cells or $CD8^+$ T cells and T cell responsiveness to mitogens (Con A and PHA) were not significantly changed after 3 weeks of oral intake of LcS preparation containing approximately 4×10^{10} viable LcS. They also showed that the NK cell activity was significantly increased, and the enhancement of NK cell activity was particularly prominent in the low-NK cell activity individuals. Interestingly, our HAM/TSP case with most obvious clinical improvement (HAM 4) showed the lowest NK activity of all 10 patients before LcS treatment. However, another HAM/TSP case (HAM6) with low levels of NK activity before LcS treatment also showed the marked enhancement of NK cell activity after LcS but this patient did not show the obvious clinical improvement as seen in HAM4. Therefore, the level of enhanced NK cell activity after treatment did not always correlate with the level of clinical improvement. Since both of these patients (HAM 4 and 6) received low dose oral prednisolone throughout study period, this may also suggest that the concomitant administration of prednisolone *is* not specifically associated with the clinical outcome against LcS administration.

In general, probiotic bacteria such as LcS favorably alter the intestinal microflora balance, inhibit the growth of harmful bacteria, promote good digestion, and may boost immune function, and possibly increase resistance to infection. Although the mechanism of increased NK cell activity after oral administration of LcS is not clear, several studies have reported an effect of LcS on the stimulation of phagocytic activity of circulating immune cell populations which participate in innate immunity. Stimulated phagocytic cells may also play a role in potentiate acquired immunity via presentation of the antigen or synthesis of cytokines. Previous histological examination in an animal study showed that LcS was taken up by M cells in Peyer's patches [40], suggesting that LcS may be degraded in gut-associated lymphoid tissue and their signal from immuno-competent cells leads to a systemic effect such as stimulation of phagocytosis, synthesis of cytokines, IgA secretion, enhancement of NK cell activity, etc. Our data indicates that NK cell numbers were not significantly changed after LcS treatment. Also, we could not detect any significant changes of frequencies and absolute numbers of all the cell phenotypes examined, including the NK inhibitory receptor NKG2A positive cells and γδT cells. It is therefore likely that increased NK cell activity might be regulated by increased expression levels of cytotoxic molecules—rather than the changes of activating receptors and/or inhibitory receptors on NK cells—although further studies are necessary to clarify this point.

Previous studies have indicated that therapies effective for HAM/TSP such as prednisolone [15,16], plasma exchange [17], and interferon-α [18–22] have problems associated with a high frequency of adverse effects; furthermore, IFN-α and plasmapheresis usually require hospitalization, are very expensive and the clinical effects usually last only a few months. Since HAM/TSP is a chronic and progressive disorder, establishment of a chronic and safe treatment is essential. Also, innate immunity is thought to be important in protecting the host against many viral infections [41], and a combination treatment of acquired immunity oriented agents like IFN-α and innate immunity oriented agents like LcS might be a more desirable approach for HAM/TSP treatment. Since previous reports have indicated that the NK cell activity was significantly decreased in HAM/TSP patients by an unknown mechanism [32,33], the augmentation of a decreased host innate immune system by LcS might be a suitable approach for treating HAM/TSP.

In conclusion, our present results suggest that oral LcS may be comparatively effective, easy and safe, and is therefore a good candidate for maintenance treatment for HAM/TSP. However, in our present study, the number of patients is small and the study was conducted in an open, uncontrolled manner. Randomized controlled studies are warranted for the evaluation of LcS in HAM/TSP treatment.

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Human T Cell Leukemia Virus Type I-Infected Patients with Hashimoto's Thyroiditis and Graves' Disease

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Context: Autoimmune thyroid diseases have been reported to be associated with human T cell leukemia virus type I (HTLV-I) infection. HTLV-I proviral load is related to the development of HTLV-I-associated myelopathy/tropical spastic paraparesis and has also been shown to be elevated in the peripheral blood of HTLV-I-infected patients with uveitis, arthritis, and connective tissue disease.

Objective: The objective of the study was to evaluate the proviral load in HTLV-I-infected patients with Hashimoto's thyroiditis (HT) or Graves' disease (GD) and ascertain the ability of HTLV-I to infect thyroid cells.

Patients and Methods: A quantitative real-time PCR assay was developed to measure the proviral load of HTLV-I in peripheral blood mononuclear cells from 26 HTLV-I-infected patients with HT, eight HTLV-I-infected patients with GD, or 38 asymptomatic HTLV-I carriers. Rat FRTL-5 thyroid cells were cocultured with HTLV-I-infected T cell line MT-2 or uninfected T cell line CCRF-CEM. After coculture with T cell lines, changes in Tax and cytokine mRNA expression were studied by RT-PCR.

Results: HTLV-I proviral load was significantly higher in the peripheral blood of patients with HT and GD than asymptomatic HTLV-I carriers. In the peripheral blood from HTLV-I-infected patients with HT, HTLV-I proviral load did not correlate with the thyroid peroxidase antibody or thyroglobulin antibody titer. After coculture with MT-2 cells, FRTL-5 cells expressed HTLV-I-specific Tax mRNA. These cocultured FRTL-5 cells with MT-2 cells expressed IL-6 mRNA and proliferated more actively than those cocultured with CCRF-CEM cells.

Conclusion: Our findings suggest the role of the retrovirus in the development of autoimmune thyroid diseases in HTLV-I-infected patients. (*J Clin Endocrinol Metab* **90: 5704–5710, 2005**)

HUMAN T CELL LEUKEMIA virus type I (HTLV-I) is a human retrovirus highly endemic in southern Japan, intertropical Africa, Melanesia, Latin America, and the Caribbean basin (1). HTLV-I is the etiological agent of adult T cell leukemia (ATL) (2) and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), an inflammatory disease of the central nervous system (3, 4), and has also been implicated in several other inflammatory disorders, such as uveitis (5), chronic arthropathy (6), pulmonary alveolitis (7), and Sjögren's syndrome (8). Furthermore, transgenic mice expressing Tax protein, a transactivator encoded by HTLV-I, develop proliferative synovitis (9) and exocrinopathy affecting lacrimal and salivary glands, features similar to those of Sjögren's syndrome in humans (10).

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Abbreviations: ATL, Adult T cell leukemia; CTLA-4, cytotoxic T lymphocyte antigen-4; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GD, Graves' disease; HAM/TSP, human T cell leukemia virus type I-associated myelopathy/tropical spastic paraparesis; HT, Hashimoto's thyroiditis; HTLV-I, human T cell leukemia virus type I; MMC, mitomycin C; PBMC, peripheral blood mononuclear cell; Tg, thyroglobulin; TPO, thyroid peroxidase; TRAb, TSH receptor antibody; WST, water-soluble tetrazolium salt.

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The possibility that HTLV-I may cause thyroid diseases was initially raised by reports of Hashimoto's thyroiditis (HT) in HTLV-I carriers and patients with HAM/TSP (11, 12). Graves' disease (GD) has also been observed in HTLV-I carriers (13, 14). Epidemiological studies have demonstrated that HTLV-I seropositivity is a risk factor for thyroid disorders in Japan. Kawai *et al.* (12) reported that the prevalence of HTLV-I antibody in HT patients resident of Tokushima and Kochi prefectures, Japan, was 6.3%, which was significantly higher than the expected frequency of 2.2%. Mizokami *et al.* (15) also reported that the prevalence of HTLV-I antibody was significantly higher in patients with either antithyroid antibody-positive chronic thyroiditis or GD than the expected frequency in Fukuoka prefecture, Japan. Mine *et al.* (16) found that the frequency of antithyroid antibodies in blood donors with HTLV-I antibody was significantly higher than that in control donors without the antibody. Akamine *et al.* (17) also found a high prevalence of positivity for thyroid autoantibodies in ATL patients and HTLV-I carriers.

Several findings support the hypothesis of the etiopathogenic role of HTLV-I in thyroid diseases: HTLV-I envelope protein and Tax mRNA have been detected in follicular epithelial cells of the thyroid tissues of a patient with HT (18); Tax mRNA was also found in infiltrating lymphocytes in the interfollicular space (18); and HTLV-I proviral DNA and

HTLV-I have been detected in thyroid tissues of patients with HT and GD (18, 19).

T lymphocytes, especially CD4+ T cells, are the main target of HTLV-I *in vivo* and carry the majority of the HTLV-I proviral load (20). The HTLV-I proviral load in peripheral blood mononuclear cells (PBMCs) is higher in patients with HAM/TSP than asymptomatic HTLV-I carriers (21), and the equilibrium set point of the proviral load is suspected to determine the development of the disease (22). We postulated that HTLV-I proviral load also influences the initiation and course of autoimmune thyroid diseases. To test our hypothesis, we measured this marker in PBMCs from HTLV-I-infected patients with HT and GD. To better understand the pathogenic mechanisms of HTLV-I-associated thyroid disorders, we determined whether HTLV-I could infect thyroid cells, and we characterized cell proliferation and cytokine gene expression in these cells after HTLV-I infection, using FRTL-5 rat thyroid cells.

Patients and Methods

Clinical samples

Blood samples were collected from 116 HTLV-I-infected patients, 38 asymptomatic carriers (33 females and five males, 21–79 yr old), 26 patients with HT (19 females and seven males, 37–80 yr old), eight patients with GD (seven females and one male, 40–59 yr old), 21 patients with HAM/TSP (17 females and four males, 31–74 yr old), and 23 patients with ATL (18 females and five males, 44–87 yr old). The diagnosis of HT was based on the presence of positive thyroid autoantibodies [thyroid peroxidase (TPO) and/or thyroglobulin (Tg)] and at least one of two additional criteria (hypothyroidism and/or goiter). Antibodies to TPO and Tg were determined by RIAs using commercially available kits (Cosmic, Tokyo, Japan). The patients with HT were treated with L-thyroxine. GD was diagnosed on the basis of history and signs of hyperthyroidism with diffuse goiter and the laboratory findings, including elevated serum T_4 and T_3 concentrations, undetectable serum TSH, and positive TSH receptor antibody (TRAb). TRAb was measured as TSH binding inhibitory Ig. One patient had ophthalmopathy. The patients with GD were treated with methimazole or propylthiouracil. Diagnosis and classification of the clinical subtypes of ATL were made based on the criteria of the Lymphoma Study Group (23) and were then confirmed in all cases by Southern blot hybridization analysis with detection of monoclonal integration of HTLV-I provirus into the genome. Diagnosis of HAM/TSP was based on the World Health Organization diagnosis guidelines (24). PBMCs donated by HTLV-I-seronegative healthy individuals (one female and two males, 25–29 yr old) served as normal controls. These control subjects did not have a history of thyroid or autoimmune diseases. PBMCs were isolated from heparinized blood by density gradient centrifugation. Seropositivity for HTLV-I was obtained by ELISA and particle agglutination assays. The screening of serum HTLV-I antibody was studied in all patients who visited our clinic at the University of the Ryukyus. All patients, HTLV-I asymptomatic carriers, and HTLV-I-seronegative healthy controls were Japanese, and they were living in Okinawa and Kagoshima prefectures (HTLV-I endemic areas), Japan. All individuals gave written informed consent for their participation.

Measurement of HTLV-I proviral load

DNA was prepared from each sample using a blood and tissue genomic DNA minikit, according to the protocol recommended by the manufacturer (Viogene-Biotek Corp., Hsichih, Taiwan) and stored at −80 C until use. The concentration of extracted DNA was adjusted to 10 ng/μl of the working solution. A quantitative real-time PCR assay was developed to measure the proviral load of HTLV-I in PBMCs. The HTLV-I copy number was referenced to the actual amount of cellular DNA by quantification of β-actin gene. The forward and reverse primers used for HTLV-I pX region were 5′-CAAACCGTCAAGCACAGCTT-3′ positioned at 7140–7159 and 5′-TCTCCAAACACGTAGACTGGGT-3′ positioned at 7362–7341. The internal HTLV-I pX TaqMan probe (5′-TTCCCAGGGTTTGGACAGAGTCTTCT-3′) was located between positions 7307 and 7332 of the genome, and carried a 5′ reporter dye FAM (6-carboxy fluorescein) and a 3′ quencher dye TAMRA (6-carboxy tetramethyl rhodamine). To quantify the human β-actin gene, the forward and reverse primers 5′-TCACCCACACTGTGCCCATCTACGA-3′ positioned at 2141–2165 and 5′-CAGCGGAACCGCTCATTGCCAATGG-3′ positioned at 2435–2411, and the β-actin TaqMan prove (5′-ATGCCCTC-CCCCATGCCATCCTGCGT-3′ positioned at 2171–2196) were used. PCR was performed with 5 μl DNA template with the use of the TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA) and target gene assay mix containing each respective forward and reverse primer and TaqMan probe. The PCR conditions were as follows: 1 cycle at 50 C for 2 min and 95 C for 10 min and 45 cycles of denaturation at 95 C for 15 sec and annealing/extension at 58 C for 1 min. PCR was carried out in triplicate for each sample. HTLV-I provirus DNA cloned into the plasmid served as the control template and the β-actin gene as the internal control. Data were quantified as mean values from the relative standard curve according to the instructions provided by the manufacturer (Applied Biosystems). Cycle numbers obtained at the log-linear phase of the reaction were plotted against a standard curve prepared with serially diluted control samples. The amount of HTLV-I proviral DNA was calculated by the following formula: copy number of HTLV-I (pX) per 1×10^4 PBMCs = [(copy number of pX)/(copy number of β-actin/2)] $\times 10^4$.

Cell culture and HTLV-I infection in vitro

FRTL-5 cells are a continuous line of rat thyroid cells and were grown in the Coon's modified Ham's F-12 medium containing 5% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS) with the addition of a mixture of six hormones: bovine thyroid-stimulating hormone (10 mU/ml), transferrin (5 μg/ml), somatostatin (10 ng/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), hydrocortisone (10 nM), and insulin (10 μg/ml). All hormones were purchased from Sigma-Aldrich (St. Louis, MO). MT-2 cells, obtained by coculturing peripheral leukemic cells from an ATL patient with normal umbilical cord leukocytes (25), were used as an HTLV-I-infected T cell line. MT-2 cells contain proviral HTLV-I DNA and produce viral particles. CCRF-CEM cells were used as the uninfected T cell line. These T cells were treated with 100 μg/ml mitomycin C (MMC) for 1 h at 37 C. After washing three times with PBS, they were cultured with an equal number of FRTL-5 cells in Coon's modified Ham's F-12 medium containing 5% FBS. The culture medium was changed on the third day after coculture. FRTL-5 cells were harvested at 3 and 7 d, followed by RNA extraction as described below.

RT-PCR

Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer, and the amount of total RNA was determined by measuring absorbance at 260 nm. First-strand cDNA was synthesized from 5 μg total cellular RNA in a 20-μl reaction volume using an RNA PCR kit (Takara Shuzo, Kyoto, Japan) with random primers. Thereafter cDNA was amplified using a multiplex PCR kit for rat inflammatory cytokine gene (Maxim Biotech, Inc., San Francisco, CA) according to the instructions provided by the manufacturer. Product sizes were 351 bp for TNFα, 294 bp for IL-1β, 453 bp for IL-6, 250 bp for TGFβ, and 532 bp for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression levels of Tax and β-actin mRNAs were analyzed as described previously (26). Product sizes were 203 bp for Tax and 548 bp for β-actin. PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining.

Cell proliferation assay

FRTL-5 cells (1×10^4 cells/well) were cultured with or without MMC-treated MT-2 or CCRF-CEM (1×10^4 cells/well) cell line in 96-well culture plates in Coon's modified Ham's F-12 medium containing 5% FBS for 1, 3, or 5 d. The data were obtained by triplicate experiments. Four hours before terminating the culture, 10 μl of the cell proliferation reagent water-soluble tetrazolium salt (WST)-8, a tetrazolium salt (Wako Chemicals, Osaka, Japan) were added to each well. At the end of incubation, absorbance at 450 nm was measured using an automated microplate reader. Measurement of the mitochondrial dehydrogenase-

mediated cleavage of WST-8 to formazan dye indicates the level of proliferation.

Statistical analysis

Data are expressed as mean ± SD. Statistical significance was analyzed by Mann-Whitney *U* test. The Spearman's rank correlation coefficient was used to describe the association between different variables. The Student's *t* test was performed for comparisons of growth of uninfected FRTL-5 cells and that of HTLV-I-infected FRTL-5 cells.

Results

Quantification of HTLV-I proviral DNA in asymptomatic HTLV-I carriers, HTLV-I-infected patients with HT or GD, HAM/TSP, and ATL

As shown in Fig. 1, we estimated the absolute copy number of HTLV-I proviral DNA per 10^4 PBMCs. First, proviral load was quantified in three healthy volunteers (seronegative), 21 HAM/TSP patients, and 23 ATL patients. The provirus was undetectable in all healthy noncarriers (Fig. 1B), whereas HAM/TSP and ATL patients were positive for HTLV-I with a proviral load of 1986 ± 198 copies (range 879-4137 copies) and 2791 ± 320 copies (range 874-6175 copies), respectively (Fig. 1A). The provirus loads in smoldering-, chronic-, acute-, and lymphoma-type ATL patients were 1561 ± 268, 2683 ± 782, 3098 ± 468, and 3248 ± 893, respectively. The copy numbers in asymptomatic carriers varied from 0.4 to 347, those of HTLV-I-infected patients with HT varied from 2 to 1076, and those of HTLV-I-infected patients with GD varied from 29 to 1222 (Fig. 1B). The mean ± SD and median of the copy number was 60 ± 11 and 39 in asymptomatic carriers. With regard to HTLV-I-infected patients with HT and GD, the values were 276 ± 53 (median 199) and 303 ± 137 (median 200), respectively. The median copy number of HTLV-I-infected patients with HT and GD was about 5-fold higher than that of asymptomatic carriers. The differences were statistically significant between asymptomatic carriers and HTLV-I-infected patients with HT and between asymptomatic carriers and HTLV-I-infected patients with GD, respectively (Mann-Whitney *U* test) (Fig. 1B). There was no significant correlation between copy number of HTLV-I proviral DNA and antibody titer of either Tg (P = 0.6535) or TPO (P = 0.4703) in HTLV-I-infected patients with HT (Spearman's rank correlation) (Fig. 2). Among the HTLV-I-infected patients with GD, the correlation between copy number of HTLV-I proviral DNA and TRAb titer was not observed (data not shown).

Detection of HTLV-I Tax mRNA in FRTL-5 cells cocultured with HTLV-I infected T cells

FRTL-5 cells were cocultured with either MT-2 or CCRF-CEM cells. After cocultivation for 3 d, FRTL-5 cells were washed extensively and exchanged with fresh medium. After the cells were cultured for further 4 d, they were washed thoroughly. At 3 and 7 d after cocultivation, FRTL-5 cells were harvested for assessment by RT-PCR for expressing HTLV-I viral antigen. Because T cell lines were pretreated extensively with MMC, these MMC-treated T cells could not proliferate, as determined by WST-8 assay. These specimens of FRTL-5 cell at 3 and 7 d of culture contained no viable MT-2 cells. As shown in Fig. 3A, FRTL-5 cells cocultured with MT-2 cells showed strong expression of Tax mRNA. In contrast, FRTL-5 cells cocultured with CCRF-CEM cells did not express Tax mRNA. To determine whether the Tax cDNA sequence was amplified from residual MT-2 cells that had been added after MMC treatment, PCR amplification of a human PTHrP exon 3 sequence was done, using these DNA samples. The human PTHrP sequence was amplified from MT-2 DNA by PCR. However, the human PTHrP sequence was not detected in any of the cocultured rat FRTL-5 cells, which suggests that residual MT-2 cells in these samples were not amplified (data not shown). These results suggest that the HTLV-I can be transmitted into FRTL-5 cells from HTLV-I producing MT-2 cells.

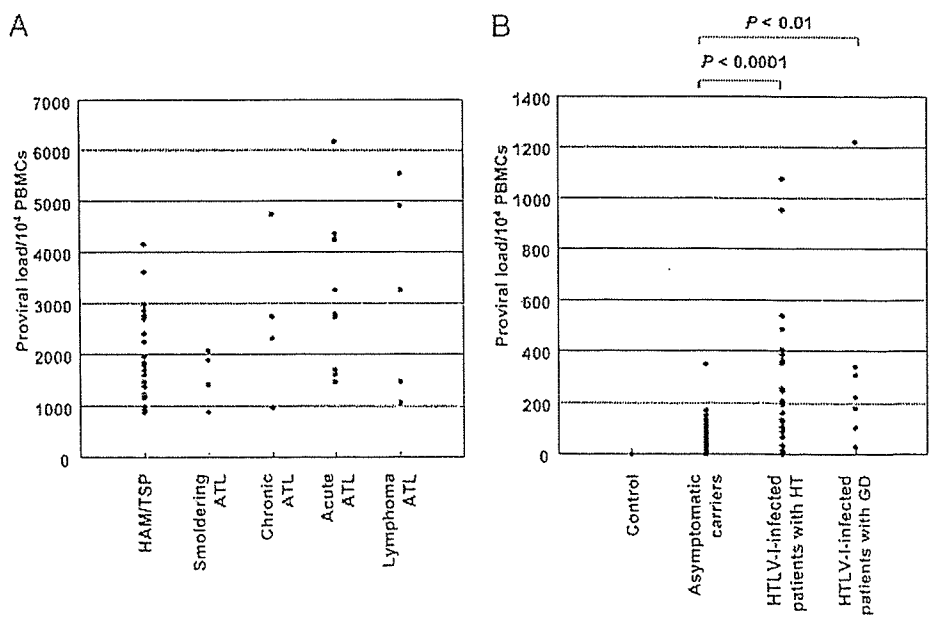

FIG. 1. HTLV-I proviral load in the peripheral blood of HAM/TSP and ATL (A) and healthy individuals without HTLV-I, asymptomatic carriers, and HTLV-I-infected patients with HT or GD (B). Data are HTLV-I copy number per 10^4 PBMCs.

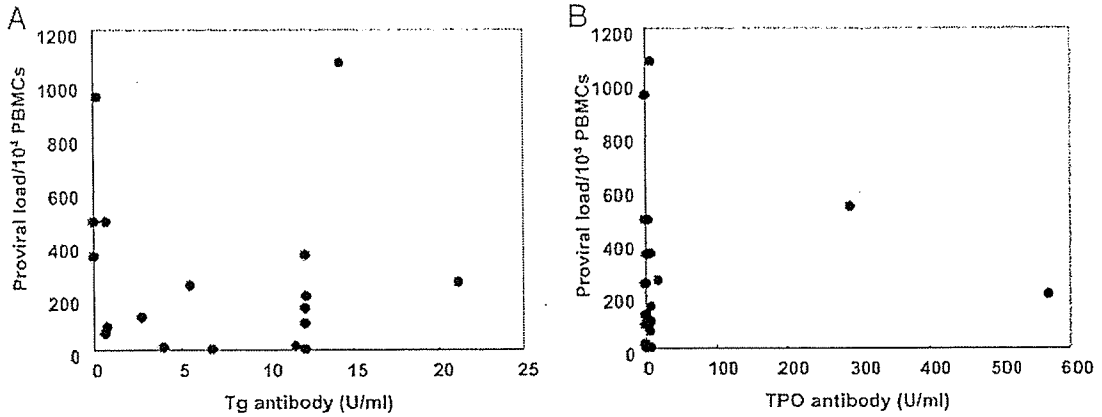

FIG. 2. Correlation between HTLV-I proviral load and antibody titer of Tg (A) or TPO (B) in HTLV-I-infected patients with HT. There was no significant correlation between copy number of HTLV-I proviral DNA and antibody titer of either Tg (P = 0.6535) or TPO (P = 0.4703).

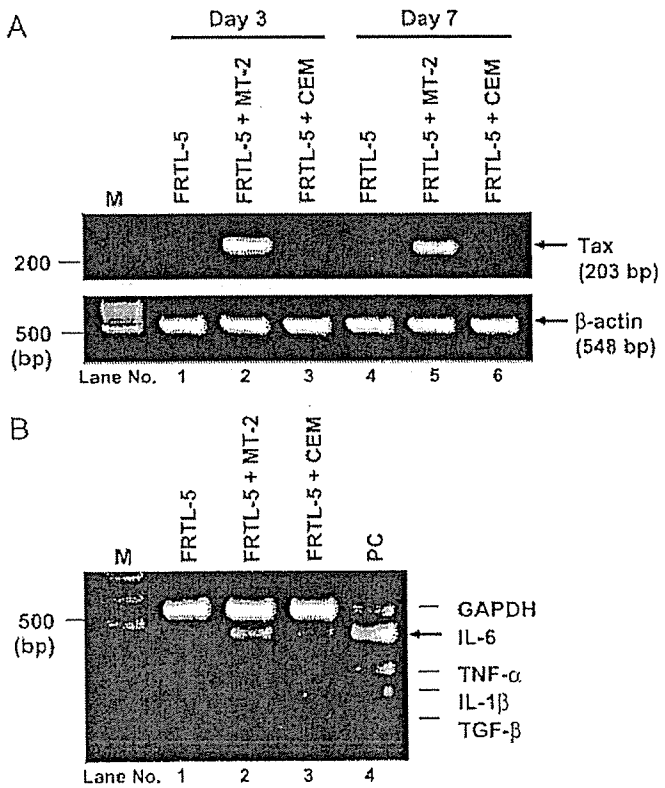

FIG. 3. HTLV-I can infect FRTL-5 cells and induce gene expression of IL-6. A, Detection of HTLV-I Tax mRNA in FRTL-5 cells by RT-PCR. FRTL-5 cells were cocultured with MMC-treated MT-2 or CCRF-CEM cells. At 3 and 7 d after cocultivation, FRTL-5 cells were harvested and then Tax mRNA expression was analyzed. Lane 1, cultured FRTL-5 cells at 3 d; lanes 2 and 3, FRTL-5 cells cocultured with MT-2 and CCRF-CEM cells at 3 d; lane 4, cultured FRTL-5 cells at 7 d; lanes 5 and 6, FRTL-5 cells cocultured with MT-2 and CCRF-CEM cells at 7 d. Human β-actin mRNA was used as a control. B, Induction of expression of IL-6 gene in FRTL-5 cells. Lane 1, cultured FRTL-5 cells at 3 d; lanes 2 and 3, FRTL-5 cells cocultured with MT-2 and CCRF-CEM cells at 3 d; lane 4, positive control. Rat GAPDH mRNA was used as a control.

Cytokine expression in FRTL-5 cells cocultured with MT-2 or CCRF-CEM cells

Tax activates not only the transcription of the viral genome but also the expression of various cellular genes. It is now clear that HTLV-I-infected T cells are capable of producing various cytokines through the transactivation of cytokine genes by the Tax protein (27). HTLV-I-infected nonlymphoid cells have also been reported to express various types of cytokines (28, 29). Therefore, we investigated the expression of inflammatory cytokines in FRTL-5 cells cocultured with MT-2 or CCRF-CEM cells by RT-PCR. RT-PCR was carried out with primer sets for IL-1β, IL-6, TNFα, and TGFβ as well as rat GAPDH. As shown in Fig. 3B, low levels of expression of IL-6 and TGFβ mRNA were detected in control FRTL-5 cells. The level of expression of IL-6 was increased in FRTL-5 cells cocultured with MT-2 cells but not in FRTL-5 cells cocultured with CCRF-CEM cells. Transcripts of IL-1β and TNFα were not detected in any of the samples.

Proliferation of FRTL-5 cells

It was reported previously that HTLV-I could infect synovial cells, resulting in their active proliferation (28). Finally, to investigate the relation of thyroid cell proliferation and HTLV-I infection, the proliferative response of FRTL-5 cells was examined by cocultivation with MT-2 cells and compared with that of FRTL-5 cells cocultured with CCRF-CEM cells using the WST-8 assay as an index of cell number. The proliferation of FRTL-5 cells at d 1, 3, and 5 was significantly increased by coculture with HTLV-I-infected T cells (Fig. 4). It was noted that MMC-treated MT-2 and CCRF-CEM cells could not proliferate.

Discussion

Although the etiology of autoimmune thyroid diseases has yet to be established, it appears to result from complex interactions between host genetic and environmental factors. The involvement of viral infection, particularly retrovirus infection, in the pathogenesis of autoimmune thyroid diseases has been demonstrated in animals and humans (30–34). However, the role of infection in precipitating autoimmune thyroid diseases remains largely hypothetical (34).

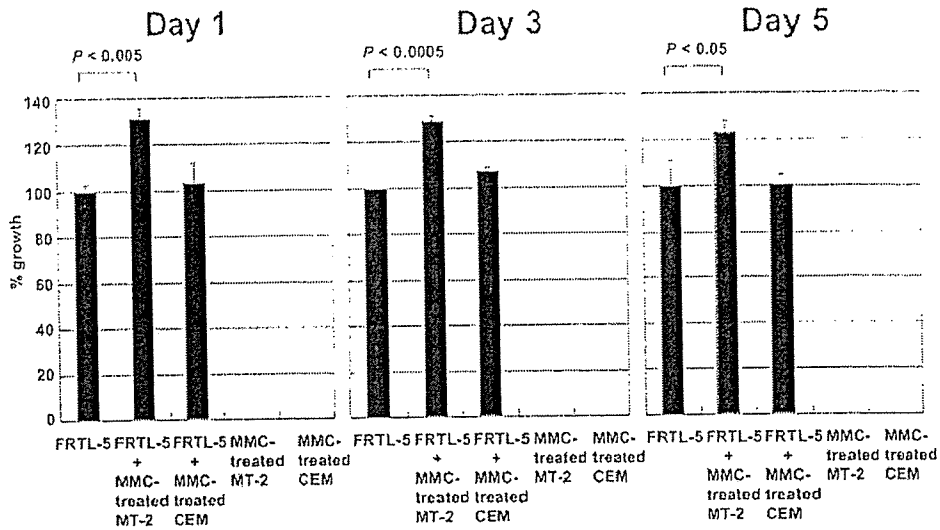

FIG. 4. Proliferation of FRTL-5 cells cocultured with MT-2 or CCRF-CEM cells. FRTL-5 cells were cultured in the presence or absence of MMC-treated MT-2 or CCRF-CEM cells for the indicated time periods. Four hours before terminating the culture, WST-8 was added to each well, and absorbance at 450 nm was measured. Data are expressed as percentage growth, compared with the uninfected FRTL-5 cells and represent the mean ± SD of triplicate measurements. MMC-treated MT-2 and CCRF-CEM cells could not proliferate.

Ciampolillo *et al.* (31) reported the presence of a HIV type 1-related DNA sequence in the thyroid and PBMCs of the patients with GD, but this finding was disputed by Humphrey *et al.* (35) and Tominaga *et al.* (36). Jaspan *et al.* (37) reported that over 85% of patients with GD have detectable serum antibodies against a human intracisternal type A retroviral particle. Furthermore, data from 35 members of three kindreds suggest that both human leukocyte antigen susceptibility and exposure to the retroviral particle are necessary for the development of GD (38). HTLV-I is considered to be implicated in the pathogenesis of autoimmune thyroid diseases in Japan, where this retrovirus is endemic, and epidemiological studies have shown an association between HTLV-I infection and thyroid disorders in Japan (12, 15–17).

The present study provides biological data suggesting the contribution of HTLV-I in the development of autoimmune thyroid diseases. Our results showed that: 1) the circulating HTLV-I proviral load was higher in HTLV-I-seropositive patients with HT or GD than asymptomatic HTLV-I carriers and lower than that in patients with HAM/TSP or ATL; 2) HTLV-I can be transmitted into thyroid cells from an HTLV-I-producing T cell line; 3) HTLV-I infection induced expression of IL-6 gene but not IL-1β, TNFα, and TGF-β in thyroid cells; and 4) HTLV-I-infected thyroid cells proliferated more actively than control cells.

The HTLV-I proviral load is thought to be a major determinant of HTLV-I-associated diseases. The proviral load is higher in the peripheral blood of patients with HAM/TSP than blood of asymptomatic carriers (21), as confirmed in the present study. It is also higher in the peripheral blood of patients with HTLV-I-associated uveitis and HTLV-I-seropositive patients with arthritis or connective tissue disease than asymptomatic carriers (39, 40). Similarly, we observed a significantly higher proviral load in HTLV-I-infected patients with either HT or GD than in HTLV-I asymptomatic carriers. Thus, a high proviral load might be involved in the pathogenesis of several other HTLV-I-associated inflammatory disorders in addition to HAM/TSP.

The unusually high proviral loads in HTLV-I infection results mainly from the Tax-driven activation and expansion of infected cells (41). The HTLV-I targets are mainly CD45RO-expressing CD4+ T lymphocytes, and the proviral load is reported to correlate with the number of memory T cells (42). Migration of HTLV-I-infected CD4+ T cells and HTLV-I-specific CD8+ cytotoxic T lymphocytes into the central nervous system is a critical step in the pathogenesis of HAM/TSP (43). Similarly, infiltration of lymphocytes plays a central role in the initiation and perpetuation of autoimmune thyroid diseases. Previous studies showed a good correlation between the degree of intrathyroidal lymphocytic infiltration and antithyroid antibody titer not only in HT (44) but also in GD (45). Although the accumulation of HTLV-I-infected T cells in the thyroid remains uncertain, HTLV-I proviral load did not correlate with antibody titer of either TPO or Tg in our study with HT. Further research using thyroid tissue from HTLV-I-infected patients is needed to support the hypothesis of the pathogenic involvement of HTLV-I-infected T lymphocytes.

HTLV-I might be transmitted from infiltrated lymphocytes to thyrocytes. We obtained evidence that thyroid cells can be infected by HTLV-I and that this infection induced gene expression of inflammatory cytokine IL-6 *in vitro*. HTLV-I Tax mRNA was detected in the FRTL-5 cells cocultured with MT-2 cells. Transcription of IL-6 is regulated by Tax protein in T cells and synovial cells (46, 47). Although the precise role of IL-6 in the pathogenesis of thyroid diseases is unknown, these results suggest the involvement of IL-6 expression in thyroid cells, which is related to Tax, in the development of inflammatory lesions caused by HTLV-I infection in the thyroid. To clarify the pathological association of thyroiditis with HTLV-I, we are attempting to detect HTLV-I proviral DNA and viral gene expression in the tissue of HTLV-I-associated thyroiditis.

The effect of HTLV-I infection on FRTL-5 growth was assessed by the WST-8 assay. Coculture with MT-2 cells increased the rate of cell proliferation. Because these effects were not observed in FRTL-5 cells cocultured with CCRF-CEM cells, they support the specific effect of HTLV-I infection on thyroid cell growth. Although several cytokines are known to modulate the proliferation of FRTL-5 cells, IL-6 had

no significant effects on the cell growth (48). Because Tax can stimulate cell growth, the active proliferation of HTLV-I-infected thyroid cells may be related to Tax expression, and goiter in patients with autoimmune thyroid diseases may be regulated by HTLV-I infection.

HTLV-I might cause a systemic immune-mediated inflammatory disease potently involving tissues other than the central nervous system, HAM/TSP being only the major syndrome. The pathological association of HTLV-I with autoimmune thyroid diseases in HTLV-I carriers still remains to be clarified. It should be noted that HTLV-I infection is not the sole cause of autoimmune thyroid diseases because HTLV-I antibody was not present in the majority of the cases. Genetic factors, involved in autoimmune thyroid diseases, include human leukocyte antigen and cytotoxic T lymphocyte antigen-4 (CTLA-4) (49, 50). It has been shown that HTLV-I infection is not associated with CTLA-4 polymorphisms in either HT or controls (51). HTLV-I infection is not regulated by genetic factor such as CTLA-4 and may affect occurrence of HT as an independent, purely environmental factor. Further studies on the effects of HTLV-I infection of thyroid tissues should help elucidate the pathobiology and pathogenesis of HTLV-I-associated thyroid diseases.

Acknowledgments

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特集

HAMの診断と治療の進歩

松﨑敏男[1)]/斉藤峰輝[1)]/納　光弘[2)]

〔KEYWORDS〕 HAM，診断，治療，HTLV-1プロウイルス量

1．はじめに

HTLV-1(human T lymphotropic virus type 1)は，主にCD4陽性Tリンパ球に感染するヒトレトロウイルスの一種である．HTLV-1-associated myelopathy(HAM)は，HTLV-1無症候性感染者(キャリアー)の一部から発症する慢性の膀胱直腸障害を伴う痙性脊髄麻痺であり，1986年に納，井形により新しい疾患単位として提唱された[1)]もので，後にカリブ海沿岸を中心に報告された熱帯性痙性脊髄麻痺(TSP)の一部と同一の疾患であることが確認された[2)]．HAM発見後すでに19年が経過し，この間に臨床像[3~5)]，発症病態[6~8)]，治療[9)]について精力的に研究が進められた．本稿では，特にHAMの臨床診断，検査所見，治療指針について，自験例をもとに最近の知見を加えて述べる．

2．HAMの現況

1993年のHAM全国調査では，HAM患者の実数は1,062名(そのうち九州700，関東33，北海道49)であった[10)]．その後，HAM患者の実数は1999年4月鹿児島で行われたHTLV国際学会のワークショップにおいて，世界で3,000人＋α，日本で1,422人と報告された[11)]．現在，鹿児島大学病院神経内科に登録されている患者数は495人である．HAM患者は，HTLV-1キャリアーの多い地域に広く分布しており，日本(特に九州，沖縄と四国，東北，北海道の一部)のほか，世界的にはカリブ諸島，アフリカ，イラン北東部，ヨーロッパの一部(ほとんどは感染地域からの移民)などに多くみられる．

3．HAMの発症形式について

男女比は1：2.3と女性に多い．平均発症年齢は45.1±16.5歳である．主な感染経路は母乳による母子垂直感染で，そのほか輸血，夫婦間伝播(ほとんど男性から女性)などがある．自験例では，若年発症(発症年齢15歳以下)は21例(4.2%)，高齢発症(同65歳以上)は74例(14.9%)で，輸血後発症は39例(7.9%)であった．家族内発症は17家族においてみられた．年間生涯発症率は無症候性キャリアー全体の0.23%である[12)]．

4．HAMの診断

1）臨床症状

HAMの診断指針を表1に示す[13)]．初発症状として歩行障害，排尿障害，しびれ感，腰痛，便秘，感覚鈍麻，手指振戦の順に多くみられる．運動障害は足の筋肉のつっぱり(痙性)による歩行障害が主なもので(痙性対麻痺，痙性歩行)，内反足となる．しだいに痙性が強くなると，小走りができない，階段昇降困難，手すり歩行，車椅子移動と症状が進行していく．歩行障害の悪化と筋力低下に伴って下肢の筋萎縮を認めることもある．排尿障害は夜間頻尿に始まり，日中の頻尿(2時間以内の排尿)，残尿，尿失禁などがみられる．残尿のため，しだいに腹部の圧迫排尿，自己導尿となり，最終的に膀胱瘻の造設を余儀なくされる症例もある．尿意がなく尿閉で発症した症例も報告されている．感覚障害は異常知覚，感覚鈍麻が主

1）MATSUZAKI Toshio, SAITO Mineki　鹿児島大学大学院医歯学総合研究科 神経病学講座神経内科・老年病学
2）OSAME Mitsuhiro　同・教授

表1　HAM/TSP診断のためのWHO診断指針(1990)

Ⅰ．臨床診断
　慢性痙性対麻痺の多彩な臨床像が初診時からそろっているとは限らず，発症初期のHAM/TSPでは単一の徴候または身体所見のみが認められることもある．
A．年齢ならびに性
　多くは孤発例で成人期発症，時に家系内発症や小児期発症，女性に多い．
B．発症様式
　通常緩徐な発症であるが，急激な発症のこともある．
C．主要な神経学的症候
　1．慢性痙性対麻痺，通常緩徐進行性．時に，初め進行した後に症状の停止する例あり．
　2．両下肢(特に近位部)の筋力低下．
　3．膀胱障害は通常は初期症状，便泌は通常後期症状，インポテンツや性欲減退も稀でない．
　4．刺痛，ジンジン感，灼熱感などのような感覚症状のほうが他覚的身体所見よりも優位．
　5．下肢に放散する下部腰痛が稀でない．
　6．振動覚はしばしば障害されるが，固有感覚はより保たれる．
　7．下肢反射亢進．しばしば足クローヌスやBabinski徴候を伴う．
　8．上肢反射亢進．しばしばHoffman徴候やTromner徴候陽性．上肢脱力は認めないこともある．
　9．下顎反射の亢進例も存在．
D．より出現頻度の少ない神経学的所見
　小脳症状・視神経萎縮・難聴・眼振・その他の脳神経障害・手指振戦・アキレス腱反射の減弱または消失．(痙攣・認識力障害・痴呆・意識障害はほとんどみられることはない)
E．HAM/TSPに伴いうる他の神経学的症候
　筋萎縮・筋束性攣縮(稀)・多発筋炎・末梢神経障害・多発神経炎・脳神経炎・髄膜炎・脳症
F．HAM/TSPに伴いうる系統的症候
　肺胞炎・ブドウ膜炎・Sjögren症候群・関節障害・血管炎・魚鱗癬・クリオグロブリン血症・単クローン性免疫グロブリン血症・成人T細胞白血病
Ⅱ．実験室的診断
　1．HTVL-Ⅰ抗体または抗原が血清ならびに髄液に存在すること．
　2．髄液に軽度のリンパ球性細胞増多をみることがある．
　3．血液あるいは髄液中に核の分葉したリンパ球を認めることがある．
　4．脳脊髄液中に軽度から中等度の蛋白増多を認めることがある．
　5．可能なら，血液あるいは脳脊髄液からのHTLV-Ⅰウイルスの分離．

HAM：HTLV-Ⅰ関連脊髄症，TSP：熱帯性痙性脊髄対麻痺　〔文献13)より引用〕

で，レベルを伴わない感覚障害(75.6%)が多い．初期より振動覚低下がみられる．自律神経障害として，頑固な便秘，皮膚乾燥，脊髄レベル以下の発汗低下がしばしば認められ，皮膚乾燥が強いと魚鱗癬様の皮膚症状を呈することもある．バビンスキー反射，チャドック反射など病的反射の陽性，腹壁反射の消失(99%)を認める[4]．HAMの治療効果の判定にも用いられる機能評価スケールを表2に示した[14,15]．脊髄症状以外の症状では，小脳症状(3.2%)，パーキンソニズム(5.5%)，筋萎縮性側索硬化症様症状(1%)[16,17]，末梢神経障害[18]が認められる．頸髄病変を有するHAMの報告もある[19]．慢性に経過するHAM患者のなかで，2年間のうちに3段階以上運動障害度が悪化する一群(急速進行群)が自験例で17例(3.4%)にみられた．急速進行群は緩徐進行群と比較すると運動障害度7(伝い歩き不能)以上の重症例が多く，下肢の痙性が強い．検査所見で，急速進行群は髄液抗HTLV-1抗体価(PA法)および髄液ネオプテリン値が高いという特徴がある[4]．

2）検査所見

血清抗HTLV-1抗体価高値，血清IgE低値，各種自己抗体陽性(リウマチ因子18.3%，抗核抗体22%，抗SS-A抗体23.4%，抗SS-B抗体9.7%，platelet associated IgG：PA IgG 30%など)，NK活性低下，末梢血HTLV-1プロウイルス量高値[20]，髄液抗HTLV-1抗体陽性を認めるほか，髄液ネオプテリン値高値，髄液内IgG産生亢進などもみられる．ATL細胞様の異型リンパ球を末梢血，髄液中に認めることもある．電気生理検査では，傍脊柱筋針筋電図で脱神経所見を，下肢SEPで中枢神経伝導速度遅延を認めるほか，下肢SSRの消失も認められる．頭部MRIでは，大脳深部白質病変を高頻度(55%)に認め

表2　HAMの機能障害の評価

運動機能障害重症度	
Grade	Disability
0	歩行・走行ともに異常を認めない
1	走るスピードが遅い
2	歩行異常(つまづき，膝のこわばり)
3	かけ足不能
4	階段昇降に手すり必要
5	片手によるつたい歩き
6	片手によるつたい歩き不能：両手なら10 m以上可
7	両手によるつたい歩き5 m以上，10 m以内可
8	両手によるつたい歩き5 m以内可
9	両手によるつたい歩き不能，四つんばい移動可
10	四つんばい移動不能，いざり等移動可
11	自力では移動不能，寝返り可
12	寝返り不能
13	足の指も動かせない

排尿障害の重症度	
頻尿	0：正常
残尿	1：わずかに存在
尿失禁	2：明らかに存在
	3：著明に存在
(残尿2：圧迫排尿，残尿3：自己導尿)	
3つの症状の合計点数で表す．	

〔文献15)より改変して引用〕

表3　HAM発症にかかわるリスク要因

- ウイルス側
  - Taxサブタイプ A は発症させやすい
- 宿主側
  1) HLA遺伝子
     - 発症抑制：HLA-A*02，Cw*08
     - 発症促進：HLA-DRB 1*0101，B*5401
  2) Non-HLA遺伝子
     - 発症抑制：IL-10 promoter-592 A/C SNP：A allele
       Vitamin D receptor exon 9 ApaI多型
       SDF-1-801A3' UTR
     - 発症促進：TNF-α-893 A/C SNP：A allele
       アグリカンVNTR 1630 bp allele
       MMP-9 promoter d(CA)n repeatの延長

SDF：stromal cell-derived factor 1，MMP：マトリックスメタロプロテアーゼ，TNF：腫瘍壊死因子，SNP：single nucleotide polymorphism

る．通常，脊髄MRIの異常を認めないが，慢性期では胸髄萎縮を，急速進行群では脊髄腫脹を認めることがある．髄液抗HTLV-1抗体価(PA法)が2倍から8倍以下の群(低力価群)が存在し，1,024倍以上の高力価群と比べて発症年齢が遅い，やや運動障害が軽い，バビンスキー反射および排尿障害の出現率が低い，血清IgGが低い，髄液ネオプテリン値が低いという特徴がある[4]．また，高力価群には急速進行群に属する症例が多く含まれている(自験例17例中10例)．最近，HAM患者の血清中に中枢神経のBetz神経細胞に反応する自己抗体である，抗heterogenous nuclear ribonuclear protein-A 1(hnRNP-A 1)抗体が高頻度に検出されるという報告があり[7]，HAMの病態への関与が示唆されている．

5．HAM発症にかかわる危険因子からみた診断

HAM発症要因として，ウイルス側および宿主側の様々な因子が解析されつつある(表3)．HTLV-1 taxのサブタイプにはサブタイプA，サブタイプBがあり，サブタイプAのウイルスに感染している個体ではHAMを発症しやすいことが報告されている[21]．一方，宿主側因子として，HLA遺伝子のうちHLA-A*02，Cw*08が発症抑制，HLA-DRB 1*0101，B*5401が発症促進に関与することが報告されている(表3)．われわれは，これらHAM発症に関与する複数のHLA，非HLA宿主遺伝子多型とHTLV-1ウイルスサブタイプを用いた多変量解析から，HAM

表4　HAM発症リスク計算式
(Best-fit logistic regression equation for the risk of HAM/TSP in the Kagoshima HTLV-1 infected cohort：n=402)

Factor, condition	ln(odds of HAM/TSP)	Odds ratio(P)
Constant	−1.716	
Age	−(0.145×age)+(0.003×age^2)	
Provirus load	+(0.460×load)+(0.487×load^2)	
TNF-α−863 A$^+$	+3.057−(4.616×load)+(1.476×load^2)	
SDF-1+801 GA	−0.808	0.45(0.042)
SDF-1+801 AA	−1.689	0.18(0.003)
HLA-A*02$^+$	−0.638	0.53(0.043)
HLA-Cw*08$^+$	−0.894	0.41(0.046)
HTLV-1 subgroup B	−1.587	0.20(0.017)

例) 60歳，$\log_{10}$(No. of tax copies/10^4 PBMCs)=2.5，TNF-α−863 A$^-$，SDF-1+801 AA，HLA-A*02$^-$，HLA-Cw*08$^+$，HTLV-1 subgroup Bの感染者の場合：ln(odds of HAM/TSP)=−1.716−(0.145×60)+(0.003×60^2)+(0.46×2.5)+(0.487×2.5^2)+3.057−(4.616×2.5)+(1.476×2.5^2)−1.689−0.894−1.587=1.14975　よってこの症例のodds of HAM/TSP=exp(1.14975)=3.157403

発症リスクを計算する式を示した(表4)[22]．この式により求めたHAM発症リスク(オッズ比)を用いて，非典型的なHAMを鑑別できる可能性がある[23]．実際に，最近，頸髄病変を認めた非典型的HAM症例のなかに，オッズ比が高値であった症例が存在したことが報告されている[19]．

6．合併症

Tリンパ球性肺胞症(70%)，シェーグレン症候群(25%)，関節症(20%)，ぶどう膜炎(15%)，多発筋炎(5%)，魚鱗癬様皮疹，ATL　8例(1.6%：くすぶり型を含むと11例：2.2%)などがある．

7．HAMの病理

神経病理所見では，胸髄中下部の外側皮質脊髄路を中心にした脱髄と神経鞘および軸索の変性脱落が認められ，血管周囲から脊髄実質に広がる炎症性単核細胞の浸潤を伴っている．胸髄以外にも，数は少ないものの大脳においても同様に単核細胞の浸潤が認められることが報告されている[24]．

8．治療

これまでに施行された治療を表5にまとめた．治療にあたり，まずHAMに伴う合併症の有無を精査することが重要である．初期治療として，特に急速進行群に対しては，メチルプレドニゾロンによるステロイドパルス療法を施行後，経口ステロイド剤による維持療法を行う．ステロイド療法には，抗炎症作用のみならず，末梢血単核球中のHTLV-1プロウイルス量を減らす効果も認められる．また，初期治療として肺合併症，網膜症がない場合には，天然型インターフェロン-α製剤(スミフェロン®)が使われる(これは，HAMに対する唯一の健康保険適用のある薬剤である)．インターフェロン-α 300万単位を30日間連日筋注し，以後週1回の投与とする．減量後に，治療開始後いったん減少したHTLV-1プロウイルス量が再び上昇することがあるので，そのような症例に対しては後療法として少量の経口ステロイド剤(5〜10 mg/日)を併用する．インターフェロン-αにはHTLV-1プロウイルス量を減らすとともに，活性化T細胞を減少させる効果がある[14]．慢性期の症例に対しては，経口ステロイド剤の少量持続療法[25,26]やビタミンC大量療法(1.5〜3 g/日)が有効である．経口ステロイド療法の際には，骨粗鬆症とステロイド糖尿に留意することが必要である．肺合併症や尿路感染を繰り返すケースでは，エリスロマイシンを使用する．関節炎を伴う症例では，サラゾスルファピリジンが有効な場合がある．痙性麻痺が強い場合は，筋弛緩剤を併用し，リハビリテーションを施行する．排尿障害に対しては，神経因性膀胱に対する治療を行う．腰・下肢痛については，原因となるものを精査したうえで，対症的にカルバマゼピン，抗うつ薬の投与や，半導体レーザーによる星状神経節近傍照射，鍼治療などの物理療法も積極的に取り入れる．将来的には，炎症細胞の中枢神

表5　現在までのHAMの治療

治療法	投与量	投与期間	施行例	有効率(A)	有効率(B)
1　副腎皮質ホルモン					
1）経口投与	10～80 mg/日	連日．隔日/1～3か月	247	78.1%	63.0%
2）大量点滴投与	500～1,000 mg/日	1～3日	14	78.6	50
3）髄注	50～70 mg/日	1～5回	5	80	40
2　血液浄化療法					
1）リンパ球除去術	約1×10⁹個/回	3～6回	9	77.8	44.4
2）プラズマフェレーシス	1回1.5～2.0 *l* の血液を免疫吸着カラムで処理	4～6回	7	42.9	42.9
3　インターフェロン-α					
1）筋注	300万単位/日	30日	32	62.5	21.9
2）吸入	100万単位/日	30日	11	81.8	27.3
4　アザチオプリン	50～100 mg/日	1～3か月	9	55.6	22.2
5　ビタミンC	1,500～3,000 mg/日	4日か5日連続投与後2日休薬/4週間	89	58.4	13.4
6　ペントキシフィリン	300 mg/日	2～4週	5	60	20
7　エリスロマイシン	600 mg/日	1～3か月	25	48	16
8　サラゾスルファピリジン	1,000～1,500 mg/日	1～3か月	24	50	12.5
9　ミゾリビン	100～150 mg/日	1～3か月	17	47.1	11.8
10　フォスホマイシン	静注4 g/日	2週間			
	その後経口2 g	2週間	14	78.6	7.1
11　TRH	静注2 mg/日	5日間	16	56.3	6.3
12　グリセオール	400～600 mg/日	3～5日間	3	66.7	0
13　ヒト免疫グロブリン	2.5～5 g/日	1～3日間	3	33.3	0
14　ダナゾール	200～300 mg/日	4～6週間	2	0	0
15　エペリゾン塩酸塩	100～150 mg/日	4～6週間	6	100	50
16　AZT+3TC	3TC 300 mg, AZT 400 mg	4～12週間	12	58.3	25

有効率(A)：やや有効以上の効果の割合，有効率(B)：有効以上の割合，TRH：甲状腺刺激ホルモン放出ホルモン

〔文献9)より引用して改変〕

経への移行を阻害するMMP(マトリックスメタロプロテアーゼ)阻害剤，TNF-αによる神経障害を抑制するための抗TNF薬，ウイルスの増殖を抑制する逆転写酵素阻害剤や，HTLV-1特異的プロテアーゼ阻害剤などの治療薬が現在検討中または開発中であり，臨床的に使用可能になるものと考えられる．

9．おわりに

HAMは，生命予後が比較的良好な疾患ではあるが，難治性で合併症も多く，患者の負担が大きいため，根治療法の開発が切望されている．現在，HAM患者会(アトムの会)が結成されており，ホームページ(http://www.minc.ne.jp/~hamtomo/)上で患者同志の情報交換が可能である．HAMに対しては，医療関係者を含めた多くの人々による治療，心のケア，福祉サービスなどの多方面にわたるサポートが必要であり，今後ともますますその充実が望まれる．

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Design and synthesis of a new polymer-supported Evans-type oxazolidinone: an efficient chiral auxiliary in the solid-phase asymmetric alkylation reactions

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Abstract—Wang resin-supported Evans' chiral auxiliary (**23**) was designed based on a novel polymer-anchoring strategy, which utilizes the 5-position of the oxazolidinone ring, and its new synthetic route applicable to multi-gram preparation in just a day was developed. Solid-phase Evans' asymmetric alkylation on **23**-derived *N*-acylimide resin and following lithium hydroperoxide-mediated chemoselective hydrolysis afforded the corresponding α-branched carboxylic acids in desired high stereoselectivities (up to 97% ee) and moderate to good overall yield (up to 70%, for 3 steps), which were comparable to those of the conventional solution-phase methods. Furthermore, recovery and recycling of the polymer-supported chiral auxiliary were successfully achieved without decreasing the stereoselectivity of the product. Therefore, this is the first successful example that the solid-phase Evans' asymmetric enolate-alkylation was efficiently performed on the solid-support, and it is concluded that the connection to the solid-support via the 5-position of the oxazolidinone ring is an ideal strategy in the solid-phase Evans' chiral auxiliary.

1. Introduction

Evans' chiral oxazolidinone is one of the efficient auxiliaries for preparing chiral building blocks necessary to synthesize molecules possessing the accurate spatial configuration of specific functional groups.[1,2] Its generality and reliability with high optical purity have already been established in a variety of efficient asymmetric syntheses of low molecular weight chiral compounds and complicated natural products.[3–5] Moreover, its potential is expanding in the study of novel asymmetric reactions.[6]

Solid-phase organic synthesis has been developed as a rapid and diversified method in organic chemistry.[7] As compared to solution-phase, the solid-phase technology provides a simple procedure 'filtration' for rapidly achieving the isolation of desired compounds or recovering expensive reagents or catalysts attached onto the solid-support for recycling. Hence, many useful reagents or catalysts, especially those used in chiral synthesis, in solution-phase methods have been intensively and successfully applied to the solid-phase methods so far.[8] However, some solid-supported chiral auxiliaries are problematic in achieving high quality of stereoselective reactions.[9] One of such well-known examples is pseudoephedrine[10] grafted onto the Merrifield resin. This solid-supported auxiliary showed lower stereoselectivity in asymmetric alkylation (approx. 85% ee) in comparison to the corresponding solution-phase experiments.

Evans' oxazolidinone has also been applied to the solid-phase stereoselective reactions such as enolate-alkylation reaction,[11] aldol reaction,[12] Diels–Alder cycloaddition[13] and 1,3-dipolar cycloaddition.[14] However, undesired results similar to those observed in the solid-supported pseudoephedrine case were reported, especially in the fundamental solid-phase asymmetric enolate-alkylation reaction which prepares optically active α-branched carboxylic acid derivatives.[11b] Indeed, maximum stereoselectivity was 90% ee in asymmetric benzylation using the auxiliary resin **1** (Fig. 1A).[15] Moreover, a side reaction was reported in the preparation of solid-supported L-serine derived chiral oxazolidinone **2**.[16] Therefore, to improve the efficiency in stereoselective reactions, we previously reported a

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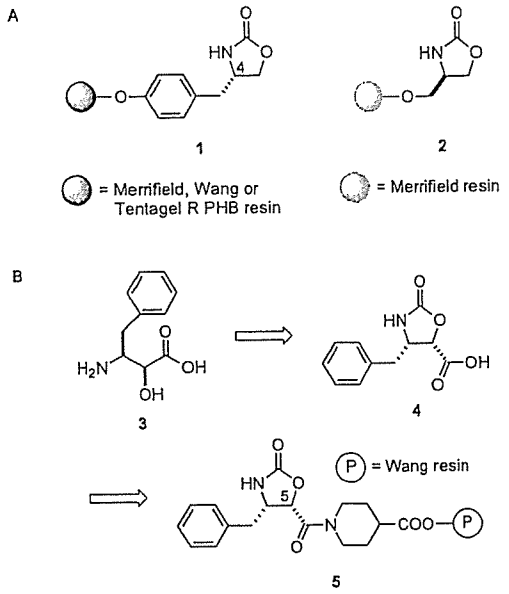


Figure 1. Reported polymer-supported Evans' chiral auxiliaries anchored at the 4-position of the oxazolidinone ring (A) and design of a new auxiliary anchored at the 5-position (B).

polymer-supported chiral Evans' oxazolidinone with a novel anchoring system onto the solid-support as a rapid communication.[17] In this article, we describe the detailed design and synthesis of the polymer-supported chiral Evans' oxazolidinone and its reusability in Evans' asymmetric alkylation.

2. Results and discussion

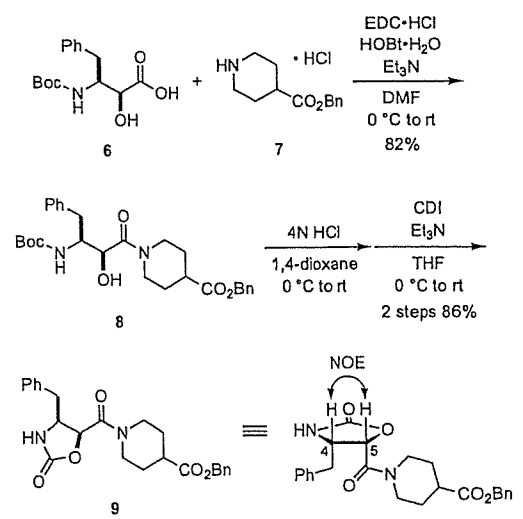
2.1. Design of a new polymer-supported chiral oxazolidinone

One of the common features of polymer-supported Evans' chiral auxiliary in all previous reports[11–14] is that a chiral 4-substituted oxazolidin-2-one was connected to the solid-support through the chiral discriminating moiety at the 4-position of the oxazolidinone ring (Fig. 1A). This made us suspect that chiral control ability of Evans' oxazolidinone is influenced by the polystyrene backbone of the solid-support, leading to the low stereoselectivity in the asymmetric alkylation.[11b] Hence, we proposed an alternative anchoring strategy, which leaves the crucial chiral discriminating moiety unmodified, and utilizes the external 5-position for connecting to the solid-support (Fig. 1B).

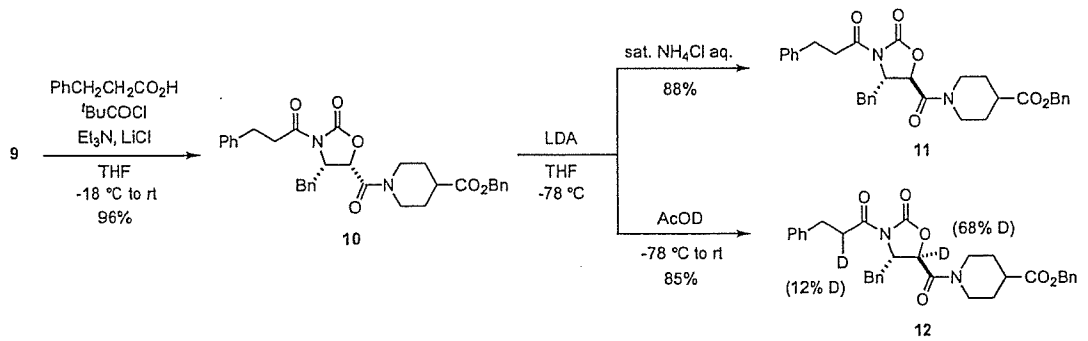
To prepare such a new oxazolidinone derivative, we focused on α-hydroxy-β-amino acids, which are routinely used in our laboratory as a core structure for the development of effective aspartic protease inhibitors.[18] The unique structure of α-hydroxy-β-amino acids, in which three different functional groups, i.e. amino, hydroxyl and carboxyl groups, are located on two adjacent asymmetric carbon atoms gave us the idea. Namely, the known oxazolidinone formation[19] at the 1,2-amino alcohol moiety of α-hydroxy-β-amino acid, (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutanoic acid 3 (allophenylnorstatine, Apns),[18] can afford a desired oxazolidinone derivative **4** with a benzyl substituent at the 4-position as a chiral discriminating group and a free carboxyl group at the 5-position which can connect with the solid-support (Fig. 1B). In addition, Burgess et al. pointed out that Wang resin had a better enantiomeric excess than Merrifield resin in asymmetric benzylation.[11b] Since Wang resin has an additional benzyl moiety which has a space from the polystyrene backbone in comparison to Merrifield resin, we planned to employ both Wang resin and, as a further spacer, a piperidine-4-carboxylic acid. Thus, in the designed solid-supported auxiliary **5**, this spacer is connected to the carboxyl group at the 5-position of the oxazolidinone moiety by a tertiary-amide bond and to Wang resin by an ester bond. This tertiary-amide bond with no amide proton is stable under both acidic and basic conditions. The ester bond between the spacer and Wang resin can be formed by the standard methods.

2.2. Evaluation of new oxazolidinone derivatives in solution-phase model experiment

To understand the efficacy of designed solid-support chiral oxazolidinone **5** as a new chiral auxiliary, we first studied a solution-phase experiment, using a model oxazolidinone derivative **9** whose C-terminal is protected by a benzyl ester to mimic Wang resin. As Scheme 1 shows, **9** was synthesized by a three-step reaction. Namely, Boc-Apns-OH **6** was coupled to benzyl piperidine-4-carboxylate **7**[20] by the EDC–HOBt (EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, HOBt: 1-hydroxybenzotriazole) method[21] to give dipeptide **8**,[22] followed by the removal of the Boc group and CDI (1,1′-carbonyldiimidazole) treatment[19] to afford the *cis*-configured oxazolidinone derivative **9** as a single isomer. During the cyclization reaction, neither aziridine nor 1,2-imidazoylamine byproduct formation was observed.[23] The *cis*-configuration of **9** was confirmed by the coupling constant[24] between H-4 and H-5, and NOE experiments (Scheme 1 and Ref. 25). Synthesized **9** had



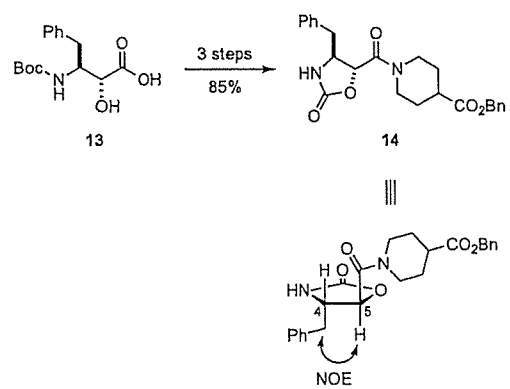
Scheme 1. Synthesis of a *cis*-configured oxazolidinone derivative 9 from Boc-Apns-OH 6.



Scheme 2. Epimerization and deuterium incorporation to the *cis*-configured carboximide 9.

coupling constants of $J_{4,5}=7.9$, 8.1 Hz, which corresponded to the representative value of the *cis*-configured oxazolidinone.

Next, oxazolidinone **9** was *N*-acylated with 3-phenylpropionic acid by the mixed anhydride method to obtain carboximide **10** (Scheme 2).[26] Although there are three α-protons in **10**, the newly introduced carboximide α-proton was expected to be most acidic. Since it was reported that the imide-selective enolization of substrates with the both carboximide and ester structures was accomplished by careful base addition,[27] and that after asymmetric reaction, *N*-acyl fragments were selectively cleaved from the auxiliary by the imide-specific lithium hydroperoxide-mediated hydrolysis,[28] we proposed **9** as a chiral auxiliary that could be recovered and reused. However, its enolate formation gave a new compound even with a careful addition of LDA (1.2 equiv) to the cooled THF solution of **10** and a subsequent stirring for 0.5 h. This compound was an epimerized *trans*-configured carboximide **11**.[29] This result suggests that the α-proton of the carboxamide moiety was predominantly deprotonated by LDA to diminish the steric repulsion caused by *cis*-configuration.[30] Indeed, quenching lithium enolate generated in situ from **10** with acetic acid-*d* (99at.% D) afforded the deuterized **12** in 85% yield. The deuterium was incorporated mainly at the α-position of the carboxamide moiety (68% D) along with the α-position of the *N*-acylimide moiety (12% D).



Scheme 3. Synthesis of a *trans*-configured oxazolidinone derivative 14 from Boc-Pns-OH 13.

This unexpected result prompted us to suggest that stable *trans*-configured oxazolidinone **14** was suitable for the auxiliary (Scheme 3). We synthesized **14** according to the procedure shown in Scheme 1, starting from Boc-Pns-OH **13** (Pns: phenylnorstatine), a 2*R* isomer of **6**, in 85% yield (3 steps). The relative stereochemistry of **14** was analyzed by NMR. Coupling constants of $J_{4,5}$ were 5.1 and 5.3 Hz, which are well consistent with the known value in *trans*-configuration[24] and a strong NOE signal was observed between H-5 and two protons at the benzylic position.[25] In addition, the most stable conformation of **14** obtained from conformational analysis showed a dihedral angle of 136.4° between two methine hydrogens (H-4 and H-5). This value and Karplus curve reasonably supported the observed coupling constant. From these observations, the configuration between H-4 and H-5 in **14** was confirmed as *trans*. Furthermore, the absolute stereochemistry of **14** was confirmed as 4*S*,5*R* by the X-ray crystal structural analysis of (*R*)-phenylethylamide **15**[31] derived from **14** (Fig. 2). In addition, it was found that the piperidine-4-carboxylic acid spacer extended outside from the oxazolidinone core, suggesting that this spacer does not interfere with the asymmetric reaction.

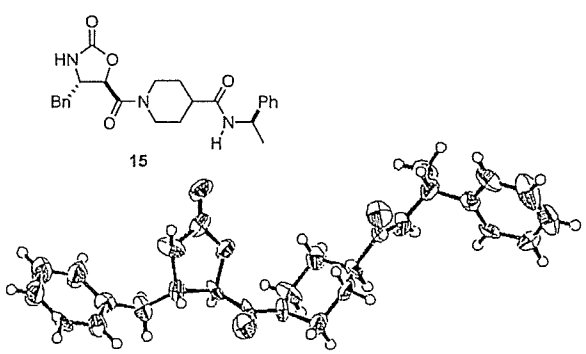
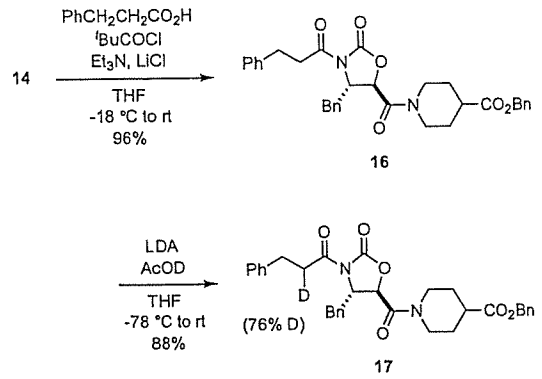


Figure 2. X-ray crystal structure of (*R*)-phenylethylamide 15.

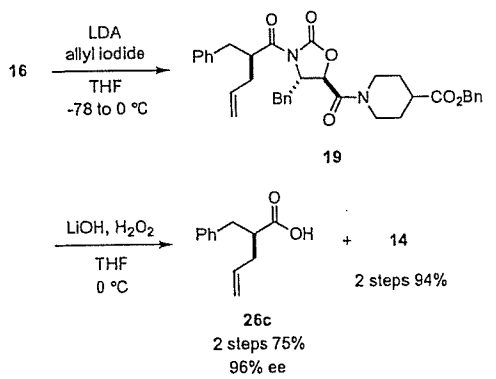
Next, we synthesized *N*-3-phenylpropionylated oxazolidinone **16** and subjected it to the deuterium labeling to confirm the enolization position (Scheme 4) by the same procedure described in Scheme 2. No particular change on TLC was observed during the enolization and the recovered product (88% yield) contained 76% of deuterized **17**, exclusively at the α-position of the desired carboximide moiety with unmodified **16**. This result suggests that the



Scheme 4. Deuterium incorporation to *trans*-configured carboximide **16**.

α-position of the imide *N*-acyl moiety in **16** has the most acidic α-proton, which is predominantly deprotonated by LDA. Self-condensation of **16** was not observed under this reaction condition.

With these positive observations, we examined the Evans' asymmetric allylation of the carboximide **16** as a model for alkylation (Scheme 5).[15] Briefly, to a solution of **16** in THF was added LDA (1.2 equiv) dropwise at −78 °C. After stirring for 0.5 h, the generated *Z*-*O*-enolate **18**[32] was treated with allyl iodide (3.0 equiv), and the temperature of the reaction mixture was gradually increased up to 0 °C over a period of 3 h. Resultant **19** was hydrolyzed by LiOOH without any purification.[28] The desired α-allylated carboxylic acid **26c** was obtained in good yield (2 steps 75%, an average of 87% for each of the two steps in the reaction sequence) and high stereoselectivity (96% ee),[33] which were comparable to the standard Evans' asymmetric allylation in solution-phase.[15] Oxazolidinone **14** was sufficiently recovered (94%) without epimerization, and no byproduct produced by the endocyclic cleavage of the oxazolidinone ring[28] was observed. These results proved that the *trans*-configured oxazolidinone **14** was effective as a chiral auxiliary and that the spacer moiety did not interfere with the asymmetric reaction.



Scheme 5. Asymmetric allylation of the *N*-3-phenylpropionylated carboximide **16**.

An energy minimization study of enolate intermediate **18** suggested that its conformation corresponds to that of the original chelation-controlled model proposed for standard Evans' chiral auxiliary system (Fig. 3).[15,34] Interestingly, this modeling also suggested that nucleophilic attack of the hydroperoxide anion to the oxazolidinone carbonyl for the endocyclic cleavage is effectively obstructed by the steric effect of the benzyl and carboxamide moieties.[35] From these data, we selected the structure of **14** originating from Pns as the candidate for solid-supported Evans' auxiliary.

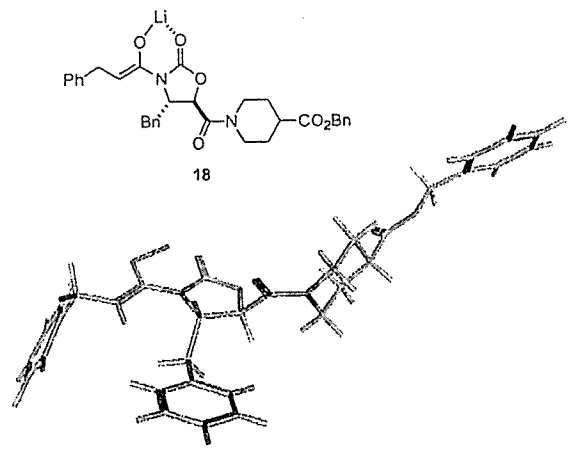


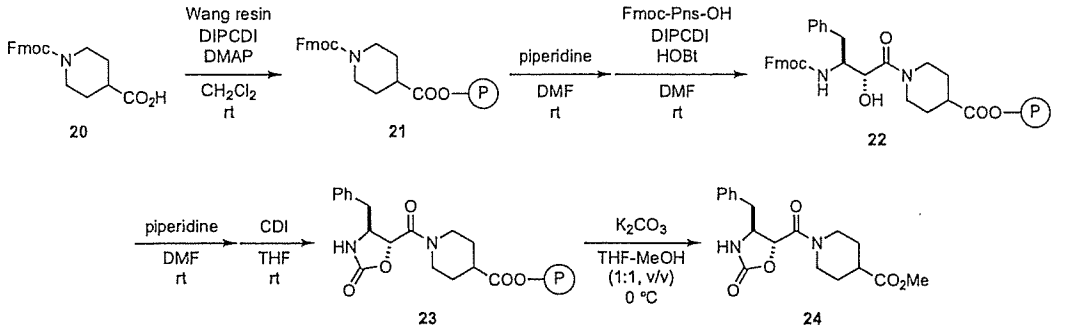
Figure 3. Energy minimization study of enolate intermediate **18**.

2.3. Solid-phase synthesis of Wang resin-supported chiral oxazolidinone 23

In our previous communication,[17] Wang resin-supported chiral oxazolidinone **23** was obtained by the carbodiimide-mediated coupling between Wang resin and the oxazolidinone-spacer unit prepared from **14** by hydrogenolysis. Since four-step solution-phase synthesis of this unit and its excess use (4 equiv) required for complete loading onto the resin were inefficient, in the present study we developed a more convenient synthetic route for **23** using Fmoc-based solid-phase method as shown in Scheme 6.[36] Namely, Fmoc-piperidine-4-carboxylic acid **20** was first loaded to Wang resin using the DIPCDI–DMAP (DIPCDI: 1,3-diisopropylcarbodiimide) method[37] in CH_2Cl_2. After Fmoc-deprotection of **21** with 20% piperidine, Fmoc-Pns-OH was coupled by the DIPCDI–HOBt method to give the dipeptide resin **22** followed by removal of the Fmoc group. The resultant 1,2-amino alcohol moiety was converted to oxazolidinone with CDI. Methanolysis of **23** with potassium carbonate in anhydrous THF–MeOH yielded the corresponding methyl ester **24** as a single isomer (95% for 6 steps). During this synthesis, neither epimerization at the 5-position nor byproduct formation such as aziridine and 1,2-aminoimidazole was observed.[23] It is noteworthy that all reactions in Scheme 6 proceeded smoothly at room temperature within a few hours, and multi-gram quantity of the oxazolidinone resin **23** with high loading yield was efficiently synthesized in just a day.

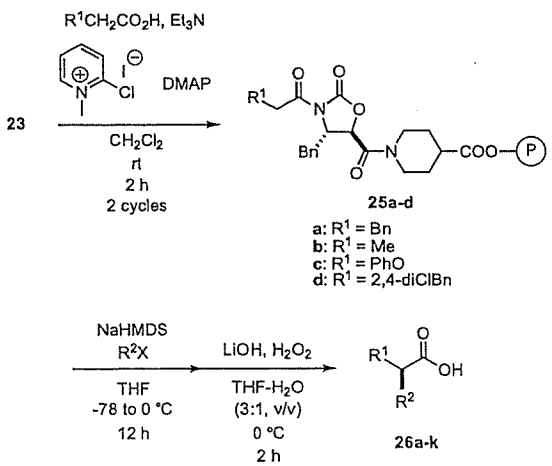
2.4. Solid-phase Evans' asymmetric alkylation with the oxazolidinone resin 23

At first, we investigated the solid-phase Evans' asymmetric allylation of the *N*-3-phenylpropionylated carboximide



Scheme 6. Solid-phase synthesis of Wang resin-supported oxazolidinone resin 23.

resin **25a**, which was prepared from **23** by Mukaiyama method (Scheme 7).[38] It was found that the use of NaHMDS (3 equiv) as a base and gradual increase of the temperature of reaction mixture up to 0 °C over a period of 12 h in the alkylation reaction were quite effective.[39] After quenching the reaction mixture with saturated NH_4Cl aq, the allylated carboximide resin was recovered, washed, then subjected to the LiOOH-mediated hydrolysis. The desired chiral α-allylated carboxylic acid **26c** was obtained with high stereoselectivity (96% ee), which was equal to the model experiment in solution-phase (Table 1, entry 3). The absolute configuration of acid **26c** was determined in comparison to the reported specific rotation,[33] suggesting



Scheme 7. Solid-phase asymmetric Evans' alkylation.

that the asymmetric alkylation on resin **25a** also proceeded in the same chelation-controlled model as the solution-phase method.[15] During the hydrolytic cleavage, the ester linkage and oxazolidinone core were stable.[40] These encouraging results urged us to understand the generality of **23** in the Evans' asymmetric alkylation reaction. Several carboximide resins **25b–d** were prepared and subjected to the similar solid-phase alkylation reactions with a series of electrophiles (R^2X).[41] The results are summarized in Table 1. Favorably, not only highly reactive alkyl halides such as MeI and BnBr but also less reactive EtI reacted sufficiently under the same reaction conditions. Hydrolytic cleavage of the resultant resin afforded the corresponding chiral α-branched carboxylic acids **26a–k** with satisfying isolated yields (50–70%, for 3 steps) and enantiomeric excesses (84–97% ee).[42] Especially, in the asymmetric benzylation of carboximide resin **25b**, stereoselectivity was found to be 97% ee (Table 1, entry 6), which was better than the value reported by Burgess et al.,[11b] and was as high enough as in the corresponding solution-phase asymmetric alkylation utilizing the standard chiral 4-substituted oxazolidin-2-one.[15] The relatively lower yield was due to the fact that the yield includes the three-step process from the oxazolidinone resin **23** to the final alkylated product **26**. We consider that yield for two steps (alkylation and hydrolysis) is similar to that of the solution-phase method, and average yield calculated for each step was reasonably acceptable (79–89%). We assume that these successful results are attributed to our new polymer-anchoring strategy based on the connection at the 5-position of the oxazolidinone ring. This liberates the chiral differentiating benzyl group from the polystyrene backbone of the resin, freeing the auxiliary

Table 1. Results of the solid-phase asymmetric Evans' alkylations

Entry	25	R^1	R^2X	26	Yield[a] (%)	ee[b] (%)
1	25a	Bn-	MeI	26a	61(85)	85
2	25a	Bn-	EtI	26b	50(79)	88
3	25a	Bn-	Allyl-I	26c	68(88)	96
4	25a	Bn-	Propargyl-Br	26d	62(85)	96
5	25a	Bn-	$BrCH_2CO_2Et$	26e	62(85)	92
6	25b	Me-	BnBr	26f	70(89)	97
7	25b	Me-	4-BrBnBr	26g	68(88)	97
8	25b	Me-	4-NO_2BnBr	26h	55(82)	97
9	25b	Me-	2,4-diClBnI	26i	65(87)	97
10	25c	PhO-	Allyl-I	26j	50(79)	96
11	25d	2,4-diClBn-	MeI	26k	59(84)	84

[a] Combined yield of 3 steps starting from oxazolidinone resin **23**. Value in the parenthesis is the average yield for each step.
[b] Determined by chiral HPLC analysis after conversion to the corresponding (*S*)-α-methylbenzylamine-derived amides.