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Provirus Load in Patients with Human T-Cell Leukemia Virus Type 1 Uveitis Correlates with Precedent Graves' Disease and Disease Activities

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We previously demonstrated the increased provirus load in the peripheral blood of patients with human T-cell leukemia virus type 1 (HTLV-1) uveitis (HU). To delineate the relevance of the increased provirus load to clinical and immunologic parameters, we studied the correlation between them. Seventy-nine HU patients (24 male and 55 female) were included in the study, with their informed consent. Plasma samples and genomic DNA of the peripheral blood mononuclear cells were isolated and the provirus load was estimated by semi-quantitative polymerase chain reaction of the gag region sequence. Serum levels of anti-HTLV-1 antibodies and soluble IL-2R were determined by electrochemiluminescence immuno assay and by ELISA, respectively. Disease activities were assessed and graded 0 to 4 according to the evaluation system. Recurrence of the disease during the follow-up period was diagnosed ophthalmologically. The provirus load was significantly higher in the HU patients after Graves' disease (GD) than in those without GD ($P<0.05$). It correlated with disease activities assessed in terms of vitreous inflammation and interval to recurrence (both $P<0.05$). In the HU patients without GD, it correlated with the serum levels of soluble IL-2 receptor ($P<0.01$), and nearly with those of HTLV-1 antibody ($P=0.063$). These correlations were not found in the HU patients after GD under methimazole treatment. The results suggested a direct involvement of HTLV-1-infected cells in the pathogenesis of uveitis, and raise the possibility that hyperthyroidism may contribute to the clonal expansion of HTLV-1-infected cells.

Key words: HTLV-1 uveitis — Provirus load — Graves' disease — Disease activities — sIL-2R

Human T-cell leukemia virus type 1 (HTLV-1) uveitis (HU) is an acute or subacute and recurrent uveitis characterized by T lymphocyte infiltration in the eye, particularly in the vitreous body, and production of interleukin (IL)-6 by the infiltrating cells.[1–6] It is associated with Graves' disease (GD) at an extraordinarily high rate.[7] Actually, one-fourth of female patients have a history of GD. Subsequent reports have confirmed our observation that, in all the patients, hyperthyroidism preceded the onset of HU,[8,9] which we described as HU after GD.[7] The chronological order of the onsets of these two diseases and the lack of epidemiological evidence for association between HTLV-1-infection and Graves' disease provide further evidence for the possible roles of hyperthyroidism or methimazole (MMI) or both in the development of HU, though the nature of these roles remains to be investigated.

The population of HTLV-1-infected cells, i.e., the provirus load, in the peripheral blood mononuclear cells (PBMC) was significantly higher in HU patients than in asymptomatic carriers.[10] An increased provirus load has also been reported in tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM).[11–16] Thus, it was suggested that the increased provirus load of HTLV-1 may be one factor in the development of inflammatory disorders caused by HTLV-1. It has also been reported that the proviral load correlates with immunologic parameters such as the serum levels of HTLV-1 antibodies and soluble IL-2 receptor (sIL-2R).[17–24] Recent reports on the analysis of the integration sites of HTLV-1 provirus suggested that the increased provirus load results from clonal proliferation of the HTLV-1-infected cells, rather than virus replication and re-infection *in vivo*.[25] However, the mechanism underlying the clonal expansion of the HTLV-1-infected cells and its relevance to the pathogenesis and pathophysiology of HTLV-1-associated diseases remain to be delineated.

In the current study, we investigated the correlation between the provirus load in the PBMC of patients with HU and clinical and immunologic parameters, in order to obtain insight into the clinical and immunologic relevance of the provirus load as well as the mechanisms underlying

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the clonal expansion of the virus-infected cells. We found that the provirus load was significantly higher in patients after GD than in those without GD, and it generally correlated with disease activities and serum levels of HTLV-1 antibody and sIL-2R.

MATERIALS AND METHODS

Patients Diagnosis of HU was made according to the criteria described previously.[4] Briefly, HTLV-1 seropositive patients with uveitis of unknown etiology after extensive ophthalmologic and laboratory examinations were diagnosed as HU. Seventy-nine patients (24 male and 55 female) were included in the study. Disease activities were assessed and graded into 0 to 4 according to the system proposed by Nussenblatt *et al*. for the evaluation of vitreal inflammatory activity in patients with uveitis.[26] Recurrence of the disease during the follow-up period was diagnosed ophthalmologically. Among 79 patients with HU, 19 (all female) developed HU after GD and were under treatment with MMI. Thyroid function tests showed that all these patients were in an euthyroid state when they were enrolled in the study. Peripheral blood samples were collected after informed consent had been obtained from the patients. The principles embodied in the Declaration of Helsinki have been adhered to by the authors.

Semi-quantitative polymerase chain reaction (PCR) of HTLV-1 provirus Our assay system for the HTLV-1 provirus load in PBMC has been described previously.[10] Briefly, PBMC samples were isolated by density gradient centrifugation using "Ficoll-Paque" (Pharmacia, Uppsala, Sweden). High-molecular-weight genomic DNA of the PBMC was extracted by means of proteinase K digestion and phenol/chloroform extraction. Using 0.5 μg genomic DNA samples as templates, HTLV-1 provirus copies were determined by semi-quantitative PCR of the gag region of the provirus.

Measurement of the serum levels of antibodies to HTLV-1 and sIL-2R The serum level of anti-HTLV-1 antibody was determined by the use of an electrochemiluminescence immuno assay (ECLIA) developed by Eisai Co., Ltd., Tokyo.[27, 28] Briefly, magnetic microparticles (Dynabeads M-450, Dynal A/S, Oslo, Norway) coupled with a combination of viral antigen purified from culture supernatant of MT-2 cell line and synthetic envelope (gp46) peptide antigen were used as a solid support. Absorbed antibodies were detected by electrochemically generated luminescence with the sandwich method using anti-human IgG (Fc) monoclonal antibody (Wako Junyaku, Osaka) coupled with ruthenium (II) tris (bipyridyl); $Ru(bpy)_3^{2+}$. The antibody level of each serum sample was expressed as a cut-off index value (C.O.I.) which is the relative level of electroluminescence compared with that of the cut-off control. C.O.I. levels between 1 and 2 were considered indeterminate, and those above 2.0 positive. Results of the ECLIA method were compared with those of the ELISA method (New Eitest ATL, Eisai, Tokyo) using 280 ELISA-positive samples collected from 77 patients with ATL, 43 with TSP/HAM and 160 asymptomatic carriers and 441 ELISA-negative samples. All the 280 ELISA-positive serum samples were shown to be positive by the ECLIA method, and 98.2% of the 441 ELISA-negative serum samples showed negative.

The serum level of sIL-2R was determined by ELISA (T Cell Sciences Inc., Cambridge, Mass.).[24]

Statistical methods Logarithmic scale transformation of the values of provirus load, interval to recurrence and serum levels of anti-HTLV-I antibody and sIL-2R was conducted, and the converted variables proved to be approximately normally distributed. Associations between the parameters of interest were examined by the use of Pearson's correlation coefficient (denoted by r) and linear regression analysis. The significance of differences between means was assessed by the use of Student's t test.

RESULTS

Provirus load in patients with HU As we have previously reported,[10] the provirus load was expressed as a percentage of the HTLV-1-infected cells in the PBMC on the assumptions that a single copy of HTLV-1 provirus is integrated in the infected T cells *in vivo*, and that 0.5 μg of genomic DNA corresponds to 0.75×10^5 cells. The mean±SD of the provirus load in the PBMC of 79 patients with HU was 3.9±4.1%, which accords well with our previous results. Because all the patients after Graves' disease in this study were female, we compared the provirus load of the female patients with HU, dividing them into those after GD (n=19) and those without GD (n=36). The geometric mean (anti-log(mean–SD)–anti-log(mean+SD)) values in the patients after GD or without GD were 3.27 (1.11–9.61) and 1.36 (0.29–6.25), respectively (Fig. 1). The difference was statistically significant (P<0.05), indicating that the HU patients after GD have a higher provirus load than those without GD.

Provirus load and activity of the uveitis HU is characterized by vitreous infiltration of inflammatory cells and a recurrent clinical course.[1–5] Therefore, to investigate the relevance of the increased provirus load to clinical manifestations, the intensity of the vitreous inflammation and the frequency of recurrence were used as clinical parameters that indicate disease activities.

When vitreous inflammation was used as a clinical parameter, 14 patients with HU of anterior uveitis type were excluded from the study because vitreous inflammation is not a good indicator for the disease activity in these patients. The provirus load of 65 HU patients was

plotted against the level of vitreous inflammation graded 0 to 4 according to the evaluation system[26] (Fig. 2). A significant correlation was found between the provirus load and the level of vitreous inflammation (r=0.341, P<0.01).

Recurrence of the uveitis was observed in 34 out of 54 patients (63.0%) who were clinically followed for more than 18 months. The relationship between the provirus load and the interval to recurrence is shown in Fig. 3. A significant but inverse correlation between these parameters was demonstrated (r=−0.351, P<0.05).

Provirus load and serum levels of HTLV-1 antibody and sIL-2R No correlation was found between the provirus load and the serum level of HTLV-1 antibody when analyzed in all the HU patients as a whole. Because patients after GD were all under treatment with MMI, which suppresses B cell activity,[29, 30] we next investigated the correlation in 42 patients without GD. The concentrations of serum HTLV-1 antibody of these patients ranged from 5.5 to 314.6 C.O.I. with a mean±SD of 104.8±81.6. The provirus load showed some correlation with the serum level of HTLV-1 antibody, but this was not statistically significant (Fig. 4) (r=0.289, P=0.063).

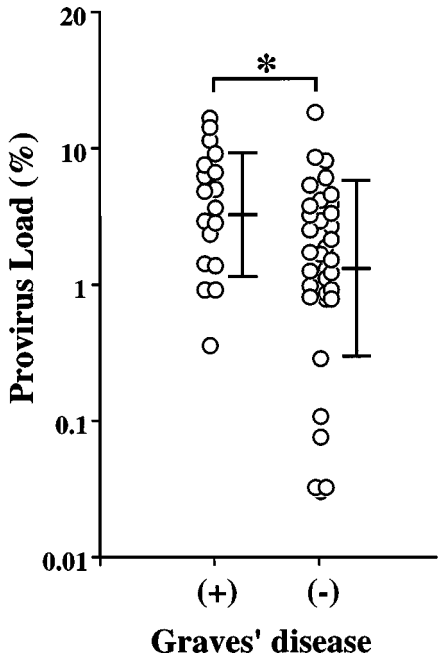

Fig. 1. HTLV-1 provirus load in the PBMC of the HU female patients after Graves' disease (19 patients) or those without it (36 patients). The mean (log x)±SD (log x) values for the population of HTLV-1-infected cells were 0.51±0.47 and 0.13±0.66, respectively. The geometric mean (anti-log (mean−SD)−anti-log (mean+SD)) values were 3.27 (1.11–9.61) and 1.35 (0.29–6.25), respectively. The difference was statistically significant (P<0.05).

The correlation between the provirus load and the serum level of sIL-2R was also investigated in the 42 patients without GD. The concentrations of serum sIL-2R

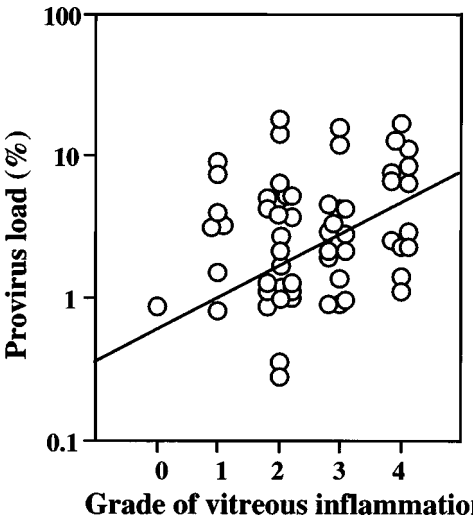

Fig. 2. Provirus load and the intensity of vitreous inflammation. The provirus load of 65 patients with HU was plotted against the level of vitreous inflammation graded 0 to 4. A significant correlation was found (r=0.341, P<0.01). The regression line is expressed as Y=0.227+1.071X where Y=log(y) and X=log(x).

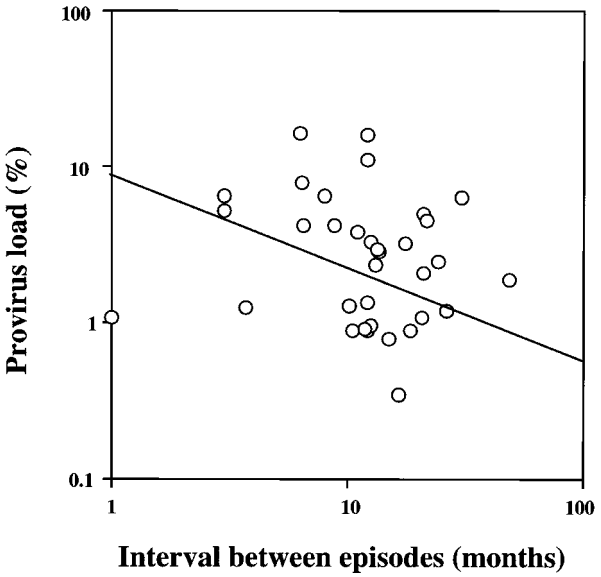

Fig. 3. Provirus load and the interval to recurrence. The interval to recurrence of 34 patients with HU who were followed for more than 18 months was plotted against the provirus load. A significant but inverse correlation was found (r=−0.351, P<0.05). The regression line is expressed as Y=0.941−0.575X where Y=log(y) and X=log(x).

ranged from 211.0 to 1,838 pg/ml with a mean±SD of 516.6±320.8 pg/ml. The provirus load showed a significant positive correlation with the serum level of sIL-2R (r=0.332, P<0.05) (Fig. 5).

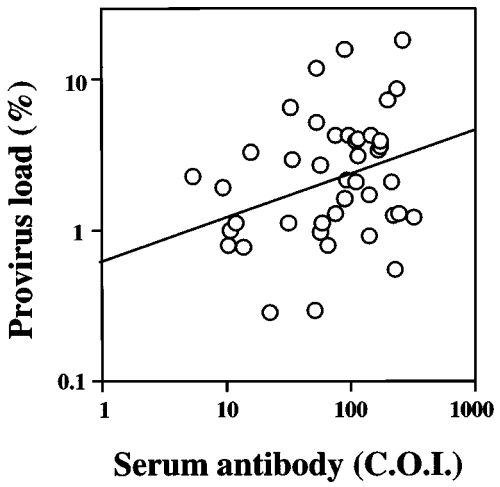

Fig. 4. Provirus load and the serum level of anti-HTLV-1-antibody. The level of serum antibody was measured by the ECLIA method described in "Materials and Methods," and is expressed as a C.O.I. The provirus load was plotted against the concentration of serum HTLV-1 antibody in 42 HU patients without GD. A positive correlation was found, but it was not statistically significant (r=0.289, P=0.063). The regression line is expressed as Y=−0.143+0.263X where Y=log(y) and X=log(x).

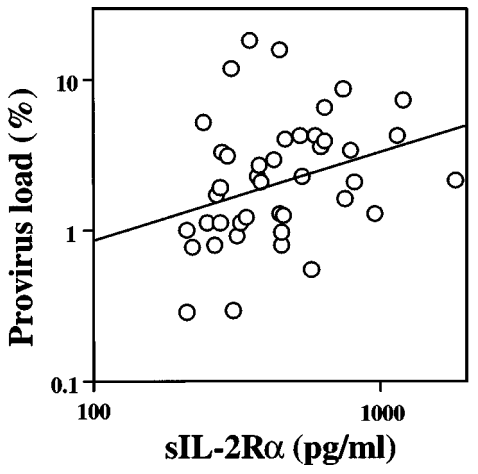

Fig. 5. Provirus load and the serum level of sIL-2R. The provirus load was plotted against the serum level of sIL-2R in 42 HU patients without GD. A significant positive correlation was found between these parameters (r=0.332, P<0.05). The regression line is expressed as Y=−1.309+0.623X where Y=log(y) and X=log(x).

These results suggested that in the HU patients the levels of antibody response to HTLV-1 antigens and activation of T cells *in vivo* are correlated with the level of provirus load, as was reported previously in asymptomatic carriers and patients with TSP/HAM.[17–24]

DISCUSSION

In the present study, we found that the provirus load is higher in HU patients after GD than in those without GD, and correlates with disease activities and serum levels of HTLV-1 antibody and sIL-2R.

Increased provirus load appears to be a common characteristic in patients with HU and those with TSP/HAM.[10–16] It is considered to result from clonal expansion of the virus-infected cells, rather than virus replication and re-infection,[25] although the mechanisms remain to be delineated. As to the virus genome, no disease-specific changes in nucleotide sequence have been found in proviruses isolated from patients with HTLV-1-related diseases and asymptomatic carriers.[31–34] The existence of a significant difference in the provirus load between patients after GD and those without GD was statistically confirmed. The autoimmune background of the patients does not appear to be necessarily associated with an increased provirus load, because patients with Sjögren's syndrome who were infected with HTLV-1 did not have an increased provirus load.[35] It may be relevant that we have found activation of the HTLV-1 long terminal repeat by thyroid hormone *in vitro* (unpublished observation), since this could contribute to clonal expansion of the infected cells through induction of the viral transcriptional transactivator Tax. The effects of MMI on clonal expansion of the virus-infected cells remain to be investigated, although recent reports do not suggest a direct effect of MMI on T cells.[36, 37]

The present work has shown that the provirus load is correlated with disease activities. This observation linked the provirus load to clinical parameters for the first time, providing support for the idea that the circulating HTLV-1-infected T cells are directly involved in the pathogenesis of HU. One possible explanation for the way in which the increased provirus load is associated with disease activities would be that the larger number of HTLV-1-infected cells would provide more chances for them to infiltrate into tissues, because adhesion molecules and inflammatory cytokines that activate vascular endothelial cells are aberrantly upregulated and constitutively expressed in HTLV-1-infected cells.[38–44] This notion is consistent with our observation of predominant T lymphocyte infiltration and accumulation of HTLV-1-infected cells in the affected eye of patients with HU.[6]

The correlation between the HTLV-1 provirus load and serum antibody has been reported previously.[20–23] In the

present study, the provirus load showed a correlation with the serum level of HTLV-1 antibody, which was quantitatively measured with the ECLIA method. The positive correlation between them was also confirmed by nonparametric analysis. The results provided further evidence for the idea that an increased number of HTLV-1-infected cells would lead to increased challenge by virus antigens expressed in amounts proportional to the number of virus-infected cells,[45] resulting in increased antibody response. In the HU patients after GD, however, no correlation was found between the provirus load and serum antibody (data not shown). This could be explained by administration of MMI.[29, 30] The results raise another possibility, i.e., that poor antibody response in the HU patients after GD might provide a better condition for reinfection of the virus, since serum antibody was shown to have a neutralizing activity.[46–48] However, this is not consistent with the clonality of the HTLV-1-infected cells in PBMC as discussed above.

The provirus load showed a significant correlation with the serum level of sIL-2R in the HU patients without GD, but not in those after GD (data not shown). Although previous reports suggested that the level of sIL-2R correlates with the number of HTLV-1-infected cells,[24, 49] this is the first demonstration of the correlation based on the quantitative analysis of provirus load, and provides supporting evidence for the interpretation that the elevated level of sIL-2R in the HTLV-1-infected individuals is due to constitutive production by the HTLV-1-infected cells.[50, 51] On the other hand, Koukkou *et al.* reported that sIL-2R is elevated in hyperthyroid patients, irrespective of the etiology, and that the level of sIL-2R was downregulated by administration of MMI, probably through normalizing the level of thyroid hormones.[52] Thus, our findings in the HU patients after GD in the present study are consistent with these observations and the interpretation thereof. Treatment with MMI would explain the lack of these correlations in the HU patients after GD, because, besides normalizing the thyroid status, MMI is reported to have suppressive effects on B and T cells *in vivo*,[29, 30] although the mechanisms involved are still unknown.

Taken together, the results in the present study underline the importance of the increased provirus load in the pathogenesis of inflammatory diseases caused by HTLV-1, and suggest a possible mechanism for the increased provirus load *in vivo* and direct involvement of the HTLV-1-infected cells in the pathogenesis of HU.

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