Acta Ophthalmologica

ACTA OPHTHALMOLOGICA 2010

Review Article

Regional Immunity of the Eye

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ABSTRACT.

This article reviews molecular mechanism of intraocular inflammation in animal models and in humans, and the immunological defence system of the eye with particular attention to ocular pigment epithelium. In experimental autoimmune uveitis (EAU), T lymphocytes, particularly $CD4^+$ T lymphocytes, play a central role in its immunopathogenic mechanisms. In humans, activated $CD4^+$ T cells also play a central role in the immunopathogenic mechanisms. This notion is demonstrated in two human diseases: one is Vogt–Koyanagi–Harada disease, and the other is human T-cell leukemia virus type 1 (HTLV-1) uveitis. Activated $CD4^+$ T cells infiltrating the eye are harmful to vision-related cells and tissues in the eye and cause sight-threatening conditions. However, the eye has regional defence systems to protect itself from these harmful activated T cells. We focus on ocular pigment epithelium (PE) and demonstrate immunoregulatory activity of iris PE and retinal PE. Iris PE suppresses activated $CD4^+$ T cells by cell-to-cell contact with a crucial role played by B7-2 molecule on iris PE and CTLA4 on T cells. The actual immunosuppressive factor being membrane bound TGF-β. In contrast, retinal PE suppresses activated $CD4^+$ T cells by soluble factors, such as soluble TGF-β and thrombospondin 1. In addition to the direct T-cell suppression by ocular PE, ocular PE has the capacity to promote activated T cells to regulatory T cells and use them as a tool to amplify the immune down regulation in the eye. The molecular mechanisms of generation of T regulatory cells by iris PE and retinal PE is also discussed.

Key words: activated $CD4^+$ T lymphocytes – cytotoxic T lymphocyte-associated antigen (CTLA)-2α – experimental autoimmune uveitis (EAU) – human T-cell leukemia virus type 1 (HTLV-1) uveitis – iris pigment epithelium – regulatory T cells – retinal pigment epithelium – transforming growth factor (TGF) β – Vogt–Koyanagi–Harada disease

Acta Ophthalmol. 2010: 88: 292–299

doi: 10.1111/j.1755-3768.2009.01757.x

Introduction

It is well known that immunogenic intraocular inflammation or uveitis is a sight-threatening disorder caused by autoimmune or infection-triggered immune mechanisms. Involvement of autoimmune mechanisms in uveitis has been speculated since a long time, but direct evidence of autoimmune mechanisms in uveitis was provided only three decades ago by Wacker & Lipton (1965) and Wacker et al. (1977) who established an animal model for autoimmune uveitis, experimental autoimmune uveoretinitis (EAU), produced by an auto-antigen isolated from soluble fraction of the retina, i.e. S-antigen. Since then, research in immunopathogenic mechanisms of uveitis has greatly progressed. These studies indicate that activated $CD4^+$ T cells play a central role in the immunopathogenic mechanisms of autoimmune uveitis in this animal model (De Kozak et al. 1976; Faure et al. 1977; Salinas-Carmona et al. 1982; Gregerson & Abrahams 1983; Mochizuki et al. 1984, 1985a; Caspi et al. 1988, 1996; Sonoda et al. 2003; Amadi-Obi et al. 2007). In the human situation, activated $CD4^+$ T cells and inflammatory cytokines produced by these activated T cells also play an essential role in the immunopathogenic mechanisms of uveitis (Sagawa et al. 1995; Sugita et al. 1996, 2006d; Ono et al. 1997; Yamaki et al. 2000b).

The eye has a unique defence system to protect intraocular tissues and cells from these pathogenic T cells. The blood ocular barrier is one of the local defence systems which anatomically protect the eye. However, once the blood ocular barrier is disrupted and activated T cells enter the eye, other defence systems or immunological barriers protect the eye. One example of such an immunological defence system is anterior chamber-associated immune deviation reported by Streilein and his associates (Kaplan 1977; Kaplan & Streilein 1978; Streilein et al. 1980; Streilein 2003). In addition, recent studies demonstrated that ocular resident cells such as ocular pigment

epithelial cells have the capacity to suppress activated T cells and further to induce regulatory T cells, which amplify the immune regulation in the eye (Yoshida et al. 2000; Ishida et al. 2003; Sugita & Streilein 2003; Sugita et al. 2004, 2006a,b,c, 2007, 2008; Futagami et al. 2007).

This review article describes the role of activated $CD4^+$ T cells in the animal model of autoimmune uveitis and in human disease as well as the molecular mechanisms of regional immunity of the eye with special attention to ocular pigment epithelial cells.

Immunopathogenic Mechanisms in an Animal Model of Autoimmune Uveitis

Wacker & Lipton (1965) and Wacker et al. (1977) isolated an auto-antigen from the soluble fraction of the retina (S-antigen). S-antigen is localized at the photoreceptor layer of the retina and the pineal gland. Immunization of experimental animals with S-antigen at a site away from the eye induces bilateral panuveitis as well as pinealitis. This model was designated as experimental allergic uveitis or, more appropriately, experimental autoimmune uveoretinitis (EAU). EAU can be induced in a variety of experimental animals which include guinea pigs (De Kozak et al. 1976; Wacker et al. 1977), rats (De Kozak et al. 1981), mice (Caspi et al. 1988; Iwase et al. 1990), and primates (Nussenblatt et al. 1981a) by immunization of retinal antigens. Today, many uveitogenic retinal antigens have been reported, such as interphotoreceptor-binding protein (Gery et al. 1990), phosducin (Dua et al. 1992), and recoverin (Gery et al. 1994).

EAU is an ideal model to dissect the immunopathogenic mechanisms of autoimmune uveitis. Early studies in the 1980s using athymic nude rats (Salinas-Carmona et al. 1982) and adoptive transfer of EAU to naïve rats by S-antigen-sensitized T cells (Mochizuki et al. 1985a) revealed that T lymphocytes, particularly $CD4^+$ helper T cells, play an essential role for EAU induction. Since then, many studies have been carried out to clarify the immunopathogenic mechanisms of EAU. Our present understanding of immunopathogenic mechanisms of EAU is briefly illustrated in Fig. 1 (Mochizuki 2009). Retinal antigen injected into animals is phagocytized by antigen-presenting cells such as macrophages and digested into small peptides, which are presented to T cells through MHC class II molecules with costimulating signals to T-cell receptor on T cells at the levels of the spleen and lymph nodes. The sensitized $CD4^+$ T cells home to the eye and recognize retinal antigens in the eye and produce various inflammatory cytokines, resulting in inflammation. T helper 1 (Th1) cells which produce Th1 cytokines such as interferon-γ are responsible for the development of EAU (Caspi et al. 1996) whereas Th2 cells which produce Th2 cytokines such as interleukin 4 (IL-4) suppress the disease (Gegg et al. 2005). More recently, it has been reported that the actual T cells responsible for EAU induction are Th17 cells (Amadi-Obi et al. 2007), which are generated by stimulation with IL-6 and TGF-β.

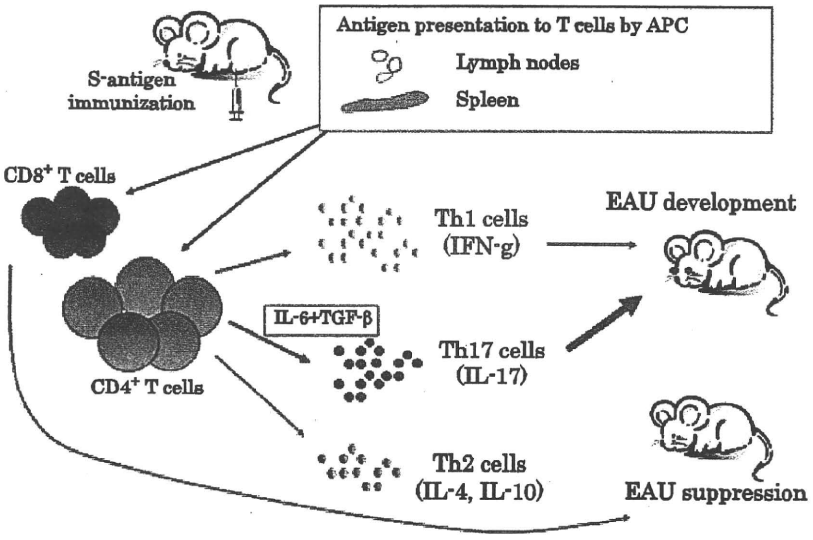

Fig. 1. Immunopathogenic mechanisms of EAU. The figure is reproduced from a reference (Mochizuki 2009, Fig. 1) with permission.

EAU is also a very useful animal model to develop various immunotherapies (Mochizuki & deSmet 1994). One of the early studies was the use of cyclosporine, a selective T-cell immunosuppressant (Nussenblatt et al. 1981b). Later, cyclosporine was compared to other conventional immunosuppressive agents in EAU (Mochizuki et al. 1985b). The knowledge drawn from these studies in EAU is applied to humans to treat non-infectious refractory uveitis, such as Behcet's disease (Nussenblatt et al. 1983; Masuda et al. 1989).

These accumulated data clearly demonstrated that activated $CD4^+$ T cells play the essential role in the immunopathogenic mechanisms of an animal model of autoimmune uveitis. This is also true in humans as described later.

Immunopathogenic Mechanisms of Uveitis in Human

Two examples of uveitis in humans will be discussed here. One is Vogt–Koyanagi–Harada (VKH) disease, a classic autoimmune disease in humans, and the other is immunogenic uveitis associated with infection of human T-cell leukemia virus type 1 (HTLV-1).

VKH disease

Clinical characteristics and histopathology of VKH disease

This disease was first reported by Vogt (1906) and described much more precisely by Koyanagi (1914) and Harada (1926).

The disease is a non-traumatic bilateral uveitis, commonly seen in pigmented ethnic groups such as Japanese, but very rare in non-pigmented ethnic groups such as Caucasians. In the early acute stage of the disease, diffuse chor-

oiditis, as demonstrated by multiple dark hypofluorescent spots by indocyanine green angiography is the primary lesions of the disease (Bouchenaki & Herbort 2001; Herbort et al. 2007), while multi-focal exudative retinal detachments are the secondary lesions. Patients also show systemic symptoms, such as headache, nausea, tinnitus, and hearing disturbance. In late stage of the disease 3–4 months after the disease onset, depigmentation occurs, such as sunset-glow fundus, alopecia, poliosis, and vitiligo. Because of these characteristic clinical features, the disease is considered to be an autoimmune disease to melanocytes.

Inomata (Inomata 1989) described the histopathology of ocular lesions in VKH disease. In the acute ophthalmic stage of the disease, numerous melanocytes are still present in the choroid surrounded by a large number of lymphocytes infiltrating the choroid. In the late stage of the disease characterized by sunset-glow fundus, the melanocytes in the choroid have completely disappeared. The ocular histopathologic features in VKH patients indicate that melanocytes in the choroid are the target of lymphocytic infiltration.

Immunopathogenic mechanisms of VKH disease

Although VKH disease was considered to be an autoimmune disease to melanocytes because of its clinical and histopathologic features, the real pathogenic auto-antigen has not been identified until Yamaki et al. (2000a,b) first established an animal model of VKH disease. They immunized animals with tyrosinase, which is a melanocyte-associated antigen and an enzyme related to melanin synthesis. Within 2 weeks after immunization with tyrosinase peptides, animals develop bilateral panuveitis characterized by lymphocytic infiltration in the iris, ciliary body, and choroid. More importantly, 8 weeks later, the animals develop depigmentation in the skin as well as in the choroid with sunset-glow fundus, similar to the features of VKH disease in humans. In addition, peripheral blood mononuclear cells (PBMC) of VKH patients respond to tyrosinase peptides (Yamaki et al. 2000b).

The question we asked was whether lymphocytes infiltrating the eye of VKH patients were sensitized to tyrosinase peptides. For that purpose, we have established $CD4^+$ T-cell clones from infiltrating cells eyes of VKH patients using the limiting dilution method (Sugita et al. 1996). Our study (Sugita et al. 2006b) has shown that $CD4^+$ T-cell clones from the eye of VKH patients respond to tyrosinase$_{450-462}$ peptides and produce inflammatory cytokines, but not to the control antigen HA peptide, an influenza virus peptide. $CD4^+$ T-cell clones from other clinical entities of uveitis such as Behcet's disease and sarcoidosis do not respond to tyrosinase peptides. The response depends on the dose and the structure of the tyrosinase peptides, indicating that the response is specific to tyrosinase peptides. These data thus indicate that the pathogenic auto-antigen of VKH disease is tyrosinase, and tyrosinase-sensitized $CD4^+$ T cells are present in the eye of VKH disease patients and produce inflammatory cytokines in response to tyrosinase.

However, it is still unknown how the lymphocytes of VKH patients are sensitized to the tyrosinase auto-antigen. We hypothesized whether tyrosinase might represent molecular mimicry to some exogenous antigens. GeneBank data analysis disclosed that only one exogenous peptide, cytomegalovirus envelop glycoprotein $H_{450-462}$ (CMV-eg$H_{290-302}$) peptides, had a structural homology to tyrosinase$_{450-462}$ peptides (Sugita et al. 2006b). Among 13 amino acids of tyrosinase$_{450-462}$ sequences, 6 amino acids sequence had a homologue and the sequence was an important motif to be recognized by HLA-class II antigen. We then, it was tested whether lymphocytes of VKH patients reacted to both tyrosinase$_{450-462}$ and CMV-eg$H_{290-302}$ (Sugita et al. 2006b). PBMC from VKH patients responded to CMV peptide as well as tyrosinase, but not to a control peptide. On the other hand, PBMC from Behcet disease and healthy donors did not respond to any of these peptides. Thus, $CD4^+$ T-cell clones from the eye of patients with VKH disease responded to both CMV peptide and tyrosinase, but not to a control antigen.

Based on these data, molecular mechanism of immunnopathogenesis of VKH disease is illustrated in Fig. 2 (Mochizuki 2009). Following cytomegalovirus infection, $CD4^+$ T cells are sensitized to CMV peptide. These CMV-sensitized $CD4^+$ T cells recognize tyrosinase expressed on melanocytes in the eye as well as in other tissues because of its molecular mimicry and cause an immunologic cross-reaction, resulting in inflammation in those tissues including the eye.

HTLV-1 uveitis

Definition of HTLV-1 uveitis

HTLV-1 uveitis is defined as an intraocular inflammation in asymptomatic carriers of human T-cell leukemia virus type 1 (HTLV-1) or Human T-lymphotropic virus type 1 (HTLV-1). In general, HTLV-1 can cause adult T-cell leukemia (ATL)/T-cell lymphoma (Hinuma et al. 1981; Yoshida et al. 1982, 1984; Yamaguchi et al. 1984) and ocular involvement, leading in these symptomatic patients either to leukemic infiltration in the eye or opportunistic infections such as cyto-

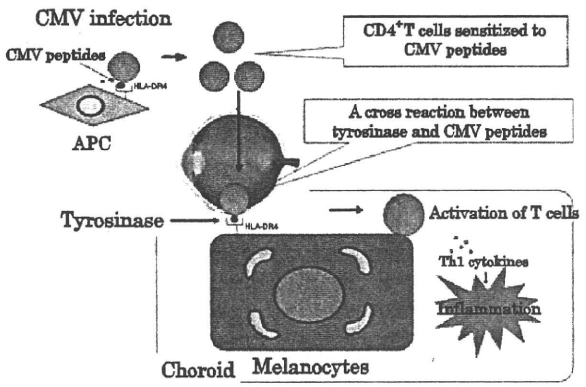

Fig. 2. Immunopathogenic mechanisms of Vogt–Koyanagi–Harada disease. The figure is reproduced from a reference (Mochizuki 2009, Fig. 2) with permission.

megalovirus retinitis (Mochizuki et al. 1996).

Diagnosis

A diagnosis of HTLV-1 uveitis is obtained when the following 2 criteria are present: (1) patients must be seropositive to HTLV-1, and (2) routine systemic and ophthalmic examinations exclude all other defined entities of uveitis.

Epidemiology

HTLV-1 is prevalent in Central Africa, Carribian Islands, South America and Kyushu Island of Japan, especially southern Kyushu Island (Mochizuki et al. 1996). To identify if HTLV-1 infection is causatively related to a certain type of uveitis, a seroepidemiological study was carried out in a HTLV-1 endemic area (southern Kyushu Island of Japan) and in less endemic area (northern Kyushu Island) (Mochizuki et al. 1992a,b). The HTLV-1 seroprevalence in patients with idiopathic uveitis was significantly higher than that in two control groups (patients with etiology defined uveitis and patients with non-uveitic ocular diseases such as cataract, glaucoma and retinal detachment). The odd ratio of idiopathic uveitis for HTLV-1 infection was estimated to be 14.6 (95% confidence interval: 5.3–40.2) in endemic area of HTLV-1, and even in less endemic of northern Kyushu Island, it was estimated to be 12.0 (95% confidence interval: 1.5–95.5) (Mochizuki et al. 1992b). This was the first clue suggesting that HTLV-1 infection is significantly related to uveitis, i.e. idiopathic uveitis in those days, and the disease is now recognized as a distinct clinical entity related to HTLV-1 and designated as HTLV-1 uveitis (HU) or HTLV-1-associated uveitis.

Clinical manifestations

Clinical studies by Yoshimura et al. (1993) and Takahashi et al. (2000) that analyzed clinical features of HTLV-1 uveitis disclosed that the disease is characterized by moderate to severe vitritis accompanied by mild iritis and mild retinal vasculitis in 1 eye (58%) or both eyes (42%). More than 60% of patients are classified as intermediate uveitis with membranous, lacework-like or dense vitreous opacities together with mild retinal vasculitis. Despite the fact that it is considered to be a viral infection, corticosteroids are very effective to treat the patients. The visual prognosis of HTLV-1 uveitis is generally good with treatments using topical and/or systemic corticosteroids, though about 60% of patients have recurrences of uveitis.

Only a few HTLV-1 uveitis patients develop HAM/TSP, but no literature has reported HU patients who develop ATL/T-cell lymphoma in their clinical course.

Immunopathogenesis of HTLV-1 uveitis

HTLV-1 uveitis is considered to be caused by inflammatory cytokines produced by HTLV-1-infected $CD4^+$ T cells which significantly accumulated in the eye of the patients. This notion is supported by the following data.

(1) The majority of infiltrating cells in the aqueous humor of HTLV-1 uveitis patients are $CD3^+$ T cells (Ono et al. 1997), and no malignant cells or leukemic cells are present based on T-cell receptor assays of the infiltrating cells (Masuoka et al. 1995). (2) PCR analysis of ocular infiltrating cells disclosed that HTLV-1 proviral DNA was detected in almost all HU patients (37 among 38 HTLV-1 patients), but not in uveitis patients because of other etiologies, who were seropositive to HTLV-1 by chance (Ono et al. 1997). (3) Viral particles were detected by transmission electron microscope in $CD4^+$ T cells in the eye of HTLV-1 uveitis patients, and HTLV-1 protein was detected by immunohistochemical staining (Sagawa et al. 1995). (4) The HTLV-1 provirus load in HTLV-1 uveitis patients is significantly higher than that of asymptomatic carriers without uveitis (Ono et al. 1995). Furthermore, the provirus load in PBMC correlates with intensity of intraocular inflammation (Ono et al. 1998). (5) The provirus load in the eye of HTLV-1 uveitis patients is significantly higher than that present in PBMC (Ono et al. 1997). (6) HTLV-1-infected $CD4^+$ T-cell clones established from infiltrating cells in eyes of HTLV-1 uveitis patients produced a large amount of various inflammatory cytokines, such as IL-1, IL-6, IL-8, TNF-α, and interferon-γ. Furthermore, addition of corticosteroids in the culture medium suppressed the cytokine production (Sagawa et al. 1995).

Regional Immunological Defence System of the Eye in Mice

As described earlier, $CD4^+$ T cells play the central role of intraocular inflammation in an animal model of autoimmune uveitis (EAU) as well as in human diseases. However, the eye has a local defence system to protect important tissues and cells inside the eye from these inflammatory cells. 'Blood ocular barrier' is the defence system, which anatomically protects the eye. The eye also has another unique defence system, consisting of an immunologic barrier called immune privilege of the eye. Streilein and his colleagues (Streilein et al. 1980; Streilein 2003) described that many ocular resident cells contribute to the immune privilege of the eye.

Here, we focused on ocular pigment epithelium (PE), because they are located at the gate of the blood ocular barrier. It seems that immune regulation works much more efficiently at the gate of the barrier rather than once activated T cells are in the eye.

Immune regulation by ocular PE

To test if ocular PE has the capacity to regulate activated $CD4^+$ T cells, ocular PE isolated from iris, ciliary body, and retina of B57BL/6 mice were cocultured with $CD4^+$ T cells in the presence of anti-CD3 antibody (Yoshida et al. 2000; Ishida et al. 2003; Sugita & Streilein 2003). It has been shown that all three ocular PE directly suppress proliferation of activated T cells. In addition, the suppression by iris PE disappears when the two cells are separated by a cell-insert membrane, indicating that immune regulation by iris PE requires cell-to-cell contact. In contract, suppression by retinal PE is not influenced by such a cell-insert membrane, suggesting that retinal PE regulates activated $CD4^+$ T cells by soluble factors. These data indicate that ocular PE directly suppresses activated $CD4^+$ T cells, but by different modes of action.

What is the biological significance of these phenomena? The microenvironment where iris PE is located is surrounded by aqueous humor, and because of this anatomic condition, soluble factors will be easily diluted by aqueous humor. Therefore, cell-

to-cell contact appears to be most efficient in such context. On the other hand, retinal PE is located in the posterior segment of the eye which is a cell dense microenvironment. Soluble factors appear to be more effective than cell-to-cell contact in such an environment.

Molecular mechanisms of direct T-cell suppression by retinal PE

To identify the immunosuppressive soluble factors participating in the immune regulation in the posterior segment of the eye, we screened gene expression of various regulatory factors in RPE and found two molecules, expressing high levels: one is transforming growth factor-β (TGF-β) and the other is thrombospondin 1, TSP-1 (Sugita et al. 2006a). Our major findings are exposed below. TGF-β, a well known potent immunosuppressive factor, is expressed on retinal PE at protein levels and secreted by retinal PE. T cells from wild-type mice are suppressed by retinal PE, whereas T cells from dominant negative TGF-β receptor mice where TGF-β signal does not enter T cells are not suppressed by retinal PE. This indicates that TGF-β signal towards T cells is essential for the T-cell suppression by retinal PE. The other soluble factor, TSP-1, is expressed on all cultured ocular PE and is secreted by the ocular PE cells, particularly retinal PE (Futagami et al. 2007). Among tested antibodies against various suppressive factors, only anti-TSP-1 antibody neutralized suppressive activity of retinal PE (Sugita et al. 2006a), indicating that TSP-1 is necessary for the suppressive activity of retinal PE. TSP-1 plays a key role to induce the active form of TGF-β in ocular PE. This notion has been confirmed by experiments using recombinant TSP-1; recombinant TSP-1 significantly enhances secretion of active form of TGF-β by R retinal PE (Futagami et al. 2007).

Figure 3 illustrates the molecular mechanisms of activated $CD4^+$ T-cell suppression by retinal PE. TSP-1 on retinal PE converts the latent form of TGF-β into its active and soluble form. Then, TGF-β signal through its receptors on T cells induces suppression of activated $CD4^+$ T cells (Mochizuki 2009).

Molecular mechanisms of direct T-cell suppression by iris PE

In the anterior segment of the eye, iris PE suppresses activated T cells using cell-to-cell contact. Therefore, the first question to be addressed here is what molecules participate in the cell-to-cell contact between iris PE and T cells. A study to screen the gene expression of the three ocular PE has shown that CD86 (B7-2), a well known costimulatory molecule, is strongly expressed on iris PE, but not on retinal PE (Sugita & Streilein 2003). Iris PE from B7-2 KO mice fails to suppress activated T cells, whereas ciliary body PE and retinal PE from B7-2 KO mice suppress T cells, indicating that B7-2 is a unique molecule for the immune regulation by iris PE (Sugita & Streilein 2003). It is well known that a ligand molecule for B7-2 is cytotoxic T lymphocyte-associated antigen (CTLA)-4. In fact, CTLA-4 is expressed on activated T cells which are in close contact with the iris PE (Sugita et al. 2004). T cells from CTLA-4 KO mice are not suppressed by iris PE, indicating that interaction between CTLA-4 on T cells and B7-2 on iris PE is necessary for the T-cell suppression by iris PE (Sugita & Streilein 2003). A key immunosuppressive factor in the anterior segment is also TGF-β, like in the posterior segment. T cells from wild-type mice are

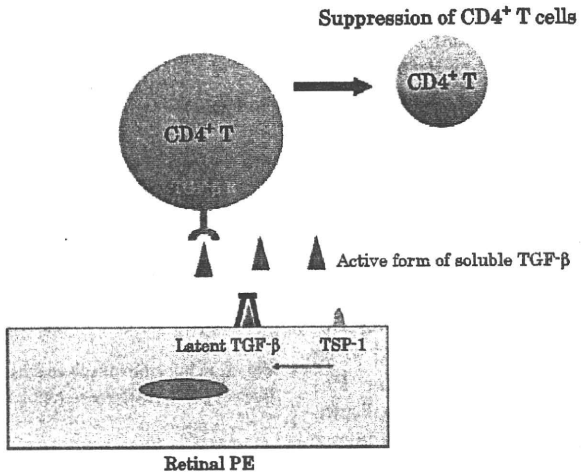

Fig. 3. Molecular mechanisms of direct T-cell suppression by retinal pigment epithelium (PE). The figure is reproduced from a reference (Mochizuki 2009, Fig. 21) with permission.

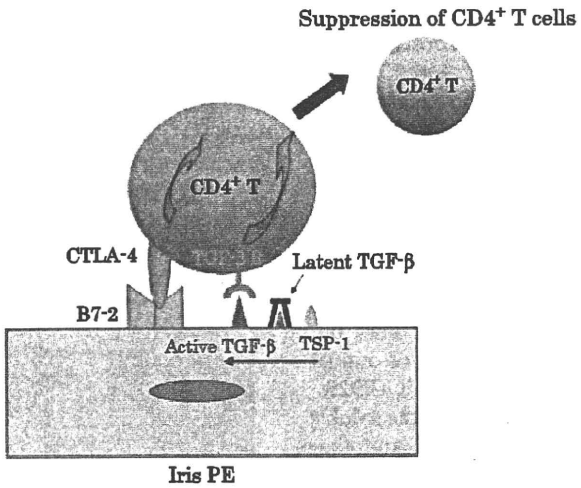

Fig. 4. Molecular mechanisms of direct T-cell suppression by iris pigment epithelium (PE). The figure is reproduced from a reference (Mochizuki 2009, Fig. 28) with permission.

strongly suppressed by iris PE, whereas T cells from dominant negative TGF-β receptor mice are not suppressed by iris PE (Sugita et al. 2006b), indicating that TGF-β signal from iris PE to T cell is essential.

Figure 4 illustrates molecular mechanisms of iris PE-induced immunosuppression (Mochizuki 2009). TSP-1 on iris PE converts the latent form of membrane-bound TGF-β to active TGF-β. Then, B7-2 molecule on iris PE and CTLA-4 on T cells combine to form cell-to-cell contact and the TGF-β signal enters T cells which induce suppression of activated $CD4^+$ T cells.

Regulatory T cells generated by ocular PE

In addition to direct suppression as described earlier, ocular PE have another unique system to amplify their immune regulatory activities by generating regulatory T cells (T reg). This has been confirmed by the following studies (Sugita et al. 2006a,b, 2008). $CD4^+$ or $CD8^+$ T cells are cocultured with ocular PE isolated from mice in the presence of anti-CD3 antibody (0.01 μg/ml). Then, PE-exposed T cells are harvested and irradiated. Responder $CD4^+$ T cells are isolated from mice and cultured with ocular PE-exposed T cells in the presence of anti-CD3 antibody (1.0 μg/ml). Influence of ocular PE-exposed T cells on proliferation of responder T cells is measured to determine if ocular PE-exposed T cells acquire regulatory activity. Proliferation of responder $CD4^+$ T cells is significantly suppressed by retinal PE-exposed T cells as well as by iris PE-exposed T cells. This indicates that both retinal PE and iris PE are able to induce T reg. However, retinal PE-generated T reg and iris PE-generated T reg are different T-cell populations. Namely, retinal PE-generated T reg is $CD4^+$ T cells, whereas iris PE-generated T reg is $CD8^+$ T cells. In contrast to these two ocular PE, ciliary body PE does not induce T reg. The profile of retinal PE-generated T Reg is $CD4^+$, $CD25^+$ and $Foxp3^+$. They produce the soluble form of TGF-β and TSP-1 and they express TGF-β receptors. On the other hand, iris PE-generated T reg is $CD8^+$ and TGF-β is membrane-binding form (Sugita et al. 2007).

We further investigated whether there were any unique molecules in retinal PE to generate T reg (Sugita et al. 2008). We used microchip gene array and found a novel factor, that is, CTLA-2α. CTLA-2 α is expressed on retinal PE, but not on other 2 ocular PE. CTLA-2 α is known to act as cathepsin L inhibitor or cysteine protease inhibitor. We investigated the function of CTLA-2 α in the T-cell regulation. Recombinant CTLA-2 α-exposed $CD4^+$ T cells suppressed proliferation of responder T cells, indicating that CTLA-2 α itself has the capacity to generate T reg. Furthermore, Foxp3 gene, a marker of regulatory T cells, was expressed on CTLA-2

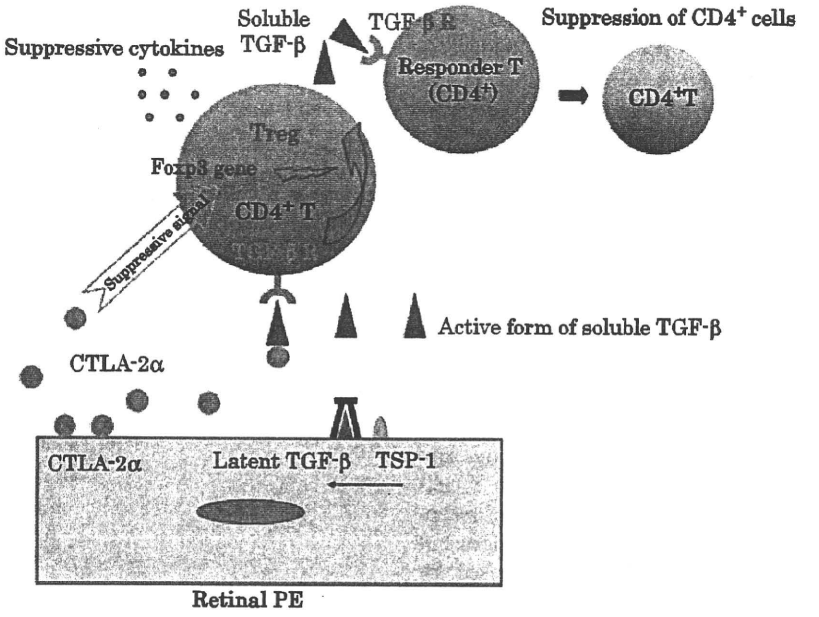

Fig. 5. Molecular mechanisms of regulatory T cells (T reg) generation by retinal pigment epithelium (PE). The figure is reproduced from a reference (Mochizuki 2009, Fig. 34) with permission.

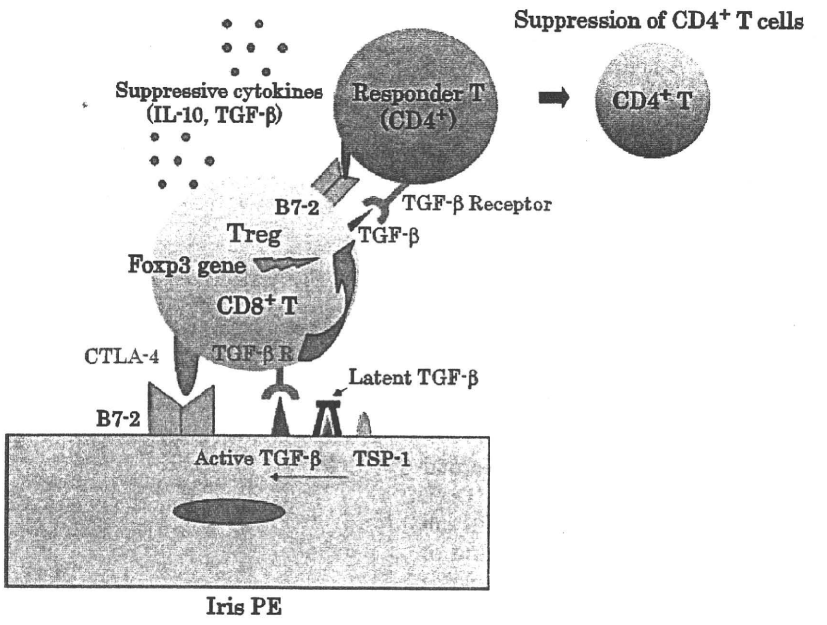

Fig. 6. Molecular mechanisms of regulatory T cells (T reg) generation by iris pigment epithelium (PE). The figure is reproduced from a reference (Mochizuki 2009, Fig. 35) with permission.

α-exposed CD25$^+$ T cells. We further examined the influence of CTLA-2 α siRNA. Retinal PE transfected with siRNA failed to convert T cells into T reg (Sugita et al. 2008). Thus, production of CTLA-2 α by retinal PE is essential to promote T reg generation.

Figures 5 and 6 illustrate the molecular mechanism of retinal PE-generated T reg and that of iris PE-generated T reg, respentively (Mochizuki 2009). TSP-1 on retinal PE stimulates latent TGF-β to change to active form of soluble TGF- β. TGF- β signals through its receptors on CD4$^+$ T cells together with CTLA-2α induce regulatory signals in activated CD4$^+$ T cells. And the CD4$^+$ T cells are transformed to Foxp3$^+$ regulatory T cells and acquire the capacity to suppress responder T cells (Fig. 5). On the other hand, iris PE reacts to CD8$^+$ T cells through B7-2.

In summary, ocular PE cells are part of the immunologic defence system of the eye. They directly suppress activated CD4$^+$ T cells. Not only that ocular PE converts other T cells to T reg, but also uses them as a tool to amplify immune regulation in the eye. However, the mode of action is different depending on the anatomic microenvironment in the eye. And specific molecules and genes precisely control the responses.

Immune Regulation in Human

Similar to mice, human iris PE directly suppresses CD4$^+$ T cells from the eye of VKH patients, and this suppression is abolished by cell-insert membrane (Horie et al. 2009). Human retinal PE also has the capacity to promote T reg generation. For example, human retinal PE-exposed T cells suppress CD4$^+$ T cells from the eye of uveitis patients, such as sarcoidosis, VKH disease, and acute retinal necrosis (Sugita S, Horie S and Mochizuki M unpublished data).

Conclusion

In conclusion, homeostasis of the eye and high quality of vision is maintained by a balance between regional immune and inflammatory processing and regional defence systems of the eye. Ocular resident cells can be injured by these activated T cells in some instances, but at the same time, ocular resident cells suppress these harmful activated T cells or even convert them to T reg and use them as a tool to amplify the immune regulation of the eye. We may use these cells and molecules for the therapy of intraocular inflammatory disorders in the near future.

References

Amadi-Obi A, Yu CR, Liu X et al. (2007): TH17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STATI. Nat Med **13**: 711–718.

Bouchenaki N & Herbort CP (2001): Indocyanine green angiography to the appraisal and management of Vogt–Koyanagi–Harada. Ophthalmology **108**: 54–64.

Caspi RR, Roberge FG, Chan CC, Wiggert B, Chader GJ, Rozenszajn LA, Lando Z & Nussenblatt RB (1988): A new model of autoimmune disease. Experimental autoimmune uveoretinitis induced in mice with two different retinal antigens. J Immunol **140**: 1490–1495.

Caspi RR, Silver PB, Chan CC et al. (1996): Genetic susceptibility to experimental autoimmune uveoretinitis in the rat is associated with an elevated Th 1 response. J Immunol **157**: 2668–2675.

De Kozak Y, Usui M & Faure JP (1976): Experimental autoimmune uveoretinitis. Ultrastructure of chorioretinal lesions induced in guinea pigs by immunization against the outer rods of the bovine retina. Arch Ophthalmol **36**: 231–248.

De Kozak Y, Sakai J, Thillaye B & Faure JP (1981): S antigen-induced experimental autoimmune uveoretinitis in rats. Curr Eye Res **1**: 327–337.

Dua HS, Lee RH, Lolley RN, Barrett JA, Abrams M, Forrester JV & Donoso LA (1992): Induction of experimental autoimmune uveitis by the retinal photoreceptor cell protein, phosducin. Curr Eye Res **11**(Suppl): 107–111.

Faure JP, de Kozak Y, Dorey C & Tuyen VV (1977): Activite de differentes preparations antigeniques de la retine dans l'induction de l'uveo-retinite autoimmune experimentale chez le cobaye. Arch Ophthalmol (Paris) **37**: 47–60.

Futagami Y, Sugita S, Vega J, Ishida K, Takase H, Maruyama K, Aburatani H & Mochizuki M (2007): Role of thrombospondin-1 in T cell response to ocular pigment epithelial cells. J Immunol **178**: 6994–7005.

Gegg ME, Harry R, Hankey D et al. (2005): Suppression of autoimmune retinal disease by lovastatin dose not require Th2 cytokine induction. J Immunol **174**: 2327–2335.

Gery I, Wiggert B, Redmond TM, Kuwabara T, Crawford MA, Vistica BP & Chader GJ (1990): Uveoretinitis and pinealitis induced by immunization with interphotoreceptor retinoid-binding protein. Invest Ophthaomol Vis Sci **27**: 1296–1300.

Gery I, Chanaud NP 3rd & Anglade E (1994): Recoverin is highly uveitogenic in Lewis rats. Invest Ophthalmol Vis Sci **35**: 3342–3345.

Gregerson DS & Abrahams IW (1983): Immunologic and biochemical properties of several retinal proteins bound by antibodies in sera from animals with experimental autoimmune uveitis and uveitis patients. J Immunol **131**: 259–264.

Harada E (1926): Beitrag zur klinishen Kenntniss von nichteitriger Choroiditis (Choroiditis diffusa acuta). J Jpn Ophthalmol Soc **30**: 356–378 (In Japanese).

Herbort CP, Mantovani A & Bouochenaki N (2007): Indocyanin green angiography in Vogt–Koyanagi–Harada disease: angiographic signs and utility in patient follow-up. Int Ophthalmol **27**: 173–182.

Hinuma Y, Nagata K, Hanaoka M, Nakai M, Matsumoto T, Kinoshita K, Shirakawa S & Miyoshi I (1981): Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. Proc Natl Acad Sci USA **78**: 6476–6480.

Horie S, Sugita S, Futagami Y, Kawaguchi T, Kamoi K, Shirato S & Mochizuki M (2009): Human iris pigment epithelium suppresses activation of bystander T cells via TGFβ-TGFβ receptor interaction. Exp Eye Res **88**: 1033–1042.

Inomata H (1989): Vogt–Konyanagi–Harada disease. In: Inomata H, Ohnishi K, Mukuno T & Ishibashi T (eds). Clinical Pathology of Retinochroidal Diseases. Tokyo: Igakushoin 88–93.

Ishida K, Panjwani N, Cao Z & Streilein JW (2003): Participation of pigment epithelium of iris and ciliary body in ocular immune privilege. 3. Epithelia cultured from iris, ciliary body, and retina suppress T cell activation by partially non-overlapping mechanisms. Ocul Immunol Inflamm **11**: 91–105.

Iwase K, Fujii Y, Nakashima I, Kato N, Fujino Y, Kawashima H & Mochizuki M (1990): A new method for induction of experimental autoimmune uveoretinitis (EAU) in mice. Curr Eye Res **9**: 207–216.

Kaplan HJ (1977): Immune response to immunization via the anterior chamber of the eye. J Immunol **118**: 809–814.

Kaplan HJ & Streilein JW (1978): Immune response to immunization via the anterior chamber of the eye. II. An analysis of F1 lymphocyte induced immune deviation. J Immunol **120**: 689–693.

Koyanagi Y (1914): Poliosis and alopecia associated with uveitis. J Jpn Ophthalmol Soc **18**: 1183–1193 (In Japanese).

Masuda K, Nakajima A, Urayama A, Nakae K, Kogure M & Inaba G (1989): Double-masked trial of cyclosporine versus colchicine and long-term open study of cyclosporine in Behcet's disease. Lancet **8647**: 1093–1095.

Masuoka K, Sagawa K, Mochizuki M, Oisumi K & Itoh K (1995): Polyclonal use of T-cell receptor α for human T-cell lymphotropic virus type 1-infected T cells. Invest Ophthalmol Vis Sci **36**: 254–258.

Mochizuki M (2009): Intraocular inflammation and homeostesis of the eye. J Jpn Ophthalmol Soc **113**: 344–378 (in Japanese).

Mochizuki M & deSmet M (1994): Use of immunosuppressive agents in ocular diseases. Prog Retin Eye Res **13**: 479–506.

Mochizuki M, Kuwabara T, Chan CC, Nussenblatt RB, Metcalfe DD & Gery I (1984): An Association between susceptibility to experimental autoimmune uveitis and choroidal mast cell numbers. J Immunol **133**: 1699–1701.

Mochizuki M, Kuwabara T, McAllister C, Nussenblatt RB & Gery I (1985a): Adoptive transfer of experimental autoimmune uveoretinitis in rats: immunopathogenic mechanisms and histological features. Invest Ophthalmol Vis Sci **26**: 1–9.

Mochizuki M, Nussenblatt RB, Kuwabara T & Gery I (1985b): Effects of cyclosporine and other immunosuppressive drugs on experimental autoimmune uveoretinitis in rats. Invest Ophthalmol Vis Sci **26**: 226–232.

Mochizuki M, Watanabe T, Yamaguchi K et al. (1992a): Uveitis associated with human T-cell lymphotropic virus type I. Am J Ophthalmol **114**: 123–129.

Mochizuki M, Watanabe T, Yamaguchi K et al. (1992b): Uveitis associated with human T lymphotropic virus type I: seroepidemiological, clinical and virological studies. J Infect Dis **166**: 943–944.

Mochizuki M, Watanabe T, Yamaguchi K & Tajima K (1996): Human T-lymphotroic virus type I associated disease. In: Pepose JS, Holland GA & Whihelmus KR (eds). Infection & Immunology. St Louis: Mosby 1366–1387.

Nussenblatt RB, Kuwabara T, de Monasterio FM & Wacker WB (1981a): S-antigen uveitis in primates. A new model for human disease. Arch Ophthalmol **99**: 1090–1092.

Nussenblatt RB, Rodgigues M, Wacker WB, Cevario SJ, Salinas-Carmona MC & Gery I (1981b): Cyclosporin A. Inhibition of experimental autoimmune uveitis in Lewis rats. J Clin Invest **67**: 1228–1231.

Nussenblatt RB, Palestine AG, Rook AH, Scher I, Wacker RB & Gery I (1983): Cyclosporin A therapy of intraocular inflammatory disease. Lancet **2**: 235–238.

Ono A, Mochizuki M, Yamaguchi K, Miyata N & Watanabe T (1995): Increased number of circulating HTLV-I infected cells in peripheral blood mononuclear cells of HTLV-I uveitis patients: a quantitative polymerase chain reaction study. Br J Ophthalmol **79**: 270–276.

Ono A, Mochizuki M, Yamaguchi K, Miyata N & Watanabe T (1997): Immunologic and virologic characterization of the primary infiltrating cells in the aqueous humor of human T-cell leukemia virus type 1 uveitis. Invest Ophthalmol Vis Sci **38**: 676–689.

Ono A, Ikeda E, Mochizuki M et al. (1998): Provirus load in patients with human T-cell leukemia virus type 1 uveitis correlates with precedent Graves' disease and disease activity. Jpn J Cancer Res **89**: 608–614.

Sagawa K, Mochizuki M, Masuoka K et al. (1995): Immunopathological mechanisms of human T cell lymphotropic virus type 1 (HTLV-I) uveitis: detection of HTLV-I-infected T cells in the eye and their constitutive cytokine production. J Clin Invest **95**: 852–858.

Salinas-Carmona MC, Nussenblatt RB & Gery I (1982): Experimental autoimmune uveitis in the athymic nude rat. Eur J Immunol **12**: 481–484.

Sonoda KH, Sasa Y, Qiao H et al. (2003): Immunoregulatory role of ocular macrophages: the macrophages produce RANTES to suppress experimental autoimmune uveitis. J Immunol **171**: 2652–2659.

Streilein JW (2003): Ocular immune privilege: therapeutic opportunities from an experiment of nature. Nat Rev Immunol **3**: 879–889.

Streilein JW, Niederkorn JY & Shadduck JA (1980): Systemic immune unresponsiveness induced in adult mice by anterior chamber presentation of minor histocompatibility antigens. J Exp Med **152**: 1121–1125.

Sugita S & Streilein JW (2003): Iris pigment epithelium expressing CD86 (B7-2) directly suppresses T cell activation *in vitro* via binding to cytotoxic T lymphocyte-associated antigen 4. J Exp Med **198**: 161–171.

Sugita S, Sagawa K, Mochizuki M, Shichijo S & Itoh K (1996): Melanocyte lysis by cytotoxic T lymphocytes recognizing the MART-1 melanoma antigen in HLA-A2 patients with Vogt–Koyanagi–Harada disease. Int Immunol **8**: 799–803.

Sugita S, Ng TF, Schwartzkopff J & Streilein JW (2004): CTLA-4$^+$ CD8$^+$ T cells that encounter B7-2$^+$ iris pigment epithelial cells express their own B7-2 to achieve global suppression of T cell activation. J Immunol **172**: 4184–4194.

Sugita S, Futagami S, Smith B, Naggar H & Mochizuki M (2006a): Retinal and ciliary body pigment epithelium suppress activation of T lymphocytes via transforming growth factor beta. Exp Eye Res **83**: 1459–1471.

Sugita S, Ng TF, Lucas PJ, Gress RE & Streilein JW (2006b): B7$^+$ iris pigment epithelium induced CD8$^+$ Tregulatory cells; both suppress CTLA-4$^+$ T cells. J Immunol **176**: 118–127.

Sugita S, Keino H, Futagami Y, Takase H, Mochizuki M, Stein-Streilein J & Streilein JW (2006c): B7$^+$ iris pigment epithelial cells convert T cells into CTLA-4$^+$, B7-expressing CD8$^+$ regulatory T cells. Invest Ophthalmol Vis Sci **47**: 5376–5384.

Sugita S, Takase H, Taguchi C et al. (2006d): Ocular infiltrating CD4$^+$ cells from patients with Vogt–Koyanagi–Harada disease recognize human melanocyte antigens. Invest Ophthalmol Vis Sci **47**: 2547–2554.

Sugita S, Futagami Y, Horie S & Mochizuki M (2007): Transforming growth factor β-producing Foxp3$^+$ CD8$^+$ CD25$^+$ T cells induced by iris pigment epithelial cells display regulatory phenotype and acquire regulatory functions. Exp Eye Res **85**: 626–636.

Sugita S, Horie S, Nakamura O et al. (2008): Retinal pigment epithelium-derived cytotoxic T lymphocyte antigen-2α induces TGFβ-producing T regulatory cells. J Immunol **181**: 7525–7536.

Takahashi T, Takase H, Urano T, Sugita S, Miyata K, Miyata N & Mochizuki M (2000): Clinical features of human T-lymphotropic virus type 1 uveitis: a long-term follow-up. Ocul Immunol Inflamm **8**: 235–241.

Vogt A (1906): Fruhzeitiges ergrauen der Zilien und Bemerkungen uber den sogenannten plotzlichen Eintritt dieser Veranderung. K Monatsbl Augenhk **44**: 228–242.

Wacker WB & Lipton MM (1965): Experimental allergic uveitis homologous retina as uveitogenic antigen. Nature **206**: 253–254.

Wacker WB, Donoso LA, Kalsow CM, Yankeelov JA Jr & Organisciak DT (1977): Experimental allergic uveitis. Isolation, characterization, and localization of a soluble uveitopathogenic antigen from bovine retina. J Immunol **119**: 1949–1958.

Yamaguchi K, Seiki M, Yoshida M, Nishimura H, Kawano F & Takatsuki K (1984): The detection of human T-cell leukemia virus provirus DNA and its application for classification and diagnosis of T-cell malignancy. Blood **63**: 1235–1240.

Yamaki K, Kondo I, Nakamura H, Miyano M, Konno S & Sakuragi S (2000a): Ocular and extraocular inflammation induced by immunization of tyrosinase related protein 1 and 2 in Lewis rats. Exp Eye Res **71**: 361–369.

Yamaki K, Gocho K, Hayakawa K, Kondo I & Sakuragi S (2000b): Tyrosinase family proteins are antigens specific to Vogt–Koyanagi–Harada disease. J Immunol **165**: 7323–7329.

Yoshida M, Miyashi I & Hinuma Y (1982): Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. Proc Natl Acad Sci USA **79**: 2031–2035.

Yoshida M, Seiki M, Yamaguchi K & Takatsuki K (1984): Monoclonal integration of human T-cell leukemia provirus in all primary tumors of adult T-cell leukemia suggests causative role of human T-cell leukemia virus in the disease. Proc Natl Acad Sci USA **81**: 2534–2537.

Yoshida M, Takeuchi M & Streilein JW (2000): Participation of pigment epithelium of iris and ciliary body in ocular immune privilege. 1. inhibition of T-cell activation *in vitro* by direct cell-to-cell contact. Invest Ophthalmol Vis Sci **41**: 811–821.

Yoshimura K, Mochizuki M, Araki S, Miyata N, Yamaguchi K, Tajima K & Watanabe T (1993): Clinical and immunological features of human T-cell lymphotropic virus type I uveitis. Am J Ophthalmol **116**: 156–163.

Received on December 6th, 2008.
Accepted on August 30th, 2009.

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Cancer Science
The official journal of the Japanese Cancer Association JCA

Intra- and inter-laboratory variability in human T-cell leukemia virus type-1 proviral load quantification using real-time polymerase chain reaction assays: A multi-center study

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(Received May 12, 2010/Revised July 20, 2010/Accepted July 22, 2010/Accepted manuscript online August 18, 2010/Article first published online September 28, 2010)

Human T-cell leukemia virus type-1 (HTLV-1) proviral load (VL) is an important determinant of viral pathogenesis and malignant evolution. Although VL has been quantified by in-house real-time quantifiable polymerase chain reaction (qPCR) technology, little is known about the harmonization among different VL assay systems. We evaluated intra- and inter-laboratory variability of VL measured at six laboratories using the same DNA samples seropositive for HTLV-1 in a two-step manner. The first study measured 60 samples by original in-house assays, finding that the median intra- and inter-laboratory coefficient of variation (CV) was 44.9% (range, 25.4–71.8%) and 59.9% (34.2–93.4%), respectively. The inter-laboratory correlation coefficients ranged from 0.760 to 0.875, indicating that VL were measured with good precision in each laboratory, but inter-laboratory regression slopes differed from 0.399 to 2.206, indicating that VL were measured with a wide variation between laboratories. To examine the effect of standardization of reference materials (RM) on the VL variability, we performed a second study using only 20 samples by substituting RM for plasmid including the HTLV-1 pX region. The median inter-laboratory CV for raw pX copy number was reduced significantly from 66.9% to 35.7%, whereas the median CV for the internal control remained almost unchanged, resulting in no improvement in inter-laboratory CV for VL. This indicates that each in-house assay system worked well with good precision, but standardizing RM alone was insufficient for harmonization. The relevant choice of not only RM, but also internal control genes for data normalization is expected to be realistic to standardize HTLV-1 VL measurement. (*Cancer Sci* 2010; 101: 2361–2367)

Quantification of the human T-cell leukemia virus type-1 (HTLV-1) provirus copy number in the genome has contributed to understanding the pathophysiology of infected cells.[1–3] Cells infected with HTLV-1 are generally thought to carry one provirus genome in their chromosomal DNA, indicating that one copy is equivalent to one cell.[4] Accordingly, because HTLV-1 proviral load (VL) directly reflects the number of HTLV-1-infected cells, it is useful and relevant to monitor dynamic changes of infected cells in individual carriers.[5] To clarify a causative relation between the pre-leukemic state of adult T-cell leukemia (ATL) and the kinetics of infected cell burden, in 2002 we established a nationwide cohort study for asymptomatic HTLV-1 carriers in Japan, designated as the Joint Study on Predisposing Factors of ATL Development (JSPFAD, http://www.htlv1.org). The main purpose of the cohort study was to measure the HTLV-1 VL of asymptomatic HTLV-1 carriers using a quantifiable polymerase chain reaction (qPCR) over a long period of time.

A variety of methods have been reported for HTLV-1 VL measurement using real-time qPCR; however, no gold standard method has been identified and validated to date because there is no standard reference material (RM) that is essential for absolute real-time qPCR for proviral DNA.[3,6–10] The variability in VL may be influenced by both biological characteristics and technical variables. From a technical point of view, some studies used a plasmid provirus as the RM for a standard calibration curve to measure the raw pX copy number before normalization, but others used HTLV-1-infected cell lines, such as a TARL-2 cell line carrying one provirus derived from infected cells in a rat.[3,4] Moreover, a variety of internal controls (IC), including β-actin, β-globin, RNase-p and CD81, have been used for the qPCR assay system. These different measurement techniques may introduce bias into the research on the association between VL and disease outcome. To address this issue, it is necessary to evaluate intra- and inter-laboratory variability of real-time qPCR assays to quantify HTLV-1 VL.

The present study demonstrated intra- and inter-laboratory variability of HTLV-1 VL in six research laboratories, and we have developed an approach to standardization.

Materials and Methods

Samples. We used a total of 80 DNA extraction samples from peripheral blood mononuclear cells (PBMC) of HTLV-1 seropositive subjects, which were stored in the core laboratory of JSPFAD at the University of Tokyo. The study protocol of the JSPFAD was approved by the respective ethical committees (http://www.htlv1.org/organization.html). Genomic DNA was isolated from peripheral blood mononuclear cells using a QIAGEN Blood kit (Qiagen, Hilden, Germany).

Study laboratories. Six research laboratories (one core laboratory, T, and five laboratories, A, B, C, D and E), all of which were members of JSPFAD, participated in this study. In each laboratory, HTLV-1 VL was routinely measured by its own in-house (home-brew) real-time qPCR system. Equipment,

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doi: 10.1111/j.1349-7006.2010.01720.x

target sites within the pX region for amplification, probe sites, standard RM and IC genes differed at each laboratory (Table 1). The original protocol in each laboratory is referred to in the references.[3,6–10] All assay systems were basically constructed based on the same concept considering the characteristics of the HTLV-1 genome. As summarized in Table 1, two types of RM were used for the standard calibration curve to measure the raw pX copy number before normalization; a plasmid provirus in four laboratories and a TARL-2 cell line carrying one provirus derived from rat-infected cells in two laboratories. Internal controls were β-actin in two laboratories, CD81 in one laboratory, β-globin in one laboratory and RNase P in two laboratories. In order to make a comparison between laboratories, we used a common procedure to express HTLV-1 VL values as pX copy numbers per 100 PBMC, based on the assumption that infected cells harbored one pX copy of the integrated HTLV-1 provirus per cell and two IC gene copies per cell.[5]

Study design. After receiving the sample DNA, each laboratory diluted them for its own in-house (home brew) real-time qPCR system. We performed two studies to evaluate variations in HTLV-1 VL copy number per 100 PBMC, pX copy number per PCR reaction containing 50 ng genomic DNA adjusted from respective assay systems, and the IC gene copy number per PCR reaction with 50 ng genomic DNA.

In the first study, 60 samples were measured by routine in-house qPCR systems in each of the six laboratories. In the second study, 20 samples were measured by the in-house assay systems only by substituting individual RM for the plasmid HTLV-1 provirus (9.0 kb provirus inserted in pUC19) that was used in the core laboratory T. The second study was performed at the same laboratories as the first study except for laboratory E. Each laboratory performed duplicate assays for each sample in both studies. All measurements were sent to laboratory A, and then the intra- and inter-laboratory variations were evaluated for each study.

Statistical analysis. Intra-laboratory variability was evaluated by calculating the mean, standard deviation (SD), median, ranges and coefficient of variation (CV = 100 × SD/mean, %) for VL measured in each laboratory. In the first study, intra-laboratory variability was evaluated according to a method described by Addona *et al.*[11]. Inter-laboratory variability, precision and accuracy of measurements were evaluated by calculating inter-laboratory CV (%) for each sample, and by fitting linear regressions. Linear regression analyses were performed between VL measured at the core laboratory T as standard and those measured at the five laboratories, from which the linear slope, Spearman's rank correlation coefficient and the coefficient of determination (R2) were evaluated. In this study, a Spearman's rank correlation coefficient >0.8 was defined as indicative of good precision, and a regression slope of 1.0 was indicative of 100% identical for VL measured in the core laboratory T and other laboratories.

In the second study, we examined whether inter-laboratory CV for VL, raw pX copies and IC gene copies could be improved by only standardizing the RM. Accuracy was evaluated by calculating practical measurements relative to the IC gene copy number (genomic dose/2). Precision was evaluated by calculating the mean of the intra-sample CV.

The Mann–Whitney test was used to compare medians between groups. A statistically significant level was set at 0.05.

Results

In the first study, six laboratory assay systems were detectable for all 60 samples with a wide range of VL. The median VL values (per 100 PBMC) for all samples in each laboratory assay were 5.4, 5.7, 12.6, 5.3, 12.3 and 3.4 copies in laboratories T, A, B, C, D and E, respectively. There was a large variation in the median VL among laboratories. The maximal difference in the median VL between laboratories was 3.7-fold (B *vs* E).

Intra-laboratory variability of VL in the first study. Figure 1A shows the characteristics of intra-laboratory variation by dividing the VL levels into three groups (low, intermediate and high) in ascending order based on the VL measured at the core laboratory T. That is, the VL of the core laboratory were prone to be linked with those of other laboratories. However, the box plots show that the distribution of measurements differed across the laboratories in each VL level. In the low and intermediate VL levels, the median VL of laboratories T, A, C and E were almost quantitatively accordant, but the values were significantly higher in laboratories B and D than in the core laboratory T (P 0.05). In the high VL levels, although a wider distribution of measured values was observed at all laboratories, there was almost quantitative accordance in the median VL between laboratories T, A and C. However, the median VL in laboratory B and D were significantly higher than in the core laboratory T, whereas that in laboratory E was lower than that in T (P 0.05).

The intra-laboratory CV (%) calculated using 20 samples at each VL level are shown in Figure 1B. The overall median intra-laboratory CV (%) was 44.9% (range, 25.4–71.8%). In the low VL level, there was no significant difference between laboratory T and the others except for laboratory B. The median intra-laboratory CV across laboratories was 44.1% (range, 32.3–58.9%). At the intermediate VL level, there was no statistically significant difference between core laboratory T and laboratories B, C and E, but the CV% in laboratory D was significantly greater than laboratory T (P 0.05). The median intra-laboratory CV across the laboratories was 34.0% (range, 25.4–44.5%). At the high VL level, the median intra-laboratory CV across the laboratories was 55.0% (range, 51.6–71.8%), despite a wide distribution of actually measured VL values (Fig. 1A), with no statistically significant difference between the core laboratory T and the rest of the laboratories, except for laboratory E (P 0.05).

Inter-laboratory variability of VL in the first study. Figure 1C shows the inter-laboratory CV (%) of measured VL for individual samples in ascending order. There was a wide variability in inter-laboratory CV by sample. The overall median inter-labo-

Table 1. In-house real-time qPCR systems for HTLV-1 VL in six research laboratories

Laboratory	Platform	RM for pX	Primer site	Probe	Template dose (ng)	IC gene	RM for IC
T	ABI Prism7000*	9.0 kb provirus inserted in pUC19	pX(tax)	Hydrolysis	50	RNase-P	Purified human genome
A	LC480**	9.0 kb provirus inserted pBR322	pX(tax)	Hydrolysis	30	β-globin	TaqMan control DNA
B	ABI Prism7700	TARL-2 rat cell line infected	pX(tax)	Hydrolysis	50	β-actin	Normal human DNA
C	LightCycler***	PCR product inserted in pGEM-T	pX(tax)	Hydrolysis	100	RNase-P	Normal human DNA
D	ABI Prism7500	TARL-2 rat cell line infected	pX(tax)	Hydrolysis	100	β-actin	Normal human DNA
E	ABI Prism7900	Cloning pX-fragment inserted in plasmid	pX(tax)	Hydrolysis	1000	CD81	CD81 PCR product inserted plasmid

*Applied Biosystems, Foster City, CA, USA; **Roche Diagnostics, Mannheim, Germany; ***Roche Diagnostics.

doi: 10.1111/j.1349-7006.2010.01720.x

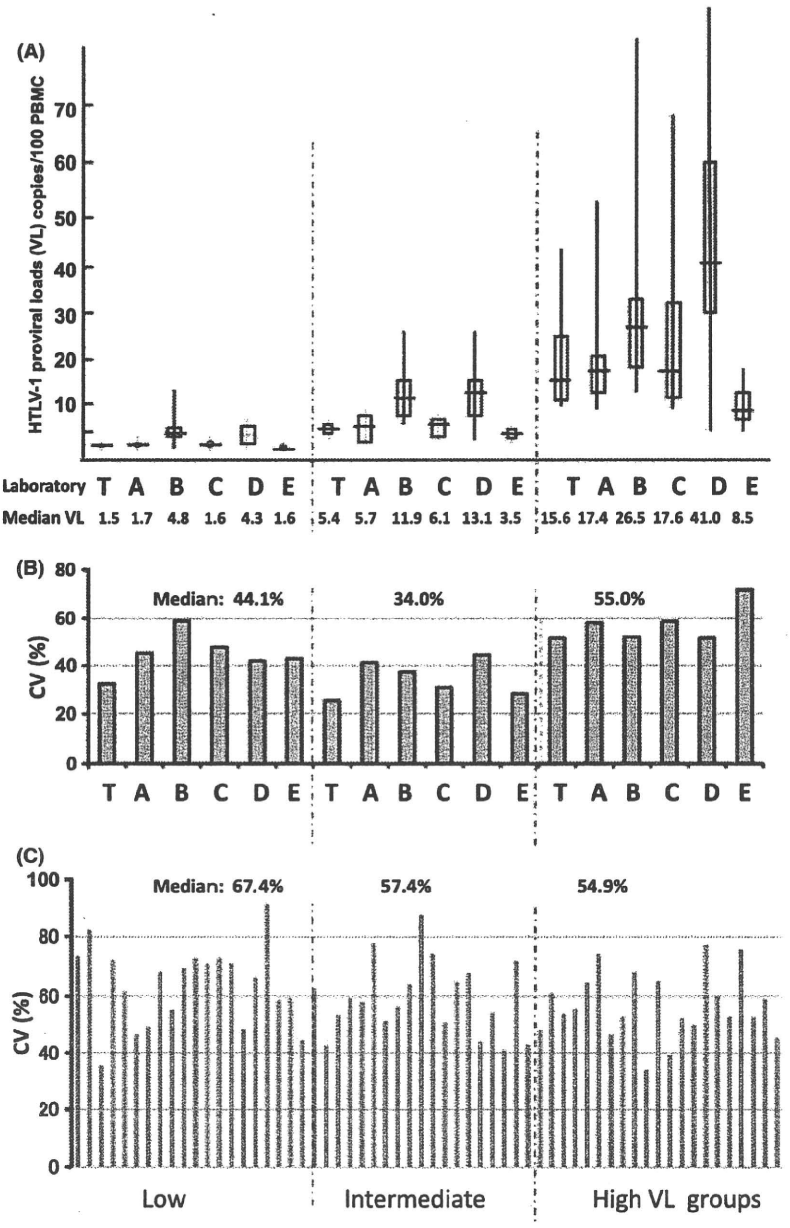

Fig. 1. Variation in human T-cell leukemia virus type-1 (HTLV-1) proviral loads (VL) quantification at the three VL levels in the first study. The three VL levels were divided based on the VL measured at a core laboratory T. Ranges of VL values at each level were as follows: low level (0–2.1, $n = 20$); intermediate level (3.0–7.7, $n = 20$); and high level (10.2–43.2, $n = 20$). (A) Intra-laboratory variability of measured VL at each VL level. The box plots show the median (horizontal line), interquartile range (box) and range (whiskers) in each laboratory (T, A, B, C, D and E). The *y*-axis shows measured VL copy numbers per 100 peripheral blood mononuclear cells (PBMC). (B) Intra-laboratory coefficient of variations (CV) (%) at three VL levels. The CV values were calculated based on the VL measured in each laboratory. (C) Inter-laboratory CV (%) for individual samples. Each CV value was calculated based on the measured VL in six laboratories.

ratory CV for 60 samples was 59.9% (range, 34.2–93.4%), consisting of 67.4% (range, 35.7–82.3%) in the low level group, 57.4% (range, 41.2–87.4%) in the intermediate group and 54.9% (range, 34.2–77.6%) in the high level group, respectively. Although inter-laboratory CV (%) was very wide by sample, there was a good correlation in the scatter plots of each VL between values measured in the core laboratory T and those measured in the other five laboratories. The fitted linear regression curves are shown in Figure 2. The inter-laboratory correlation coefficients ranged from 0.760 to 0.875, indicating that VL were measured with good precision in each laboratory. However, inter-laboratory regression slopes differed among laboratories. The slopes of laboratories A and C were close to 1.0 (0.992 and 0.984, respectively), indicating that the measured VL in the two laboratory systems were similar to values that were measured in the core laboratory T. However, the slopes of laboratory B and D were greater than 1.0 (1.393 and 2.206, respectively), indicating that the VL measured were always higher than in the core laboratory T. In contrast, the slope of laboratory E was 1.0 (0.399), indicating that the VL were always lower than in the core laboratory T. However, it is well recognized that the difference in slope influences all samples equally when a good correlation is observed as a systematic deviation.[12] Therefore, in this paper we derived an original "adjustment coefficient" in order to standardize data by calculating an inverse value of the slope (1/slope) for each laboratory. The "adjustment coefficient" for each laboratory ranged from 0.453 to 2.51 (Table 2).

Inter-laboratory variability after sharing RM in the second study. We considered that the large variation among assay systems in the first study might be related to some factor affecting universal measurements of samples, such as the RM and normalization, which are essential for qPCR. Therefore, we conducted the second study.

In the second study, the median VL of 20 samples in five laboratories (T, A, B, C and D) were 4.9, 6.6, 2.7, 4.5 and 3.4

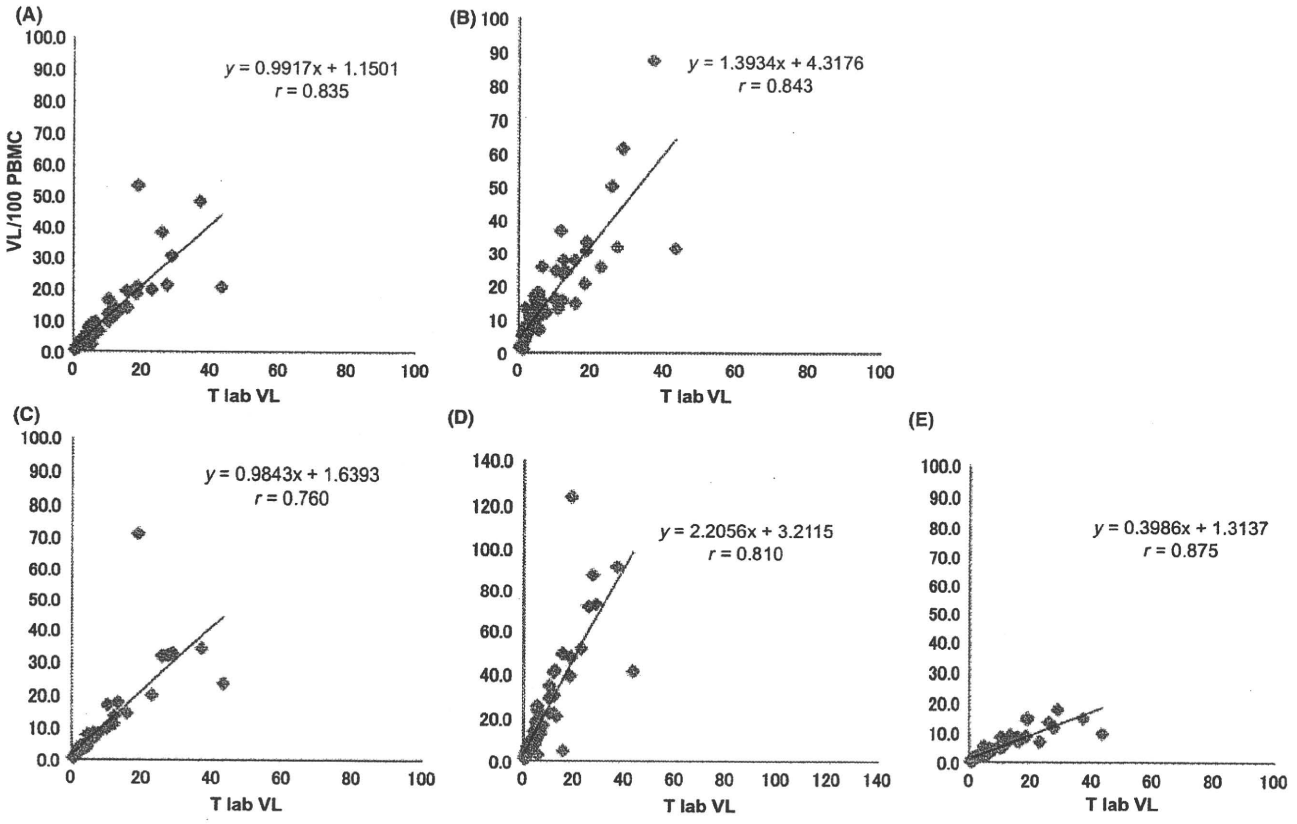

Fig. 2. Inter-laboratory comparison of human T-cell leukemia virus type-1 (HTLV-1) proviral loads (VL) in linear regression analysis. Scatter plot of VL of 60 samples between the core laboratory T and the other five laboratories (A), (B), (C), (D) and (E). PBMC, peripheral blood mononuclear cells.

copies per 100 PBMC, respectively. The maximal difference in the median VL was 2.4-fold (A *vs* B). The median inter-laboratory CV for VL, raw pX copy number and IC gene copy number before normalization (the first study) and after normalization (the second study) are shown in Figure 3A–C, respectively. The median inter-laboratory CV for VL was slightly reduced (from 59.9% to 48.2%) with no statistical significance. The median CV for raw pX copy number was reduced significantly from 66.9% to 35.3% (P 0.05), whereas those for the VL and IC gene copy number remained statistically unchanged. We also performed linear regression analyses in a similar way to the first study. Data from the first and second studies are summarized in Table 3. This discrepancy of inter-laboratory CV between the raw copy number and VL appeared to account for poor IC accuracy and precision. Therefore, we next examined the effect of the IC gene copy assays for inter-laboratory CV of VL.

Table 2. Summary of inter-laboratory variability of VL in the first study and an original adjustment coefficient for standardization

Index of inter-laboratory variability	Laboratory				
	A	B	C	D	E
Correlation coefficient†	0.835	0.843	0.760	0.810	0.875
Slope†	0.992	1.393	0.984	2.206	0.399
Adjustment coefficient (=1/slope)	1.008	0.718	1.016	0.453	2.506

†These indexes were derived from linear regression analyses for 60 measured VL between each of the five laboratories (A, B, C, D, E) and the core laboratory T set as a reference (=1.0).

Quality of IC gene copy assays. To confirm whether a large variation in IC gene copy measurements is involved in the lack of improvement of standardization of VL in each assay system, we evaluated the measurement quality of the IC gene copy assay in each laboratory. The "IC accuracy" was defined as the measured copy number relative to the expected copy number with an input genomic DNA dose of 50 ng. This amount corresponds to approximately 16 600 copies based on one copy per 3 ng of genomic DNA. The "IC precision" was evaluated by using the median CV (%) of 60 measurements by qPCR with the respective IC gene. The results are summarized in Table 4, showing that both "IC accuracy" and "IC precision" were superior in RNase P, β-globin and CD81 compared with β-actin.

Discussion

Many studies have reported that a VL is linked to the pathogenesis of a virus. The HTLV-1 VL is thought to be equivalent to the HTLV-1-infected cell number.[13,14] However, in contrast to RT-qPCR for a large amount of transcripts, it is difficult to accurately discriminate a small concentration of the HTLV-1 provirus at a level of 10^{-2} to $^{-3}$.[12] The reliability of in-house assay systems has been previously evaluated as the quality of reproducibility, but little information is known about the intra- and inter-laboratory variations in HTLV-1 VL measured by different in-house qPCR systems that are set up independently at each laboratory. It is desirable that all assay systems can

doi: 10.1111/j.1349-7006.2010.01720.x

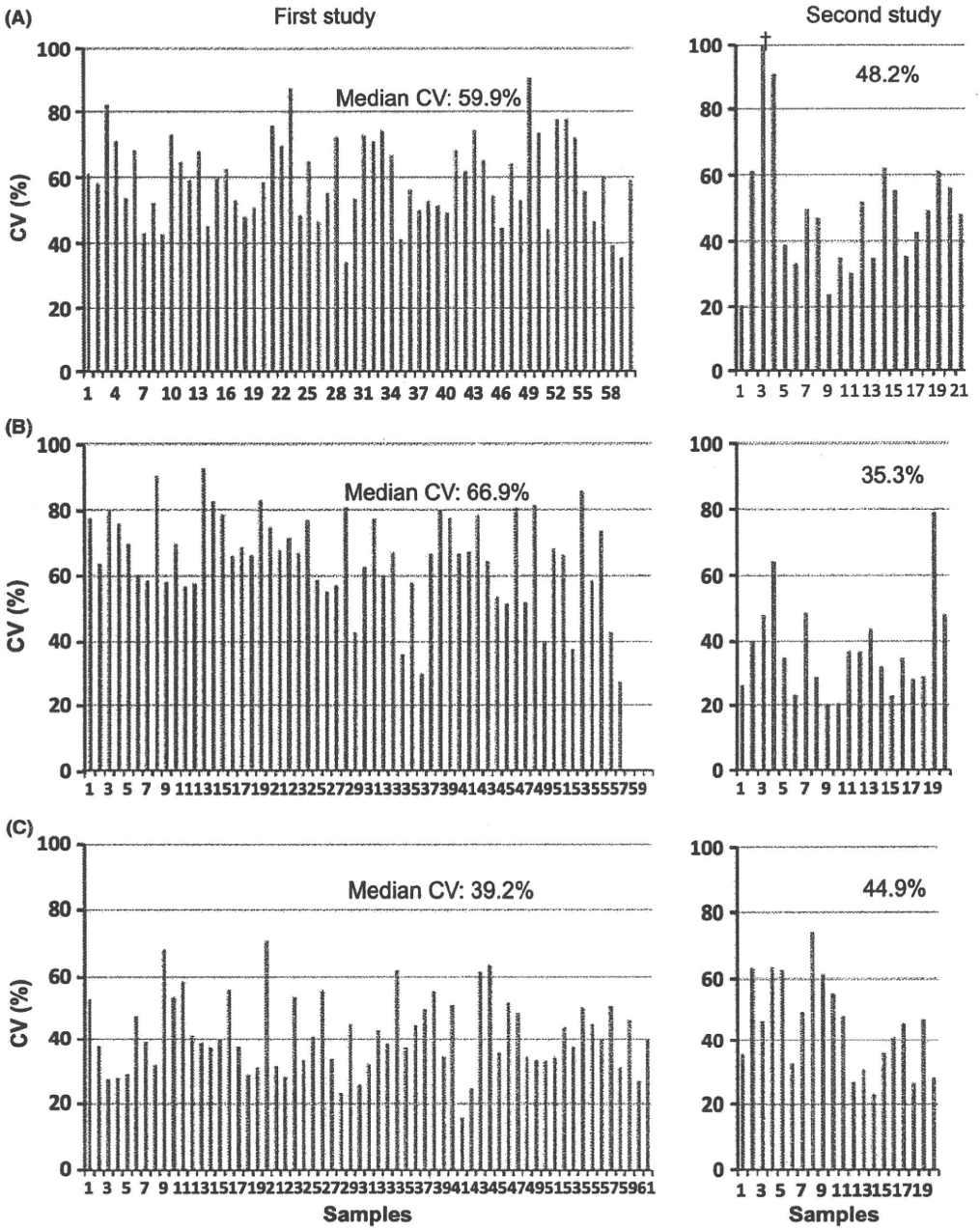

Fig. 3. Comparison of inter-laboratory coefficient of variations (CV) in individual samples for (A) proviral loads (VL), (B) raw pX copy number before normalization and (C) IC gene copy in the first and second studies. The median CV for only the raw pX copy number significantly decreased compared with that of the first study (66.9 versus 35.3%, P 0.05). †The data was scaled over (144.7%).

measure VL accurately anytime and anywhere,[15,16] but to our knowledge there is no information on this point.

In this paper, we discovered three interesting findings in the first study. First, an extremely wider variation than we expected was seen in the measured values for HTLV-1 VL, especially in the group of high VL samples, from much lower copies to 100 copies or more per 100 PBMC. This is probably explained by the biological characteristics of the HTLV-1 virus, such as defects or mutations in target regions of primers and probes, or multiple integration of the proviral genome.[17] Second, we found a large difference in the actually measured VL between laboratories with a maximal difference of 3.7-fold in the median value. Third, the inter-laboratory correlation coefficients were excellent between the core laboratory T and each of the other laboratories. The second and third findings together indicate that each in-house assay system works well individually, but there was a systematic deviation from the expected values due to the difference in assay systems (Table 1). Such systematic deviation can be easily adjusted with an additional factor like our original "adjustment coefficient (an inverse value of the slope)" (Table 2) to compensate the data. However, such compensation alone is not enough to standardize the HTLV-1 VL measurement and to explain the wide intra- and inter-laboratory variability. Therefore, we performed the second study.

Table 3. Summary of inter-laboratory variability of VL in the first and second studies

	First study	Second study	*P*-value
CV%, median (range)			
VL	59.9 (34.2–93.3)	48.2 (20.0–144.3)	
Raw pX copy/50 ng DNA dose	66.9 (30.1–91.6)	35.7 (20.5–79.0)	0.05
IC gene copy/50 ng DNA dose	39.2 (16.2–71.2)	44.9 (23.0–74.0)	
Index of linear regression analysis†			
VL			
Correlation coefficient	0.824	0.815	
Slope	1.195	1.05	
pX raw copy/50 ng DNA dose			
Correlation coefficient	0.761	0.811	
Slope	1.513	1.055	
PCR efficiency			
pX	1.976	1.981	
IC	1.967	1.98	

†Indexes were derived from linear regression analyses for measured VL between each of the five laboratories and the core laboratory T set as a reference (=1.0).

Table 4. Accuracy and precision for internal control (IC) gene copy assays in each laboratory

	IC gene used in laboratory†					
	T (core)	C	B	D	A	E
	RNP	RNP	β-actin	β-actin	β-globin	CD81
IC accuracy‡						
Measured/Expected	1.345	0.869	2.522	1.816	1.051	1.041
IC precision§						
CV (%)	11.8	13.2	35.1	32.6	11.4	13.8

†Rnase-P(RNP), β-globin and CD81 were expected to be at a tolerable level, but not β-actin. ‡"IC accuracy" was defined as the measured copy number relative to the expected copy number with an input genomic DNA dose of 50 ng. §"IC precision" was evaluated by using median coefficient of variation (CV) (%) of 60 measurements by quantifiable polymerase chain reaction with the respective IC gene.

In the second study, we standardized the RM for pX using plasmid provirus DNA for each in-house assay system, based on our inference that the main cause of the universal difference in the VL by laboratory might be due to the difference in RM. The inference was confirmed as presented in Table 3, where the inter-laboratory CV for raw pX copy number was reduced significantly after the RM standardization. Moreover, we found that IC gene measurements were also important for standardization of VL measurements as presented in Table 4, showing that both "IC accuracy" and "IC precision" were superior in RNase P, β-globin and CD81 compared with β-actin. Nevertheless, the most relevant IC gene for HTLV-1 provirus quantification remains unclear.

In general, quantitative variations by qPCR are affected by many factors, such as biological variations, process variations, systemic variations and other biased variations. Now a key question arises: which grade of intra- or inter-laboratory CV is acceptable for measurement of HTLV-1 VL? Of the in-house qPCR for HTLV-1 VL assays, most studies have described only a test performance by evaluating intra- and inter-assay variability using the same sample. For example, a report by Nagai[18] states that acceptable intra- and inter-assay CV values are around 25%. On the other hand, there are few studies on intra- and inter-laboratory variability indispensable for HTLV-1VL standardization. We searched the literature for qPCR assays in herpes viruses that were similar to our study. The studies reported that the median CV values of intra- and inter-laboratory variations about 20 and 40% for Cytomegalovirus (CMV)[19], and about 40 and 135 for Epstein-Barr virus (EBV)[20], respectively. Interestingly, intra-laboratory CV were 40% and significantly smaller than inter-laboratory CV. Although their study designs were not always the same as ours, the grade of median intra-laboratory CV values for HTLV-1, 44.9% (range, 25.4–71.8%), in our study was a little larger than that for CMV and EBV, whereas the grade of median inter-laboratory CV value for HTLV-1 in our study, 59.9% (range, 34.2–93.4%), was smaller than that for the two viruses. However, the grades could not be simply compared between HTLV-1 and other viruses because the viruses were biologically different to each other and the VL assay systems were also different.

Similarly, the grade of intra-laboratory CV for genomic genes have been reported to be 25–30% based on the quality of reproducibility within a single in-house assay.[5,9] Compared with the grade of the previous data, our intra-laboratory CV of 44.9% (range, 25.4–71.8%) seemed to be greater. This may come from the difference in the study design and analyzing procedures; previous studies focused on evaluating the quality of reproducibility of one sample in a single assay, whereas the present study focused on evaluating how much variability is present in the measurement of the same 20 samples in three VL levels in each laboratory, as described by Addona *et al.*[11] Therefore, the most relevant grade of intra-laboratory CV for HTLV-1 provirus quantification remains unclear.

Regarding a grade of inter-laboratory variation for measurement of HTLV-1 VL, we were unable to compare this with other studies because the present study is the first to investigate the variation. We have shown that the grade of median inter-laboratory CV% was 59.9% (range, 34.2–93.4%), and the value decreased as the measured value increased from low to high (Fig. 1C), which presumably reflects the effects of stochastic phenomena operative at a low input template copy number. So far, most healthy carriers have a low VL of less than three copies per 100 PBMC,[21] and it is considered that a VL of 5–10 copies per 100 PBMC is the critical level to be at risk of adult T-cell leukemia.[22] Therefore, we need a measure of high accuracy and precision for real-time qPCR of proviral DNA equivalent to the actual number of infected cells. Taken together, it is desirable that both grades of intra- and inter-laboratory CV for HTLV-1 VL measurement is 20%. The present study indicates that the 20% of inter-laboratory CV for measurement of VL is feasible, because the inter-laboratory CV for the pX copy number reduced by one-second (66.9% to 35.7%). Unfortunately, although it was not achievable in this series, as summarized in Table 4, poor IC accuracy and precision were responsible for insufficient improvement of inter-laboratory CV for VL. Accordingly, if high accuracy and precision of IC, such as RNP, beta-globin and CD81 is selected, 20% of inter-laboratory CV for VL seems to be realistically feasible.

In conclusion, in this study, we have shown that there is a variety in real-time qPCR assay systems, and a wide variability in intra- and inter-laboratory values for quantifying HTLV-1 VL. To improve the accuracy and precision of the quantification, standardization of HTLV-1 VL using appropriate RM (plasmid DNA) and relevant IC genes is necessary.

Acknowledgment

This work was supported by the Japanese government.

doi: 10.1111/j.1349-7006.2010.01720.x

Disclosure Statement

The authors have no conflict of interest.

Abbreviations

CV	coefficient of variation
HTLV-I	human T-cell leukemia virus type-I
IC	internal control
PCR	polymerase chain reaction
qPCR	quantifiable polymerase chain reaction
RM	reference material
VL	proviral load

References

1 Asquith B, Bangham CR. Quantifying HTLV-I dynamics. *Immunol Cell Biol* 2007; **85**: 280–6.
2 Yamano Y, Nagai M, Brennan M *et al.* Correlation of human T-cell lymphotropic virus type 1 (HTLV-1) mRNA with proviral DNA load, virus-specific CD8 (+) T cells, and disease severity in HTLV-1-associated myelopathy (HAM/TSP). *Blood* 2002; **99**: 88–94.
3 Kamihira S, Dateki N, Sugahara K *et al.* Real-time polymerase chain reaction for quantification of HTLV-1 proviral load: application for analyzing aberrant integration of the proviral DNA in adult T-cell leukemia. *Int J Hematol* 2000; **72**: 79–84.
4 Yoshida M. Discovery of HTLV-1, the first human retrovirus, its unique regulatory mechanisms, and insights into pathogenesis. *Oncogene* 2005; **24**: 5931–7.
5 Miley WJ, Suryanarayana K, Manns A *et al.* Real-time polymerase chain reaction assay for cell-associated HTLV type I DNA viral load. *AIDS Res Hum Retroviruses* 2000; **16**: 665–75.
6 Watanabe M, Ohsugi T, Shoda M *et al.* Dual targeting of transformed and untransformed HTLV-1-infected T cells by DHMEQ, a potent and selective inhibitor of NF-kappaB, as a strategy for chemoprevention and therapy of adult T-cell leukemia. *Blood* 2005; **106**: 2462–71.
7 Takenouchi N, Yamano Y, Usuku K *et al.* Usefulness of proviral load measurement for monitoring of disease activity in individual patients with human T-lymphotropic virus type I-associated myelopathy/tropical spastic paraparesis. *J Neurovirol* 2003; **9**: 29–35.
8 Kamihira S, Dateki N, Sugahara K *et al.* Significance of HTLV-1 proviral load quantification by real-time PCR as a surrogate marker for HTLV-1-infected cell count. *Clin Lab Haematol* 2003; **25**: 111–7.
9 Nagai M, Yamano Y, Brennan MB, Mora CA, Jacobson S. Increased HTLV-I proviral load and preferential expansion of HTLV-I Tax-specific CD8+ T cells in cerebrospinal fluid from patients with HAM/TSP. *Ann Neurol* 2001; **50**: 807–12.
10 Tanaka G, Okayama A, Watanabe T *et al.* The clonal expansion of human T lymphotropic virus type 1-infected T cells: a comparison between sero-converters and long-term carriers. *J Infect Dis* 2005; **191**: 1140–7.
11 Addona TA, Abbeatiello SE, Schilling B *et al.* Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nat Biotechnol* 2009; **27**: 633–41.
12 Bustin SA, Vandesompele J, Pfaffl MW. Standardization of qPCR and RT-qPCR. *Genet Eng Biotechnol News* Aug 1, 2009; (Vol. 29, No. 14): http://www.genengnews.com/article.
13 Umeki K, Hisada M, Maloney EM, Hanchard B, Okayama A. Proviral loads and clonal expansion of HTLV-1-infected cells following vertical transmission: a 10-year follow-up of children in Jamaica. *Intervirology* 2009; **52**: 115–22.
14 Watanabe T. HTLV-1-associated diseases. *Int J Hematol* 1997; **66**: 257–78.
15 Bustin SA. Why the need for qPCR publication guidelines? – The case for MIQE. *Methods* 2010; **50**: 217–26.
16 Bustin SA, Benes V, Garson JA *et al.* The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009; **55**: 611–22.
17 Matsuoka M. Human T-cell leukemia virus type I (HTLV-I) infection and the onset of adult T-cell leukemia (ATL). *Retrovirology* 2005; **2**: 27.
18 Nagai M, Matsumoto W, Kodama D *et al.* Analysis of HTLV-1 proviral load in 202 HAM/TSP patients and asymptomatic HTLV-1 carriers: high proviral load strongly predisposes to HAM/TSP. *J Neurovirol* 1998; **4**: 586–93.
19 Pang XL, Fox JD, Fenton JM *et al.* Interlaboratory comparison of cytomegalovirus viral load assays. *Am J Transplant* 2009; **9**: 258–68.
20 Preiksaitis JK, Pang XL, Fox JD *et al.* Interlaboratory comparison of Epstein–Barr virus viral load assays. *Am J Transplant* 2009; **9**: 269–79.
21 Bangham CR. The immune control and cell-to-cell spread of human T-lymphotropic virus type 1. *J Gen Virol* 2003; **84**: 3177–89.
22 Iwanaga M, Watanabe T, Utsunomiya A *et al.* Human T-cell leukemia virus type I (HTLV-1) proviral load and disease progression in asymptomatic HTLV-1 carriers: a nationwide prospective study in Japan. *Blood* 2010; **116**(8): 1211–9.

Human T-cell leukemia virus type I (HTLV-1) proviral load and disease progression in asymptomatic HTLV-1 carriers: a nationwide prospective study in Japan

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Definitive risk factors for the development of adult T-cell leukemia (ATL) among asymptomatic human T-cell leukemia virus type I (HTLV-1) carriers remain unclear. Recently, HTLV-1 proviral loads have been evaluated as important predictors of ATL, but a few small prospective studies have been conducted. We prospectively evaluated 1218 asymptomatic HTLV-1 carriers (426 males and 792 females) who were enrolled during 2002 to 2008. The proviral load at enrollment was significantly higher in males than females (median, 2.10 vs 1.39 copies/100 peripheral blood mononuclear cells [PBMCs]; $P < .001$), in those 40 to 49 and 50 to 59 years of age than that of those 40 years of age and younger ($P = .02$ and .007, respectively), and in those with a family history of ATL than those without the history (median, 2.32 vs 1.33 copies/100 PBMCs; $P = .005$). During follow-up, 14 participants progressed to overt ATL. Their baseline proviral load was high (range, 4.17-28.58 copies/100 PBMCs). None developed ATL among those with a baseline proviral load lower than approximately 4 copies. Multivariate Cox analyses indicated that not only a higher proviral load, advanced age, family history of ATL, and first opportunity for HTLV-1 testing during treatment for other diseases were independent risk factors for progression of ATL. (*Blood*. 2010;116(8): 1211-1219)

Introduction

Human T-cell leukemia virus type I (HTLV-1), the first human retrovirus to be identified, is etiologically associated with adult T-cell leukemia (ATL), HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP), and HTLV-1 uveitis/HTLV-1–associated uveitis (HU/HAU).[1-3] Worldwide, endemic areas for the virus are unevenly distributed, which include southwest Japan, the Caribbean islands, South America, and a part of Central Africa.[4] In Japan, the number of HTLV-1 carriers was estimated to be approximately 1.2 million people during the late 1980s.[5] The majority of HTLV-1 carriers remain asymptomatic throughout their lives. The lifetime risks of developing ATL and HAM/TSP are estimated to be approximately 2.5% to 5%[6,7] and 0.3% to 2%,[8,9] respectively.

Several molecular biologic studies have reported that various cellular dysfunctions induced by viral genes (eg, *tax* and *HBZ*), genetic and epigenetic alterations, and the host immune system may be involved in the leukemogenesis of ATL.[10-12] Clinical and epidemiologic studies have also reported a variety of possible risk factors for ATL, including vertical transmission of HTLV-1 infection, male gender, a long latent period, increased leukocyte counts or abnormal lymphocyte counts, and higher levels of anti–HTLV-1 antibody titers and soluble interleukin-2 receptor-α.[13-19] However, there are no clear determinants that separate those who develop ATL from those who remain healthy carriers.

Recently, HTLV-1 proviral load levels have been evaluated as important predictors of development of ATL and HAM/TSP. Some cross-sectional studies showed that HTLV-1 proviral load levels were higher in ATL and HAM/TSP compared with asymptomatic HTLV-1 carriers.[20,21] However, the proviral load levels of asymptomatic HTLV-1 carriers exhibited a very wide range,[20,22,23] and these levels may vary by sex, race, habitats, and comorbidities.[24] The proviral load levels of asymptomatic HTLV-1 carriers were also examined serially in some prospective studies; however, the

Submitted December 9, 2009; accepted April 20, 2010. Prepublished online as *Blood* First Edition paper, May 6, 2010; DOI 10.1182/blood-2009-12-257410.

The online version of this article contains a data supplement.

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number of reported cases was very small.[25-28] Although these previous studies suggest a possible important role for HTLV-1 proviral load in the development of ATL and HAM/TSP, the association between HTLV-1 proviral load and diseases development remains unclear.

The identification of risk factors for developing ATL among virus carriers is necessary to prevent these diseases in HTLV-1 endemic areas. To investigate detailed viral- and host-specific determinants of disease development, larger and longer prospective studies are warranted. In 2002, we established a nationwide cohort study for asymptomatic HTLV-1 carriers in Japan named the Joint Study on Predisposing Factors of ATL Development (JSPFAD).[29] The main objective of this project is to establish reliable predisposing factors for developing ATL by prospectively following a large number of asymptomatic HTLV-1 carriers. Here, for the first-time, we report the study method, baseline demographic characteristics, and distribution characteristics of baseline HTLV-1 proviral load of asymptomatic HTLV-1 carriers. We have also evaluated progression to ATL and its risk predictors.

Methods

Participants and study design

The JSPFAD is a nationwide prospective study of HTLV-1 carriers, which was approved by the Ministry of Education, Culture, Sports, Science and Technology of Japan. The project was established in August 2002 by Japanese clinicians and basic researchers of 41 institutions composed of 14 university hospitals and 27 educational hospitals located in various areas of Japan (supplemental Appendix, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Objectives of the project are to establish reliable predisposing factors for development of ATL by prospectively following a large number of asymptomatic HTLV-1 carriers. This includes performing clinical examinations and biomarker assays, as well as establishing a biomaterial resource bank of plasma, viable peripheral blood mononuclear cells (PBMCs), frozen PBMCs pellets, and genomic DNA from PBMCs of HTLV-1–infected persons for the future evaluations with new molecular biology techniques.

Hematologists at the collaborating institutions were responsible for enrolling participants after receiving approval from their Institutional Review Boards. The study protocol was approved by the Ministry of Education, Culture, Sports, Science and Technology of Japan. Eligible participants were those who had known of their HTLV-1 infection and had confirmed the HTLV-1–positive serology at any of the medical institutions. Potential participants visited any of the collaborating institutions directly or via the website of the JSPFAD (www.htlv1.org/). They received adequate explanations for the enrollment procedure from the hematologists at the collaborating institutions. Enrollment was conditional on participants giving written informed consent in accordance with the Declaration of Helsinki. The primary participants were asymptomatic HTLV-1 carriers. A small number of patients with definite ATL, HAM/TSP, and HU/HAU were also enrolled as controls.

Data collection and sample storage

After providing written informed consent, participants were expected to fill out a questionnaire regarding demographic information, to provide peripheral blood samples, and to periodically visit the institution for follow-up. After reconfirming the asymptomatic HTLV-1 carrier status of the participants, hematologists at the collaborating institutions assigned a unique identification number to each participant and subsequently sent all materials (individual questionnaire sheets, clinical data, and blood samples drawn into ethylenediaminetetraacetic acid and heparin tubes) to the JSPFAD office (Department of Medical Genome Sciences, Laboratory of Tumor Cell Biology, Graduate School of Frontier Sciences, University of Tokyo, Japan).

The self-administered questionnaire included items on demographic characteristics, birthplaces of the participants and their mothers, family history regarding HTLV-1 status and HTLV-1–associated diseases, length of marriage, partner's HTLV-1 status, first opportunity for HTLV-1 testing, and histories of disease manifestations other than HTLV-1–associated diseases. Additional questionnaire items, information on prior blood transfusion, and smoking habits (present, past, or nonsmoking) were also included after April 2008.

Clinical data included information on the date of visit, complete blood cell count, differential cell counts (including abnormal lymphocytes per 100 leukocytes), lactate dehydrogenase, HTLV-1 serologic test, comorbidities other than HTLV-1–associated diseases, and the development of any HTLV-1–associated diseases during follow-up. Blood samples were collected at enrollment, annually thereafter (in principal), and as needed. Blood samples sent to the study office at the University of Tokyo were separated into plasma, PBMCs, and genomic DNA and then used for viral marker assays at the University of Tokyo or stored for the biomaterial bank at the Japanese Red Cross Fukuoka Blood Center.

Viral marker assays

HTLV-1 proviral load of PBMC samples was measured by real-time polymerase chain reaction (PCR) using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems Japan), as previously described with minor modifications.[30,31] Genomic DNA from PBMCs was isolated using a QIAGEN Blood Kit (QIAGEN). Quantitative real-time PCR was performed using multiplex PCR with 2 sets of primers specific for the HTLV-1 provirus and the human gene encoding the RNase P enzyme. The primers and the probe for the gene encoding RNase P were purchased from Applied Biosystems; those for the pX region of the HTLV-1 provirus were described previously.[30,31] Genomic DNA of normal control PBMCs mixed with a plasmid DNA, which contained almost the whole genome of the HTLV-1 provirus (*SacI* site of 5′-LTR to *SacI* site of 3′-LTR), was used as control template. The copy number of the plasmid DNA was calculated based on the size and weight of the plasmid DNA, as measured by spectrophotometry. The proviral loads were expressed as copy numbers per 100 PBMCs, based on the assumption that infected cells harbored 1 copy of the integrated HTLV-1 provirus per cell. Samples with a higher proviral load (> 20 copies/100 PBMCs) were subjected to Southern blot analysis to examine the clonality of the infected cells. Assays to detect the integrated band of HTLV-1 provirus genome were described previously.[32] Genomic DNA samples (10 mg) were digested with *PstI* or *Eco*RI restriction enzymes and were size-fractionated on 0.7% agarose gels. They were then transferred onto a nylon membrane by the Southern blot technique. Hybridization to randomly primed ^{32}P-labeled DNA probes for the whole proviral genome (*SacI* to *SacI* fragment of the HTLV-1 proviral genome) was performed, followed by appropriate stringency washing steps and autoradiography. Soluble interleukin-2 receptor was measured by a commercial laboratory (SRL Inc) using an enzyme-linked immunosorbent assay (Endogen) and reported as units per milliliter.

Statistical analysis

Analyses were performed for participants who enrolled as of December 2008. Age at enrollment was categorized into 5 groups: younger than 40, 40 to 49, 50 to 59, 60 to 69, and 70 years or older. Geographic location was divided into 4 areas: northern (Hokkaido and Tohoku), metropolitan (Tokyo, Osaka, and Nagoya), southern (Kyushu and Okinawa), and others (supplemental Figure 1). First opportunity for HTLV-1 testing was divided into 3 categories: by screening for HTLV-1 (regional-mass, multiphasic, blood donor, and maternal screenings), by the presence of HTLV-1–infected family members (including spouse), and by the patient status under treatment for diseases unrelated to HTLV-1. A positive family history was considered to be present when participants had information on first-degree relatives (parents, siblings, or offspring) who were HTLV-1 carriers or had HTLV-1–associated diseases (ie, ATL, HAM/TSP, and HU/HAU). Any leukemia and/or lymphoma other than ATL were also taken into consideration. A positive comorbidity at enrollment was considered to be present when any information on diseases other than HTLV-1–associated diseases

was available at enrollment. HTLV-1 proviral loads (copy numbers/100 PBMCs) were used as a continuous variable (raw and the power-transformed data) or by categorizing them into quartiles. We applied a square-root transformation to the raw data of proviral loads to reduce the skewness. Continuous data were presented as median (range) values and compared using a Mann-Whitney test. Categorical data were compared using a χ^2 test or Fisher exact test. We calculated person-years of follow-up for each participant from the date of enrollment to the date of ATL diagnosis, the date of last follow-up, or September 30, 2009, whichever came first. Cumulative progression to ATL was estimated using Kaplan-Meier curves. To estimate the effect of baseline HTLV-1 proviral load and selected demographic factors on ATL development, we performed Cox proportional hazards analyses, and expressed as hazard ratios (HR) and 95% confidence intervals (CI), which were calculated by robust sandwich variance estimates. To check for possible incompleteness in the multivariate model, we also performed analyses using sub-datasets. All statistical analyses were performed using SAS Version 9.1 (SAS Institute Japan) with a 2-tailed significance level of .05.

Results

Baseline demographic characteristics

From August 2002 to December 2008, 1259 participants of asymptomatic HTLV-1 carriers were enrolled in this study. However, HTLV-1 proviral load was not measured for 41 participants. Thus, a total of 1218 participants (426 males and 792 females) were included in this analysis. Demographic characteristics of the participants at enrollment are shown in Table 1. The median ages at enrollment in the cohort were 59.6 years (range, 6.9-92.8 years) for males or 58.3 years (range, 17.8-90.3 years) for females. The largest percentage of study participants was from the southern area, which is a well-known HTLV-1 endemic area in Japan, followed by the metropolitan area. The southern area also had the largest percentage for birthplaces for most participants and their mothers.

One-half of the participants came to know of their HTLV-1 infections through screening for HTLV-1, and one-fourth was informed of their infections while receiving treatments for diseases other than HTLV-1–associated diseases. More than half of the participants did not know their family status of HTLV-1 infection. Only 119 female participants knew about the HTLV-1 infection status of their husbands, of whom 53 (45%) of the husbands were positive for HTLV-1 (data not shown). However, we were not able to obtain reliable information on male-to-female transmission for the female participants. We obtained information on comorbidities at enrollment from 257 participants, of which 45 had comorbid infectious diseases (eg, strongyloidiasis, chronic bronchitis, hepatitis C virus infection, lymphadenitis), 29 had autoimmune diseases (rheumatoid arthritis, chronic thyroiditis, Sjögren syndrome, and other autoimmune or chronic inflammatory diseases), 80 had a variety of definite malignant diseases other than ATL (non-Hodgkin lymphoma, acute myeloid leukemia, gastric cancer, lung cancer, or other malignancies), 16 had skin diseases, and 87 had other common diseases (eg, hypertension, diabetes).

Distributions of baseline HTLV-1 proviral load

Figure 1 shows distribution of baseline HTLV-1 proviral load in 1218 participants. There was a wide range of skewness in the raw data, with a median of 1.60 copies/100 PBMCs (range, 0-55.8 copies/100 PBMCs; 25th-75th percentile, 0.29-4.54 copies/100 PBMCs; Figure 1A). The square-root transformation reduced the skew in the raw data, with a median of 1.26 copies/100

Table 1. Baseline demographic characteristics of asymptomatic HTLV-1 carriers

Variable	Male, no. (%)	Female, no. (%)
Total	426	792
Age, y		
Younger than 40	48 (11.3)	119 (15.0)
40-49	70 (16.4)	130 (16.4)
50-59	99 (23.2)	174 (22.0)
60-69	88 (20.7)	172 (21.7)
70 or older	121 (28.4)	197 (24.9)
Place of enrollment		
Northern area	10 (2.3)	32 (4.0)
Metropolitan area	75 (17.6)	144 (18.1)
Southern area	333 (78.2)	597 (75.4)
Other areas	8 (1.9)	19 (2.4)
Birthplace of participants		
Northern area	18 (4.2)	33 (4.2)
Metropolitan area	30 (7.0)	80 (10.1)
Southern area	240 (56.3)	400 (50.5)
Other areas	20 (4.7)	54 (6.8)
Unknown	118 (27.7)	225 (28.4)
Birthplace of participants' mothers		
Northern area	16 (3.8)	32 (4.0)
Metropolitan area	13 (3.1)	39 (4.9)
Southern area	247 (58.0)	426 (53.8)
Other areas	28 (6.6)	64 (8.1)
Unknown	122 (28.6)	231 (29.2)
First opportunity for HTLV-1 testing		
Screening for HTLV-1	209 (49.1)	452 (57.1)
Regional mass screening	77	164
Multiphasic screening	24	44
Blood donor screening	108	128
Maternal screening	0	116
Revelation of HTLV-1–positive family	33 (7.7)	101 (12.7)
During treatment of other diseases	117 (27.5)	148 (18.7)
Unknown	67 (15.7)	91 (11.5)
Family history of HTLV-1–associated diseases*		
Absent	98 (23.0)	154 (19.5)
Absent for a first-degree relative but having an infected spouse	6 (1.4)	23 (2.9)
Carrier only	27 (6.3)	74 (9.3)
HU/HAU only	2 (0.5)	1 (0.1)
HAM	2 (0.5)	7 (0.9)
ATL	34 (8.0)	74 (9.3)
Leukemia or lymphoma	9 (2.1)	26 (3.3)
Unknown family history	248 (58.2)	433 (54.7)
Comorbidity†		
Absent	331 (77.7)	630 (79.5)
Present	95 (22.3)	162 (20.5)
Infectious diseases	20	25
Autoimmune diseases	3	26
Malignant diseases	36	44
Skin diseases	8	8
Other disease	28	59

HTLV-1 indicates human T-cell leukemia virus type 1; HU, HTLV-1 uveitis; HAU, HTLV-1–associated uveitis; HAM, HTLV-1 myelopathy; and ATL, adult T-cell leukemia.

*Family history was restricted to a first-degree relative. "Present" indicates that participants have a parent, sibling, or offspring diagnosed with HTLV-1-associated diseases. Family members with HAM and HU/HAU were included into the category of "HAM." Family members with ATL and HAM and/or HU/HAU were included into the category of "ATL."

†Comorbidity indicates that participants have any diseases other than HTLV-1–associated diseases at enrollment.

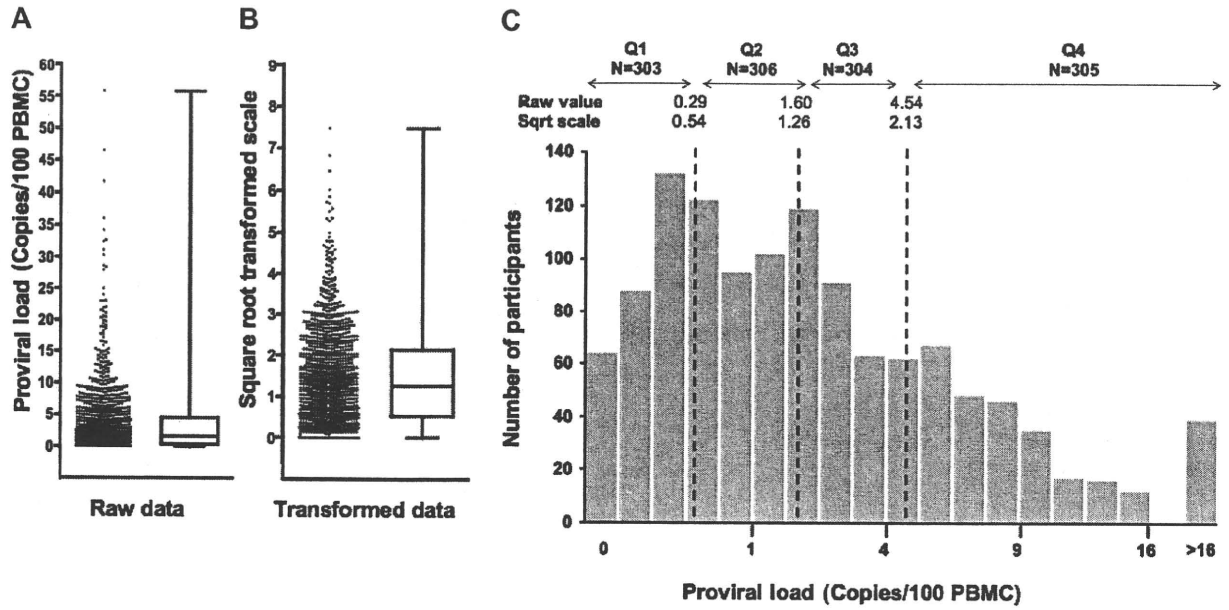

Figure 1. Distribution of baseline HTLV-1 proviral load levels among 1218 asymptomatic HTLV-1 carriers. (A) Scatter plot of raw data of proviral load (left) and the vertical box and whiskers plot (right): the box delineates 25th percentile (0.29 copies/100 periopheral blood mononuclear cells [PBMCs]), median (1.60 copies/100 PBMCs), and 75th percentiles (4.54 copies/100 PBMCs), and the whiskers delineate the minimum (0 copies/100 PBMCs) and maximum (55.8 copies/100 PBMCs). (B) Scatter plot of square-root transformed values of the raw proviral load (left) and the vertical box and whiskers plot (right): the box delineates 25th percentile (0.54 copies/100 PBMCs), median (1.26 copies/100 PBMCs), and 75th percentiles (2.13 copies/100 PBMCs), and the whiskers delineate the minimum (0 copies/100 PBMCs) and maximum (7.47 copies/100 PBMCs). (C) The frequency of participants in the quartile distributions of proviral load. Q1 indicates quartile 1 (< 25th percentile); Q2, quartile 2 (25th percentile to median); Q3, quartile 3 (median to 75th percentile); Q4: quartile 4 (> 75th percentile); Sqrt, square-root transformation; and N, number of participants.

PBMCs (range, 0-7.47 copies/100 PBMCs; 25th-75th percentile, 0.54-2.13 copies/100 PBMCs; Figure 1B). Figure 1C shows the frequency of participants in each quartile of proviral load.

The median proviral load and a frequency of subjects in each quartile of proviral load by demographic characteristics are shown in Table 2. Males and females were significantly different in proviral load levels, with a median value of 2.10 copies/100 PBMCs (range, 0-46.6 copies/100 PBMCs) for males and that of 1.39 copies/100 PBMCs (range, 0-55.8 copies/100 PBMCs) for females ($P < .001$). Males were probably distributed in the highest quartile of proviral load level than females.

Among age groups, the median proviral load of those 40 to 49 and 50 to 59 years of age was significantly higher than that of those less than or equal to 40 years ($P = .02$ and $P = .007$, respectively). Both age groups were probably distributed in the highest quartile of proviral load levels. Because we found a significantly different median proviral load by sex, we additionally evaluated the proviral load level by age group in each sex. The highest median value was found in those 50 to 59 years of age (2.89 copies/100 PBMCs) in males, but in 40 to 49 years of age (1.49 copies/100 PBMCs) in females, although there were no statistical differences by age group for both sexes (data not shown).

Among the categories for the first opportunity for HTLV-1 testing, the proviral load level was significantly higher ($P = .002$) in participants informed of their infection during treatment for diseases unrelated to HTLV-1 compared with those who came to know of their infection by screenings (Table 2). Participants informed of their infection during treatment for diseases unrelated to HTLV-1 were probably distributed in the highest quartile of proviral load levels. There was no difference in the proviral load level between those who came to know of their infection by the presence of HTLV-1–positive family members and those who came to know of their infection by screenings.

When we evaluated the proviral load level by family history status, participants who had no family history of HTLV-1 infection, who had only HTLV-1 carriers in the family, who had only an HTLV-1 carrier husband, and who had only HU/HAU in the family were grouped together as a reference category. The proviral load levels of those with a family history of HAM/TSP (median 3.85 copies/100 PBMCs) and ATL (median 2.32 copies/100 PBMCs) were significantly higher ($P = .01$ and $P = .005$, respectively) compared with those of the reference group (Table 2). Indeed, those with a family history of HAM/TSP and ATL were probably distributed in the third and fourth quartiles of proviral load levels. Of interest, the median proviral load level of those with a family history of leukemia or lymphoma was also significantly higher ($P = .009$) compared with those of the reference group.

Among the categories for comorbidity, there was no statistical difference in the proviral load levels when we simply compared between those with and without comorbidity at enrollment (data not shown). However, when we compared those without comorbidity and those with infectious diseases at enrollment, the median proviral load of the latter was significantly higher than that of the former ($P = .05$; Table 2).

Prognosis

During a median follow-up period of 1.0 year (range, 0-6.6 years) and a total of 1981.2 person-years, 14 (1.1%) participants (4 males and 10 females) progressed to overt ATL (2 acute, 2 lymphoma, and 10 smoldering types; Table 3). The incidence rate of ATL was 7.1 per 1000 person-years for all types of ATL and 2.0 per 1000 person-years for the aggressive types (acute and lymphoma) of ATL. The median duration from date of enrollment to date of diagnosis of ATL was 13.8 months (range, 2.8-64.4 months). The cumulative probability of progression to ATL was reached 4.8% (95% CI, 1.9%-11.8%) at 5.4 years (Figure 2).

Table 2. HTLV-1 VL levels by demographic characteristics

Demographic characteristics	No.	Median VL (range) (copies/100 PBMCs)	Frequency of subjects by VL level, n (% of row) Quartile 1 (VL: < 0.29)‡	Quartile 2 (VL: 0.29-1.60)	Quartile 3 (VL: 1.60-4.54)	Quartile 4 (VL: ≥ 4.54)
Total		1.60 (0-55.8)	303	306	304	305
Sex						
Male	426	2.10 (0-46.6)*	84 (19.7)	100 (23.5)	93 (21.8)	149 (35.0)
Female	792	1.39 (0-55.8)†	219 (27.7)	206 (26.0)	211 (26.6)	156 (19.7)
Age, y						
Younger than 40	167	1.37 (0-16.4)†	49 (29.3)	43 (25.8)	50 (29.9)	25 (15.0)
40-49	200	1.77 (0-41.7)*	43 (21.5)	52 (26.0)	51 (25.5)	54 (27.0)
50-59	273	1.84 (0-36.1)*	64 (23.4)	64 (23.4)	63 (23.1)	82 (30.4)
60-69	260	1.56 (0-46.6)	66 (25.4)	66 (25.4)	61 (23.5)	67 (25.8)
70 or older	318	1.52 (0-55.8)	81 (25.5)	81 (25.5)	79 (24.8)	77 (24.2)
First opportunity for HTLV-1 testing						
Screening	661	1.46 (0-55.8)†	182 (27.5)	160 (24.2)	175 (26.5)	144 (21.8)
Revelation of HTLV-1–positive family	134	1.45 (0-46.6)	31 (23.1)	40 (29.9)	39 (29.1)	24 (17.9)
During treatment for other diseases	265	1.93 (0-41.7)*	56 (21.1)	66 (24.9)	57 (21.5)	86 (32.5)
Unknown	158	2.08 (0-30.3)*	34 (21.5)	40 (25.3)	33 (20.9)	51 (32.3)
Family history of HTLV-1–related diseases						
Absence or carrier/HU/HAU only	385	1.33 (0-32.4)†	100 (26.0)	105 (27.2)	100 (26.0)	80 (20.8)
HAM/TSP	9	3.85 (1.2-9.4)*	0	1 (11.1)	5 (55.6)	3 (33.3)
ATL	108	2.32 (0-46.6)*	18 (16.7)	26 (24.1)	33 (30.6)	31 (28.7)
Leukemia or lymphoma	35	2.47 (0-12.8)*	3 (8.6)	9 (25.7)	11 (31.4)	12 (34.3)
Unknown family history	681	1.55 (0-55.8)	182 (26.7)	165 (24.2)	155 (22.8)	179 (26.3)
Comorbidity						
Absence	961	1.65 (0-55.8)†	241 (25.1)	234 (24.4)	244 (25.4)	242 (25.2)
Infectious diseases	45	2.75 (0-28.6)*	7 (15.6)	8 (17.8)	13 (28.9)	17 (37.8)
Autoimmune diseases	29	1.33 (0-41.7)	10 (34.5)	7 (24.1)	4 (13.8)	8 (27.6)
Malignant diseases	80	1.57 (0-19.4)	19 (23.8)	21 (26.3)	23 (28.8)	17 (21.3)
Skin diseases	16	0.60 (0.07-14.6)	6 (37.5)	5 (31.3)	3 (18.8)	2 (12.5)
Other disease	87	1.17 (0-22.0)	20 (23.0)	31 (35.6)	17 (19.5)	19 (21.8)

HTLV-1 indicates human T-cell leukemia virus type 1; VL, HTLV-1 proviral load; PBMCs, peripheral blood mononuclear cells; HU, HTLV-1 uveitis; HAU, HTLV-1–associated uveitis; HAM, HTLV-1 myelopathy; TSP, tropical spastic paraparesis; and ATL, adult T-cell leukemia.

*Mann-Whitney test revealed a statistically significant difference in the VL level compared with the reference group.

†Reference group.

‡The VL was categorized based on quartile cutoff points (the 25th, 50th, and 75th percentiles of the VL distribution) in 1218 HTLV-1 carriers. The unit of VL was copies/100 PBMCs.

The median proviral load at enrollment for these 14 participants was 10.3 copies/100 PBMCs (range, 4.17-28.58 copies/100 PBMCs), which was significantly higher than those who did not develop ATL (1.56 copies/100 PBMCs; range, 0-55.8 copies/100 PBMCs; $P < .001$). Of interest, the median proviral load level at enrollment was significantly higher for those who developed smoldering types of ATL than for those who developed aggressive types of ATL (11.4 and 5.1 copies/100 PBMCs, respectively, $P = .02$), whereas the median entry age was significantly younger for the former than for the latter (59.8 and 73.9 years, respectively, $P = .02$). Distribution of the 14 participants who developed ATL by demographic characteristics and by quartile of proviral load levels is shown in Table 4. Among 14 ATLs, 13 occurred in the highest quartile of baseline proviral load (> 4.54 copies/100 PBMCs) and 1 occurred in the third quartile (1.60-4.54 copies/100 PBMCs), whereas no ATL developed in quartiles 1 and 2 (< 1.60 copies/100 PBMCs). A high frequency of ATL was also seen in older age group, those with first opportunity for HTLV-1 testing during treatment of other diseases and those with a family history of ATL. Therefore, we decided to include the baseline HTLV-1 proviral load (the square-root transformed continuous value), age, first opportunity for HTLV-1 testing, and family history into Cox hazard analyses as covariates to test the effects on the development of ATL.

We identified that baseline proviral load was strongly associated with the risk of progression to ATL on both univariate and multivariate Cox analyses. In the multivariate analysis, the adjusted HR for the square-root transformed proviral load per unit increase was 3.57 (95% CI, 2.25-5.68; Table 5). We also found that advanced age, family history of ATL, and first opportunity to learn of HTLV-1 infection during treatment of other diseases were independently associated with the development of ATL, after adjusting the effect of proviral load. The adjusted HR for developing ATL per 5-year increase of age from 40 years was 1.67 (95% CI, 1.12-2.50). HTLV-1 carriers having a family history of ATL had 12 times higher risk of developing ATL compared with those not having the history (adjusted HR = 12.1; 95% CI, 2.26-64.7), and those who came to know their HTLV-1 infection during treatment for other diseases had 4 times higher risk of developing ATL compared with references (adjusted HR = 4.16; 95% CI, 1.37-12.6), although the CIs were wide because of the smaller group sizes (Table 5). Of interest, male gender was not a significant risk factor for developing ATL, even though the median proviral load was significantly higher in males than in females (Table 2).

Because the distribution of proviral load was skewed even after the value was square-root transformed, it was possible that ATL events in subjects with skewed high proviral loads contributed to results. To check the possibility, we performed a multivariate analysis using a sub-dataset that excluded subjects with skewed proviral load (> 16 copies in Figure 1C; n = 39, including 3 who developed ATL). Nevertheless, we observed similar results as the original dataset, although age factor was no longer statistically