibition of spontaneous HTLV-1 antigen expression in PBMCs from HAM/TSP patients was performed as follows. PBMCs from HAM/TSP patients after depletion of CD8$^+$ cells were cultured *in vitro* at 1×10^6 cells/ml in 20 U/ml IL-2-containing RPMI medium at 37°C in a 24-well plate (BD) in the presence of various anti-HTLV-1 mAbs, HAM-IgG, or controls. After 24 h, cells were harvested and an aliquot stained with anti-CD3, CD4, or CD8 mAb, followed by fixation and subsequent intracellular Tax staining. The frequency and absolute cell numbers of Tax-positive cells were analyzed by flow cytometry (FCM) using the Flowcount (Coulter). The remaining cells were further cultured for 2 ~ 6 weeks with a change of media with or without antibody every 3 ~ 4 days. If necessary, cultures were split into 1:2 or 1:4.

The elimination of HTLV-1 antigen-expressing cells was tested as follows. The IL-2-dependent HTLV-1-infected T cell lines established from PBMCs of normal donors (2×10^5 cells/ml) were cocultured with autologous fresh PBMCs (2×10^6 cells/ml) in 20 U/ml IL-2-containing RPMI medium in triplicate in a round-bottom 96-well microtiter plate (BD) in the presence or absence of various antibodies. After initial coculture for 3 days, these cultures were split, and one was cultured in the presence and the other in the absence of fresh PBMCs and antibodies for 3 days. If necessary, these cells were further treated with antibodies and fresh PBMCs every 3 days. These cell cultures were periodically monitored for changes in the levels of Tax-expressing cells and levels of p24 production.

Flow cytometry (FCM) and enzyme-linked immunosorbent assay (ELISA)

For the detection of HTLV-1 antigen-expressing cells, sample cells were analyzed using polychromatic FCM. Briefly, live cells were Fc receptor-blocked with 2 mg/ml pooled normal human IgG in FACS buffer [phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) and 0.1% sodium azide] for 10 min on ice, and prestained with fluorescent dye-labeled mAbs for 30 min. After washing with FACS buffer, the cells were fixed in 4% paraformaldehyde (PFA) in PBS for 5 min at room temperature followed by permeablization and washing in 0.5% saponin + 1% BSA (Sigma) containing FACS buffer. The cells were incubated with 0.1 μg/ml of Cy5-labeled anti-Tax antibody (clone Lt-4) for 30 min. Negative control cells were stained with Cy5-Lt-4 in the presence of 50 μg/ml of unlabeled Lt-4. These cells were analyzed using a FACSCalibur (BD) and the data obtained were analyzed using the Cell Quest software (BD). Typical staining of HTLV-1-infected T cell lines with Lt-4 and LAT-27 is also shown in Supplementary Fig. S1 (Supplementary Data are available online at www.liebertpub.com/aid).

Production of HTLV-1 was determined by the measurement of the HTLV-1 core p24 antigen levels in the culture supernatants using our in-house formulated and standardized ELISA kit using a pair of anti-HTLV-1 p24 mAbs. The sensitivity of this assay was determined to be 0.5 ng/ml of p24 (data not shown).

ADCC assay

HTLV-1-immortalized target cells from healthy donors were labeled with ^{51}Cr for 60 min as described previously[37] and mixed with varying ratios of fresh PBMCs (varying effector-to-target cell ratios) in the presence or absence of various antibodies for the indicated period of time in 20 U/ml IL-2-containing medium. Appropriate controls were included with each assay including target cells cultured in media alone (spontaneous release) and in 0.5 N HCl (100% release). After brief centrifugation, supernatants were harvested and ^{51}Cr activity in each sample was determined using a gamma counter. The net percentage ^{51}Cr release was calculated using standard methods as follows (cpm in experiment – cpm in medium)/(cpm in 0.5 N HCl – cpm in medium) × 100. In some experiments, PBMCs were depleted of CD4$^+$, CD8$^+$, CD14$^+$, CD16$^+$, CD19$^+$, or CD56$^+$ cells using appropriately conjugated immunomagnetic beads and tested for their effector activity.

Statistical analysis

Data were tested for statistical significance by the Student's *t* test using Prism software (GraphPad Software).

Results

HTLV-1 neutralizing activities of LAT-27 and human anti-HTLV-1-IgG in vitro

The syncytium inhibition assay has been generally used to evaluate HTLV-1 neutralization titers of anti-HTLV-1