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

Yuetsu Tanaka,[1] Yoshiaki Takahashi,[1] Reiko Tanaka,[1] Akira Kodama,[1] Hideki Fujii,[1] Atsuhiko Hasegawa,[2] Mari Kannagi,[2] Aftab A. Ansari,[3] and Mineki Saito[4]

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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[1] Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan.
[2] Department of Immunotherapeutics, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan.
[3] Department of Pathology, Emory University School of Medicine, Atlanta, Georgia.
[4] Department of Microbiology, Kawasaki Medical School, Okayama, Japan.

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 antibodies. To optimize the syncytium inhibition assay, we screened various coculture combinations of HTLV-1-producing cells with a variety of HTLV-1-negative target cells, and selected the HTLV-1-producing T cell line ILT-M1 and the HTLV-1-negative T cell line Jurkat. Overnight coculture of the ILT-M1 and Jurkat cells resulted in the generation of numerous large syncytia (Fig. 1). Using this assay system, we titrated the syncytia-blocking activity of monoclonal LAT-27 and polyclonal IgG purified from pooled plasma from HAM patients (HAM-IgG). HAM-IgG was used as a positive anti-HTLV-1 antibody control because it contained high titers of antibodies against HTLV-1 antigens (Supplementary Fig. S2). The minimum concentrations required for the "complete" inhibition of syncytia formation by LAT-27 and HAM-IgG antibodies were calculated to be 5 μg/ml and 50 μg/ml, respectively (Supplementary Fig. S3). To adjust for decay in antibody activities during cultivation at 37°C, we used LAT-27 and HAM-IgG at concentrations of 10 and 100 μg/ml, respectively, in all subsequent experiments.

To confirm the gp46 specificity of LAT-27 and HAM-IgG in this syncytium inhibition assay, an affinity-column-purified gp46 antigen[36] was added to an aliquot of either LAT-27 or HAM-IgG solution prior to cocultivation. Controls consisted of incubating an aliquot of the cocultures in media alone (shaded bars denoted by 0) or media containing 10 μg/ml of gp46 (dark bars also denoted by 0). As shown in Fig. 2, HAM-IgG incubated in media alone clearly inhibited syncytia formation in a dose-dependent manner (at 12.5 ~ 100 μg/ml). However, preincubation of the HAM-IgG at 12.5 ~ 100 μg/ml with 10 μg/ml of affinity-purified gp46 resulted in significant reversal of inhibition, suggesting that gp46 was the main target for the neutralization activity present in the human anti-HTLV-1 antibodies. Similar results were obtained when LAT-27 instead of HAM-IgG was preincubated with gp46 (data not shown).

LAT-27 as reported previously[29] and HAM-IgG completely inhibited HTLV-1-mediated T cell immortalization of normal activated T cells *in vitro* at concentrations of 10 μg/ml and 100 μg/ml, respectively (Fig. 3).

Effect of antibodies on spontaneous HTLV-1 antigen expression in vitro

To evaluate the role of anti-gp46 neutralizing antibodies against T cells carrying endogenous HTLV-1 from naturally infected donors, we cultured freshly isolated PBMCs from HAM/TSP patients in the presence or absence of various antibodies for 24 h. To exclude any potential effects of CD8$^+$ CTL that are present within the PBMCs from the HAM/TSP patients,[10] PBMCs were depleted of CD8$^+$ T cells prior to the assay. For quantitation of the frequencies of HTLV-1 antigen-expressing cells, we stained an aliquot of the cells for the expression of intracellular Tax antigen utilizing our standardized anti-Tax mAb, which has generally been used to detect HTLV-1-infected cells.[10,38] At a concentration of 10 μg/ml, LAT-27 reduced the frequency of Tax$^+$ cells (Fig. 4A). This reduction was antigen and epitope specific since neither the isotype control rat IgG2b mAb (anti-HCV envelope) nor the anti-gp46 nonneutralizing mAb (LAT-25) and the other anti-gp46 nonneutralizing mAbs (clones LAT-12 and MET-3) that compete with LAT-27 in an antibody binding assay showed any detectable inhibitory effect (data not shown). The reduction in the frequency of Tax$^+$ cells by LAT-27 was partially reversed by a mixture of anti-CD16 and anti-CD32 mAbs when added at the initiation of the assay, suggesting an involvement of Fc receptors in this reduction assay.

As shown in Fig. 4B, after prolonged culture (2 weeks) the suppressive effect of LAT-27 became more evident since there remained few if any Tax$^+$ cells in the LAT-27-treated cultures of PBMCs from each of the HAM patients tested. A similar suppressive effect was observed for HAM-IgG but not normal human IgG (Fig. 4B). It should be noted that in the present culture conditions, similar to what has been generally observed for the PBMC cultures from HTLV-1-infected

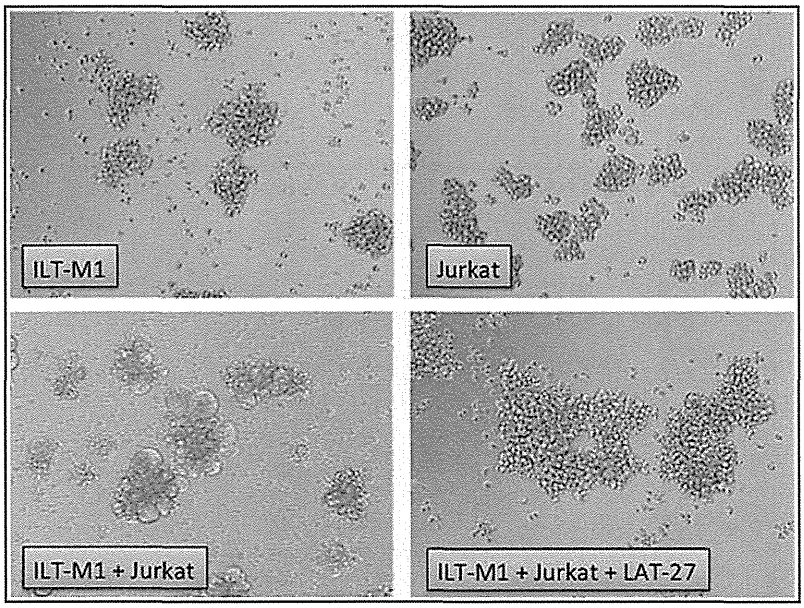


FIG. 1. Human T cell leukemia virus type-1 (HTLV-1)-mediated syncytia formation. HTLV-1$^+$ ILT-M1 and HTLV-1$^-$ Jurkat cells were either cultured alone or cocultured at a cell-to-cell ratio of 1:1 in the presence or absence of 10 μg/ml LAT-27 for 18 h. Syncytia were microscopically observed using an inverted microscope at a magnification of 100×. Representative data from three independent experiments are shown.

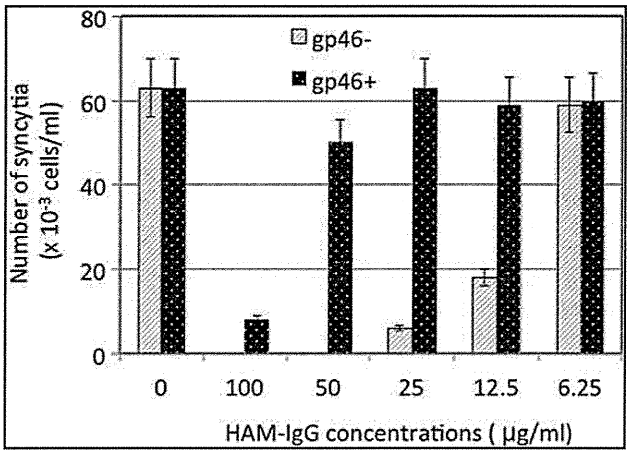


FIG. 2. Anti-gp46 antibodies are major HTLV-1 neutralizing antibodies in HAM-IgG. HAM-IgG at graded concentrations (0 ~ 100 μg/ml) was preincubated with either affinity-purified gp46 antigen (black bars) at 10 μg/ml for 10 min or incubated with medium alone (gray shaded bars, labeled as "gp46-") and tested for syncytia inhibition activity. The numbers of syncytia were manually counted using a "Burker-Turk" hemocytometer. Representative data from three independent experiments are shown.

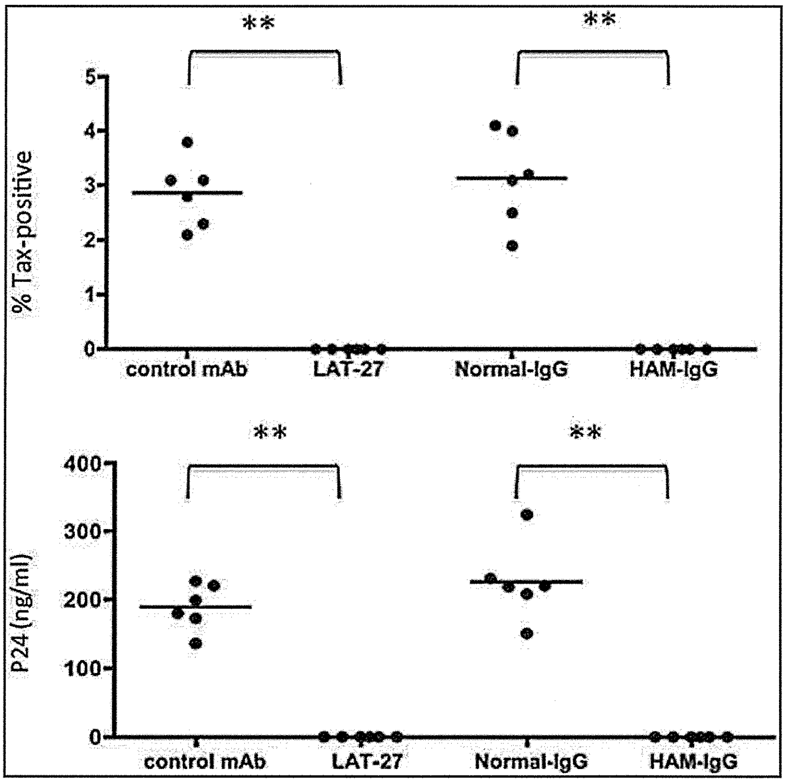
donors, the frequency of Tax$^+$ cells gradually decreased during 2 weeks in culture even in IL-2 medium alone and thus it was not likely due to an effect of the addition of the control rat isotype IgG or normal IgG. Spontaneous immortalization of T cells by HTLV-1 was observed in the PBMC cultures from two-thirds of the HAM patients treated with medium alone, isotype control, or normal IgG, but not in those treated with LAT-27 or HAM-IgG, as judged 6 weeks after culture (data not shown).

Importantly, neither LAT-27 nor HAM-IgG (data not shown) showed any detectable blocking effects on HTLV-1 Tax expression (Fig. 5) and p24 antigen production (data not shown) in long-term cultured HTLV-1-infected cell lines. It is known that the levels of Tax-positive cells vary depending on the cell lines being utilized due to DNA methylation, hypoacetylation of histones, or epigenetic changes of provirus.[39] Thus, we reasoned that the addition of the neutralizing antibodies blocks *de novo* expansion of HTLV-1 infection and/or eliminates the HTLV-1 gp46 expressing T cells via an FcR-dependent pathway in combination with effector cells contained within the PBMCs.

FcR-dependent elimination of HTLV-1-infected cells by antibodies

Due to the limitations on the availability of PBMC samples from HAM/TSP patients, we established a number of IL-2-dependent HTLV-1-infected CD4$^+$ T cell lines from PBMCs of normal donors to determine whether ADCC was involved in the suppression of HTLV-1-infected cells. These HTLV-1$^+$CD4$^+$ T cell lines were cocultured with autologous fresh PBMCs in the presence or absence of various antibodies including F(ab′)$_2$ of LAT-27, which showed HTLV-1 neutralization at a minimum concentration of 2.5 μg/ml (data not shown). HAM-IgG was included as an ADCC-positive control. After 3 days in culture, these cells were stained for cell surface CD4 and intracellular Tax antigen, and analyzed on a gated population of cells that displayed high forward and side scatters, which included a majority of the HTLV-1-infected

FIG. 3. LAT-27 and HAM-IgG completely block HTLV-1-mediated T cell immortalization *in vitro*. Activated peripheral blood mononuclear cells (PBMCs) from normal donors were seeded into six wells of 96-well U-bottom plates (1×10^5 cells/0.1 ml/well) and cocultured with an equal number of mitomycin C-treated ILT-M1 cells in the presence or absence of 10 μg/ml of LAT-27 or rat isotype control, 100 μg/ml of normal human IgG, or HAM-IgG. Half of the medium was replaced every 3 ~ 5 days with new similar fresh media, and if necessary, cultures were split into 1:2. Each data point reflects the frequency of Tax$^+$ cells or the levels of p24 in the culture supernatants of each well 6 weeks after culture. Data shown are representative of three independent experiments. The differences between the controls and the experimental data were highly significant, denoted as $**p < 0.01$. The negative control used for LAT-27 was an isotype control (rat IgG2b anti-HCV).



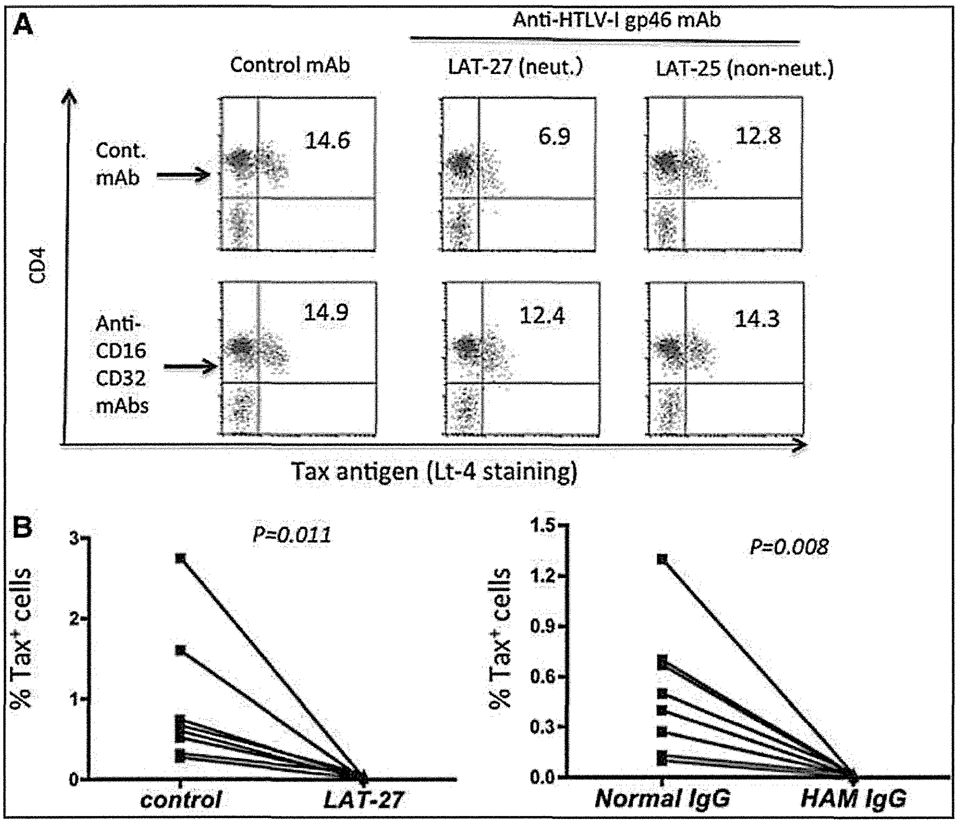


FIG. 4. Reduction of Tax-expressing cells in *in vitro* cultures of PBMCs from HAM patients in the presence of HTLV-1 neutralizing monoclonal antibody (mAb). **(A)** PBMCs from HAM patients were depleted of CD8$^+$ T cells and cultured *in vitro* for 24 h at 1×10^6 cells/ml in interleukin (IL)-2-containing medium in the presence or absence of 10 μg/ml antibodies indicated in the figure. The cells were then stained for cell surface CD4 and intracellular Tax antigen as described in the Materials and Methods section. The numbers in each dot-plot show the percentage of CD4$^+$ Tax$^+$ cells. The mixture of antibodies against human Fc receptors (FcR) (anti-CD16 and CD32) was added to block FcR function. Data shown are representative of three independent experiments using PBMCs from different donors. **(B)** PBMCs from HAM patients ($n=8$) were depleted of CD8$^+$ T cells and cultured *in vitro* in IL-2-containing medium in the presence of (1) LAT-27 or an isotype control mAb at 10 μg/ml or (2) HAM-IgG or normal human IG at 100 μg/ml for 2 weeks. The cells were stained for Tax antigen and the total percentage of Tax$^+$ cells was calculated. The control used for LAT-27 was an isotype control (rat IgG2b anti-HCV mAb). The negative control mAb for anti-CD16 and CD32 was mouse IgG1 against HIV-1 (clone 2C2).

CD4$^+$ T cells but not normal PBMCs. However, no detectable reduction of Tax$^+$ cells was observed in the cultures treated with either LAT-27 or HAM-IgG cocultured in the presence of PBMCs (data not shown). Thus, these cells were washed and cocultured again for an additional 3 days with the same antibodies and fresh PBMCs.

As shown in Fig. 6A, although fresh PBMCs alone reduced the frequency of Tax$^+$ cells to some extent, a marked net reduction was seen in the presence of LAT-27 and HAM-IgG. In a similar fashion, the production of HTLV-1 p24 in the culture supernatants was markedly reduced by LAT-27 and HAM-IgG in the presence of autologous PBMCs. As shown in Fig. 6B, when these cultures were exposed one more time to the same antibodies and fresh PBMCs, LAT-27 IgG and HAM-IgG, but not F(ab′)$_2$ of LAT-27 or normal IgG, further reduced the frequency of Tax$^+$ cells. These data suggest that the addition of LAT-27 as well as HAM-IgG eliminates the HTLV-1 gp46 antigen-expressing cells via an FcR-dependent manner while blocking the spread of HTLV-1 to new target cells including fresh PBMCs in the same cell cultures *in vitro*. The involvement of complement-dependent cytotoxicity was ruled out because the fetal calf serum used in the present study was heat inactivated prior to use.

ADCC against HTLV-1-infected cells by LAT-27

To examine whether LAT-27 could mediate ADCC in the present culture conditions, IL-2-dependent HTLV-1-infected T cells established from normal donors were labeled with ^{51}Cr and cocultured with fresh autologous PBMCs in the presence or absence of antibodies. Significant ADCC activity was induced by HAM-IgG, but not LAT-27, by 6 h (data not shown). However, after 24 h at a high effector-to-target cell ratio, LAT-27, but not the F(ab′)$_2$ fragment of LAT-27, showed significant cytotoxicity ($p<0.01$) (Fig. 7A). When the effector PBMCs were depleted of either CD16$^+$ or CD56$^+$ cells, but not CD14$^+$ or CD19$^+$ cells, the ADCC activity mediated by either LAT-27 or HAM-IgG was significantly reduced ($p<0.01$) (Fig. 7B and C). These data suggest that the CD16$^+$ CD56$^+$ subpopulation of PBMCs [representing natural killer (NK) cells] were most likely the main effector cells involved in the cell lysis. These results

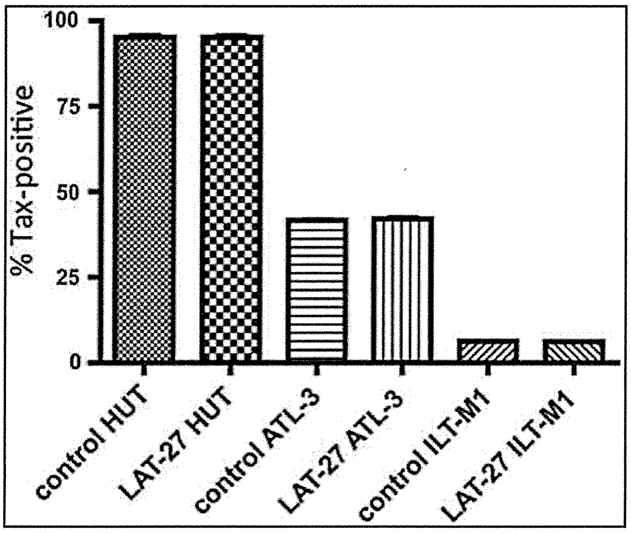


FIG. 5. LAT-27 alone does not affect long-term cultured HTLV-1-infected T cells. A standard HTLV-1-infected cell line HUT-102 (HUT), an IL-2-dependent CD4$^+$ T cell line (ATL-3, generated from an ATL patient), and an IL-2-dependent CD8$^+$ T cell line (ILT-M1) were cultured in the presence of 10 μg/ml of either LAT-27 or isotype control (control) for 4 days, and the frequencies of Tax$^+$ cells were determined by flow cytometry ($n = 3$).

demonstrate that the monoclonal LAT-27, similar to the polyclonal HAM-IgG, is able to induce ADCC against HTLV-1-infected cells by autologous NK cells while protecting the spread of new infection with HTLV-1.

Discussion

The present study demonstrates that the monoclonal anti-HTLV-1 gp46 antibody clone LAT-27 generated by our laboratory mediates both HTLV-1 neutralization and HTLV-1-specific ADCC, and such ADCC activity might be capable of eliminating HTLV-1-infected T cells *in vitro* in the presence of autologous fresh PBMCs. Although fresh PBMCs alone showed a partial but significant inhibitory activity against HTLV-1-infected cells during prolonged *in vitro* cultivation, the data obtained here suggest that the HTLV-1-specific ADCC activity is the direct mechanism for this eradication. Similar suppressive activities were demonstrated for human IgG from HAM patients. This mechanism may explain the previous findings reported by Tochikura *et al.*[28] on the HTLV-1 suppressing activity of human anti-HTLV-1 antibodies. Furthermore, this mechanism may also explain in part why HTLV-1 antigen-expressing cells are not found *in vivo* in anti-HTLV-1 antibody-positive individuals. Although it is not known where and when HTLV-1 is produced *in vivo* in the infected individual, the continued presence of CD8$^+$ T cells and antibodies specific for HTLV-1 indicates that HTLV-1 should be expressed periodically. Based on the results presented in this article, it might be possible that HTLV-1 expression occurs upon T cell stimulation in the periphery, but as soon as the cells express HTLV-1 gp46 antigen they might be instantly killed by the combination of anti-HTLV-1 ADCC-inducing antibodies and activated NK cells.

We submit that the addition of fresh PBMCs to the autologous HTLV-1-producing T cell cultures may result in it becoming readily infected and immortalized by HTLV-1. Thus, it is clear that the presence of neutralizing antibody is essential for the prevention of new infection of PBMCs and since ADCC effector mechanisms are functional during this time period, their contribution to the control of infection deserves merit. Interestingly, the ADCC induced by LAT-27 progressed slowly and the elimination of Tax$^+$ cells became evident only after two consecutive exposures every 3 days in the present cell culture conditions. Since there was heterogeneity of the intensity of gp46 expression among cells in a single HTLV-1-infected cell line (data not shown), the findings suggest that the lysis of such gp46$^{\text{low}}$ cells by ADCC requires a prolonged incubation period. Alternatively, since the repeated exposure against PBMCs resulted in an accumulation of live PBMCs, it is possible that a large number of effector fresh PBMCs might be required for the complete eradication by LAT-27, possibly due to the relatively low affinity of LAT-27 for human FcR.

Cell depletion experiments in the present study showed that the effector cells involved in the HTLV-1-specific ADCC in fresh PBMCs were either CD16$^+$ or CD56$^+$ cells, representing the cytolytic human NK cell subset, although it remains to be confirmed with purified NK cells. Because there are abundant circulating NK cells in the periphery in healthy donors, these findings strongly suggest that the HTLV-1-specific ADCC responses in the presence of neutralizing antibodies might have a role in controlling HTLV-1 *in vivo* in concert with HTLV-1-sepecific CTL responses in healthy HTLV-1 carriers. This view is supported by the findings that the ADCC effector function of PBMCs is lower in both HAM/TSP and ATL patients than healthy HTLV-1 carriers or normal donors,[17,40] suggesting that defects in functional ADCC activities may contribute to the onset of HTLV-1-related diseases.

The level of ADCC of HTLV-1$^+$ cells by LAT-27 was weaker than that induced by human polyclonal anti-HTLV-1 IgG. This might be due to the fact that LAT-27 is of rat origin and recognizes a single epitope on the gp46 (amino acids 191–196)[29] in contrast to the fact that HAM-IgG is of human origin and consists of high titers of polyclonal antibodies against multiple epitopes on gp46. In addition, it has been shown that mouse and rat IgG exhibit different ADCC activities with human NK cells depending on their subclasses, and that rat IgG2b (the subclass of LAT-27), but not IgG2a, triggers effective ADCC with human NK cells.[41] Along these lines, it is possible that a humanized form of LAT-27 utilizing the human IgG1- or IgG3-Fc portion as a backbone would be far more effective than even the rat IgG2b of LAT-27.

This hypothesis has been confirmed by preliminary experiments using humanized LAT-27 consisting of human IgG1, which was generated in collaboration with Dr. Shimizu of IBL Inc. (Tanaka *et al.*, unpublished observations). In addition, epitope specificity and/or the affinity of anti-gp46 antibodies may also be involved in determining the ADCC-inducing activities. For example, LAT-25, which belongs to the rat IgG2b subclass and recognizes a C-terminal region of the gp46, did not eradicate HTLV-1$^+$ cells (Fig. 7). Similarly, Kuroki *et al.* showed that a human mAb recognizing gp46 amino acids 191–196 (similar to the epitope recognized by LAT-27) could induce ADCC, but another human mAb

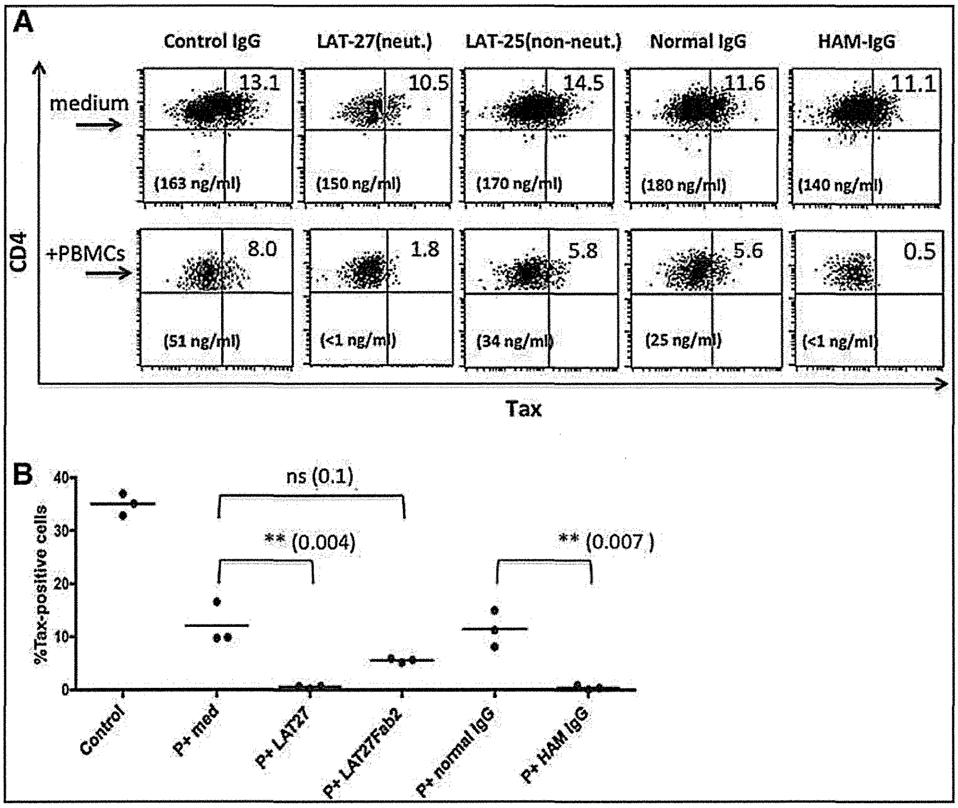


FIG. 6. Elimination of Tax$^+$ cells and reduction of HTLV-1 p24 production in IL-2-dependent HTLV-1-infected T cells cocultured with autologous PBMCs in the presence of LAT-27 or HAM-IgG. **(A)** IL-2-depnedent CD4$^+$ HTLV-1-infected T cells established from the PBMCs of normal donors were repeatedly exposed to autologous PBMCs (+PBMCs) in the presence of 10 μg/ml of LAT-27 or isotype control, or 100 μg/ml of HAM-IgG, or normal human IgG twice at 3 day intervals. Two days after the second exposure, the high forward and side scatter gated populations of cells that contained a majority of the HTLV-1$^+$ cells but not PBMCs were analyzed for the frequencies of CD4$^+$ Tax$^+$ cells. Percentages of CD4$^+$ Tax$^+$ cells are shown in the upper right quadrant. The numbers in parentheses show the levels of HTLV-1 p24 produced in the culture supernatants. Data shown are representative of three independent experiments using PBMCs from different donors. **(B)** As shown in **(A)**, IL-2-dependent CD4$^+$ HTLV-1-infected T cells were cultured *in vitro* either alone (control) or exposed to autologous PBMCs (P+) in the presence of 10 μg/ml of LAT-27 or F(ab′)$_2$ LAT-27, or 100 μg/ml of normal human or HAM-IgG in triplicate wells with three supplementations provided at 3 day intervals. Two days after the third exposure, the cells were examined for the frequencies of CD4$^+$ Tax$^+$ cells. Data shown are representative of three independent experiments using HTLV-1-infected cells and PBMCs from different donors.

recognizing the gp46 amino acids 187–193 could not, even though the two mAbs bind similarly to the cell surface of HTLV-1-infected cells and belong to the ADCC-inducing human IgG1.[22]

It remains to be determined whether there are clonal populations of human IgGs that can mediate both the neutralization and ADCC against HTLV-1. So far, it has been shown that the two activities could be operating separately by different epitope-specific human mAbs against gp46.[22] Recently, Kuo *et al.*[24] showed that both neutralizing and non-neutralizing mouse anti-gp46 mAbs can activate neutrophils and mediate its burst activity in the presence of an HTLV-1-infected MT-2 cell line, and concluded that HTLV-1-specific ADCC capacity is not coupled to the neutralizing capacity of the antibody. Thus, these articles highlight the finding of LAT-27 as a special antibody. Analyses of the conformational and antigenic structure of gp46 expressed on the cell surface will be necessary to address this issue further.

Another possible target for ADCC on HTLV-1-expressing cells is the envelope gp21; however, it has been unclear whether human anti-gp21 antibodies function in ADCC. In addition, the recent finding that the glycosylation of Fc-IgG plays an important role in anti-HIV-1 ADCC effector mechanisms[42] suggests that this issue needs to also be considered in the evaluation of anti-HTLV-1 gp46 antibodies and for vaccine formulations in general. Nevertheless, it is clear that the simultaneous operation of neutralization and ADCC by single or polyclonal antibodies is essential to recognize and eliminate HTLV-1$^+$ cells since not only T cells but also the NK cells are permissive to HTLV-1 infection.[43]

The present study also showed that fresh PBMCs had a partial and significant but not complete suppressive activity against autologous HTLV-1-infected cells in the absence of anti-HTLV-1 antibodies. Our preliminary experiments indicate that monocytes might be involved in this partial suppression because PBMCs depleted of CD14$^+$ cells, but not of NK cells, were no longer suppressive in the absence of LAT-27 (data not shown). Since HTLV-1-infected T cells are continuously activated due to the Tax antigen, one possible mechanism is a monocyte-dependent cell death (MDCD)

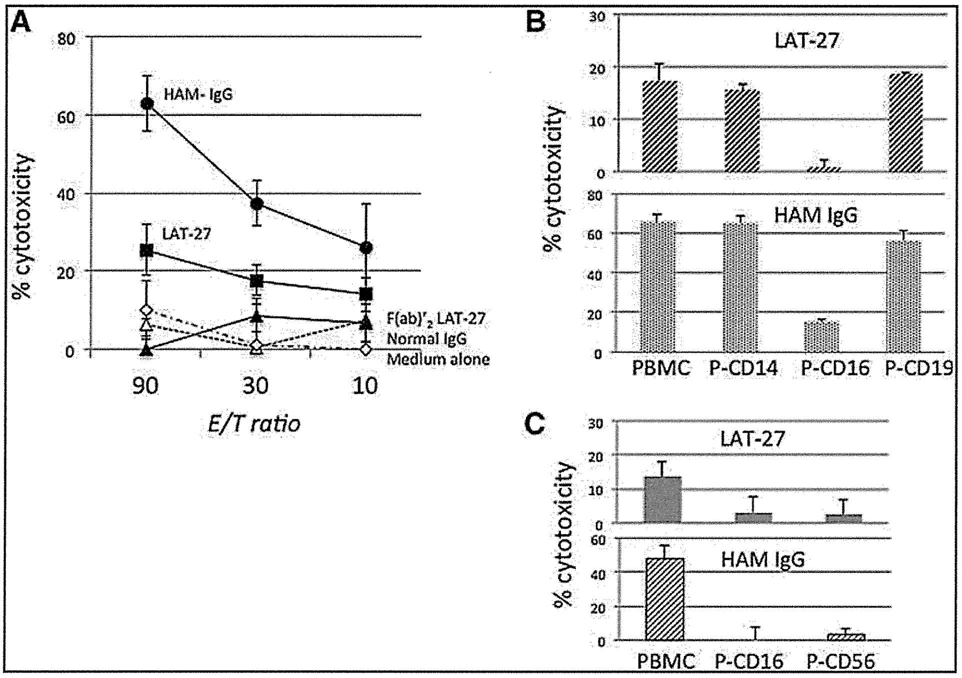


FIG. 7. The CD16$^+$ CD56$^+$ PBMCs mediate antibody-dependent cellular cytotoxicity (ADCC) in the presence of LAT-27 or HAM-IgG. **(A)** ^{51}Cr-labeled HTLV-1-infected cells were cocultured *in vitro* with autologous fresh PBMCs at various E/T ratios in the presence or absence of 10 μg/ml of LAT-27 or F(ab′)$_2$ LAT-27, or 100 μg/ml of normal human or HAM-IgG for 24 h. Each coculture was performed in triplicate, and the amount of radioactivity in the culture supernatants was determined. Data shown are representative of three independent experiments. **(B, C)** Effector PBMCs before or after depletion of CD14$^+$, CD16$^+$, CD19$^+$, or CD56$^+$ cells were assayed for ADCC activity against autologous HTLV-1-infected cells in the presence of LAT-27 (10 μg/ml) or HAM-IgG (100 μg/ml) in triplicate wells in the 24 h ^{51}Cr-release assay. Data shown are representative of two independent experiments.

against activated autologous T cells.[44] Further studies are in progress to address this mechanism.

Based on the data presented herein, it is suggested that humanized LAT-27 mAb might have potential as a passive vaccine against HTLV-1 infection for HTLV-1-uninfected individuals at high risk of HTLV-1 infection, including babies born to HTLV-1 carriers and drug abusers who are also at high risk of HIV infection, and for HTLV-1 carriers whose anti-HTLV-1 neutralizing and ADCC-inducing antibody titers are low. One concern is the potential interference of LAT-27 activity by other nonneutralizing or non-ADCC-inducing antibodies that may interfere with the binding of LAT-27 to gp46. We have performed some experiments and obtained data showing that LAT-12, which blocked the binding of LAT-27 to HTLV-1-infected cells, did not interfere with either LAT-27-mediated syncytium blocking[29] and/or the eradication of HTLV-1-infected cells with autologous PBMCs (Supplementary Fig. S4). It seems likely that the binding affinities of neutralizing antibodies to gp46 expressed on actively living cells are higher than those of nonneutralizing antibodies. Thus, validation of humanized LAT-27 in animal models is currently one of our objectives.

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Y.Tak. and A.H. carried out the ADCC assays. R.T. and A.K. produced, purified, labeled monoclonal antibodies, confirmed their specificities, and made in-house EILSA for p24. M.S. participated in the determination of proviral loads and performed the statistical analysis. M.K. established HTLV-1-infected cells from patients and participated in the design of the study. A.A.A. participated in the design of the study and helped to draft the manuscript. Y.T. conceived the study, participated in its design and coordination, carried out the coculture assays, and drafted the manuscript. All authors read and approved the final manuscript.

Author Disclosure Statement

No competing financial interests exist.

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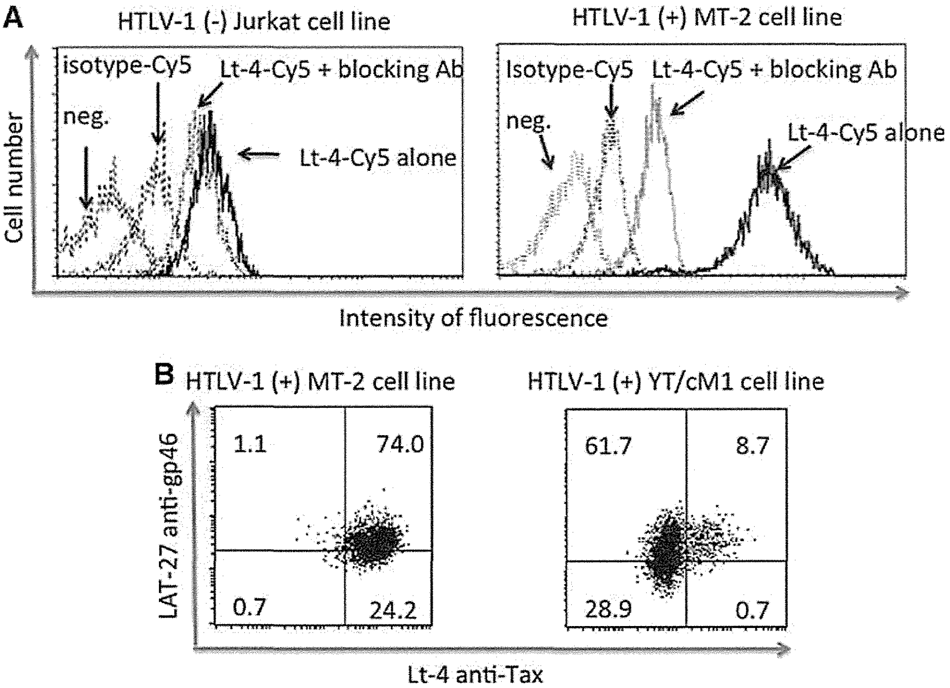
Address correspondence to:
Yuetsu Tanaka
Department of Immunology
Graduate School of Medicine
University of the Ryukyus
207 Uehara, Nishihara-cho
Okinawa 903-0215
Japan

E-mail: yuetsu@s4.dion.ne.jp

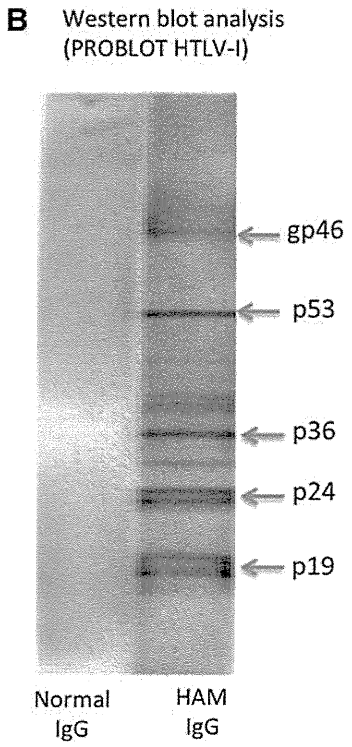
Supplementary Data



SUPPLEMENTARY FIG. S1. Flow cytometry of Tax and HTLV-I gp46 antigens. **(A)** Tax-specific and nonspecific staining by Cy5-labeled Lt-4. HTLV-I-negative Jurkat cells and HTLV-I-positive MT-2 cells were stained with either Cy5-labeled Lt-4 or Cy5-labeled mouse isotype control (IgG3) in the presence or absence of a 500 times excess of nonlabeled Lt-4 (blocking Ab). **(B)** Typical dual staining of MT-2 and another HTLV-I-immortalized T cell line (YT/cM1) with FITC-LAT-27 and Cy5-Lt-4. Negative controls for the two mAbs were obtained from cells stained in the presence of a 500 times excess of nonlabeled homologous blocking mAbs as explained above.

A HTLV-I-coated gelatin particle agglutination assay (SERODIA®HTLV-I)

Sample (1 mg/ml)	Titer
Normal-IgG	< 8 x
HAM-IgG	4,096 x



SUPPLEMENTARY FIG. S2. Characterization of anti-HTLV-I antibody profile of HAM-IgG. (**A**) Purified HAM-IgG at 1 mg/ml was serially diluted and subjected to a commercial anti-HTLV-I agglutination assay (SERODIA®HTLV-I, Fujirebio Inc.). Titers were expressed as the reciprocal dilution that showed a positive reaction. (**B**) Using a commercial anti-HTLV-I western blot assay, IgG (10 µg/ml) from pooled plasma of normal donors and HAM patients was examined for HTLV-I antibodies.