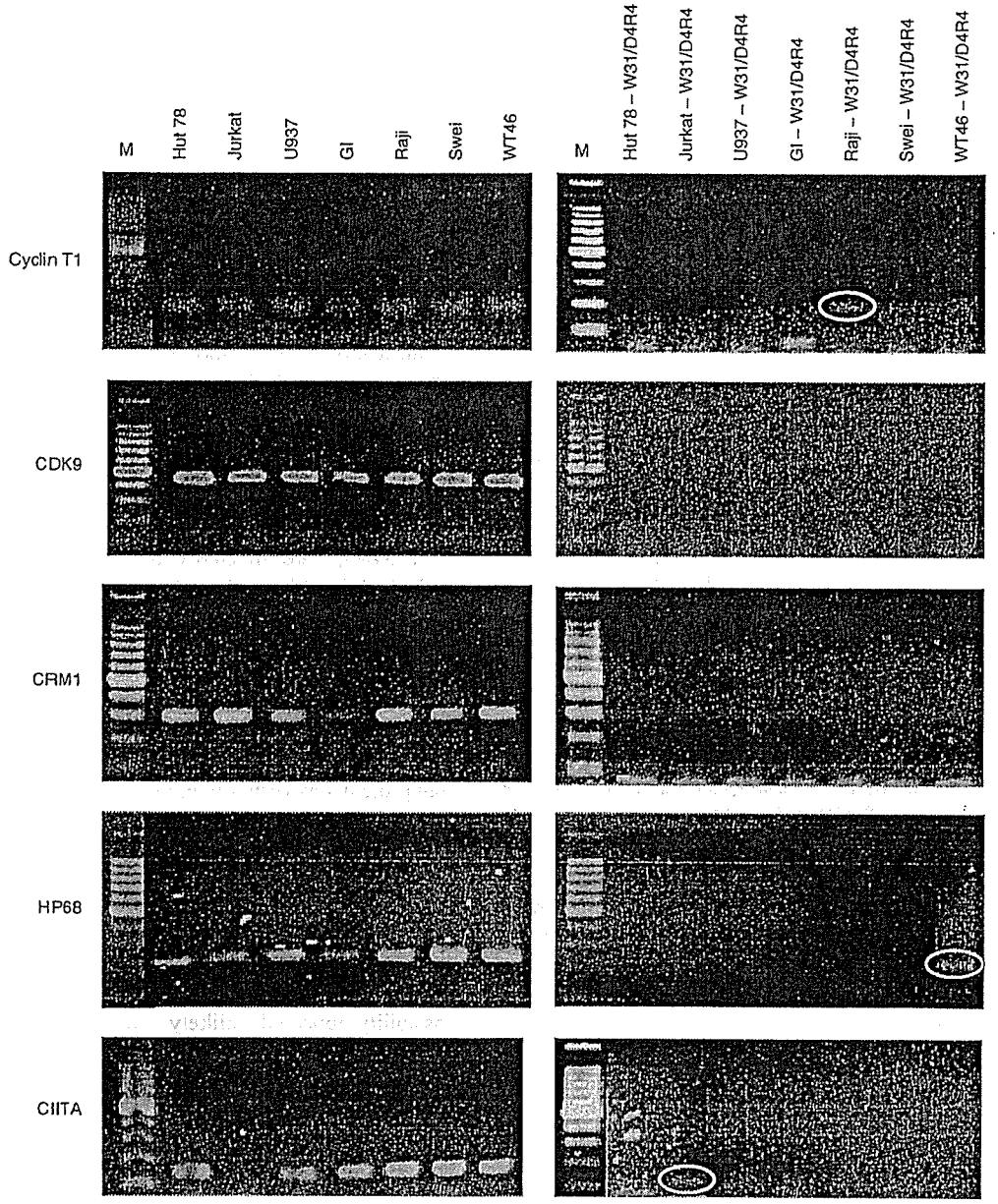

Fig. 4. Expression profiles of human genes in parental human cell lines (left) and hybrid cells (right). Expression of cyclin T1, CDK9, CRM1, HP68, and CIITA was examined by RT-PCR. Human cell lines were fused with HIV-1-infected W31/D4R4 cells. Total RNAs from hybrid cells were extracted at day 21 after fusion. The same hybrid cells were used for in Fig. 3 and this figure. The asterisk indicates that Jurkat cells did not express detectable amounts of CIITA. Ovals indicate specific PCR products. M, 100-bp ladder marker.

cells ceased to express all of the human genes examined, but still showed increased production of p24. This suggests that human molecules that facilitate HIV-1 replication are not limited to cyclin T1, CDK9, CRM1, HP68, and CIITA. There may be unknown human molecules that compensate for the loss of cyclin T1, CDK9, CRM1, HP68, and CIITA and promote HIV-1 replication.

Interestingly, fusion with Jurkat cells consistently failed to increase p24 production. This was not because the efficiency of cell fusion was low in Jurkat since we confirmed that all of the human cell lines examined in this study fused with W31/D4R4 cells with almost the same efficiency (data not shown). It was initially reported that CIITA might promote HIV-1 replication by functioning as a substitute of Tat during an initial post-infection period (Saifuddin et al., 2000). However, more recent work indicates that overexpression of CIITA inhibits viral replication by blocking the function of Tat (Okamoto et al., 2000; Accolla et al., 2002). In this regard, it is interesting to note that human CIITA, which was not expressed in Jurkat, began to be expressed after fusion with rat cells. Transcriptional induction of CIITA might be involved in poor production of p24 in W31/D4R4–Jurkat hybrid cells.

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A slight but significant increase in p24 concentration (10 pg/ml) was found in the supernatants of W31/D4R4–WT46 hybrid cells (Fig. 3B). Interestingly, expression of human HP68 was detected only in this hybrid cells. These results are consistent with the previous reports that human HP68 is a cellular protein important for capsid assembly (Zimmerman et al., 2002; Lingappa et al., 2006). Based on these findings, we suggest that introduction of human HP68 should be considered when designing animal models of HIV-1 infection.

In summary, we performed cell fusion experiments using HIV-1-infected rat fibroblasts and uninfected human cell lines of T-cell, B-cell, and macrophage lineages. Our results indicate that human cellular factors supporting HIV-1 replication are distributed in all of these cell lineages. Identification of additional factors affecting HIV-1 replication, the presence of which was suggested by the cell fusion experiments (Fig. 4), would be important to understand the replication cycle of HIV-1 and to develop countermeasures to control HIV-1 infection.

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プロバイオティクスの臨床応用

HTLV-1-associated myelopathy (HAM)

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はじめに

HTLV-1(ヒトT細胞白血病ウイルス1型)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関連脊髄症(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炎症性の膀胱直腸障害，感覚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]．後にカリブ海沿岸を中心に報告された抗HTLV-I抗体陽性の熱帯性痙性脊髄麻痺(TSP)と同一の疾患であることが確認されている[2]．

1999年時点の報告では，HTLV-1キャリアー数は世界で220万人，日本で120万人であり，HAM患者の実数は世界で3,000人＋α，日本で1,422人と報告された．外国の文献ではHTLV-Iキャリアーが1100万～2000万人とも推測されている[3]．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，沖縄を含む西日本，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．わが国におけるHAMの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．

HAM患者の男女比は1：2.3と女性に多く，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，ほかに輸血，夫婦間伝播(ほとんど男性から女性)などがある．HAMの主症状として下肢の痙性による運動障害，頻尿，残尿，尿失禁といった排尿障害，レベルを伴わないジンジン感などの感覚障害，自律神経障害として，頑固な便秘，皮膚乾燥，障害を受けた脊髄レベル以下の発汗低下が認められる．検査所見で，血清抗HTLV-1抗体価高値，血清IgE低値，NK活性低下[4,5]，末梢血中HTLV-1プロウイルス量高値[6]，髄液中抗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．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(発症後2年で運動障害度が3段階進行する)では脊髄腫脹を認めることがある[7]．プロウイルス量と疾患の活動性にはある程度の相関があり，約半数以上が治療後も進行する[8]．

初期治療として，ステロイド療法，天然型インターフェロン-α製剤が使われる．症状が固定した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やビタミンC大量療法(1.5～3g/日)，erythromycin(600mg/日)やsalazosulfapyridineが使われる．しかし，シェーグレン症候群，呼吸器障害，関節症，筋炎，成人T細胞性白血病などの合併症があること，ステロイド抵抗性の症例が20%ほど存在することなどの問題点があり，

表1　HAM患者10例に対するLcS投与前後の臨床効果

症例	年齢（歳）	性別	罹病期間（年）	末梢血抗HTLV-I抗体価（PA法）	末梢血HTLV-Iプロウイルス量（投与前）	末梢血HTLV-Iプロウイルス量（投与後）	併用薬剤	便秘の改善	痙性スコア（投与前）	痙性スコア（投与後）	運動障害（投与前）	運動障害（投与後）	排尿障害スコア（投与前）	排尿障害スコア（投与後）	全体の評価
HAM1	34	女性	19	×131,072	1,757	1,397	VC 375mg/日	有	2	1	4	3	2	0	有効
HAM2	62	男性	14	×32,768	634	777	PSL 5mg/日 VC 375mg/日	有	＋1	0	6	6	3	0	有効
HAM3	50	女性	17	×2,048	907	779	なし	有	2	1	5	4	3	0	有効
HAM4	45	女性	15	×16,384	2,942	471	PSL 5mg/日 VC 375mg/日	有	＋1	0	5	3	6	3	著効
HAM5	60	女性	7	×8,192	204	194	PSL 5mg/日 VC 375mg/日	有	3	4	4	4	1	0	やや有効
HAM6	47	男性	18	×2,048	716	849	PSL 10mg/日 VC 375mg/日	有	2	1	6	6	5	4	やや有効
HAM7	46	男性	24	×16,384	278	361	なし	有	3	1	4	3	2	0	有効
HAM8	55	女性	10	×65,536	2,882	524	なし	有	2	1	4	3	6	3	有効
HAM9	41	女性	17	×65,536	1,073	1,263	なし	有	3	0	2	2	3	1	有効
HAM10	57	女性	13	×32,768	245	387	なし	無	2	1	4	4	2	1	やや有効

HTLV-Iプロウイルス量：コピー/10^4末梢血リンパ球
VC：ビタミンC，　PSL：プレドニゾロン

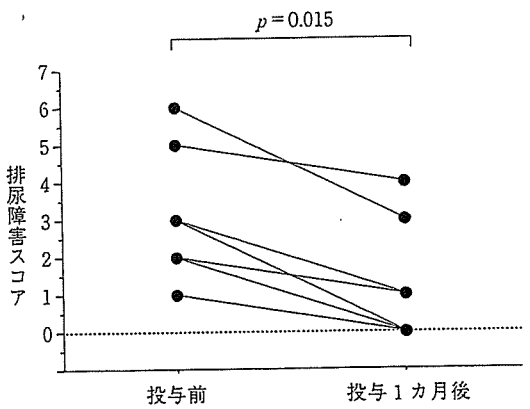

図1　*Lactobacillus casei* Shirota株
投与前後の排尿障害スコアの変化

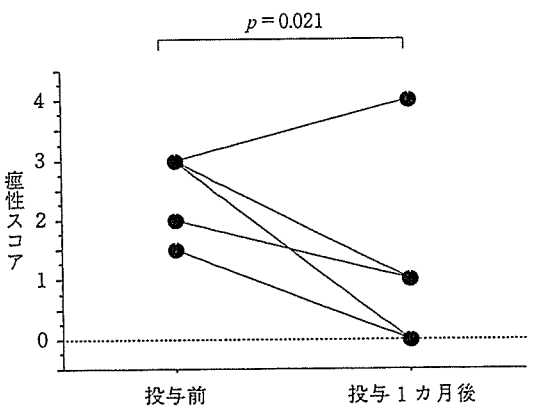

図2　*Lactobacillus casei* Shirota株の
投与前後の痙性スコア

いまだ根治療法がなく，特に病期が進んだ慢性期の症例に対する有効な治療法が確立されていないのが現状である[9]．

Lactobacillus casei Shirota株(LcS)は人の腸内から分離され，葉酸を利用して増殖する乳酸菌である．長い間，発酵乳を産生させるのに使われてきたが[10]，LcSにはNK細胞活性を増加させる作用が報告されている．そこで，健常人に比較してNK活性が低いと報告されている[4,5]HAM患者に対するLcSの治療効果を検討した．

■対象と方法

鹿児島大学臨床倫理委員会の承認を受け，インフォームドコンセントを得たHAM患者10例(男性3例，女性7例)を対象とした．対象患者の平均年齢は49.7±8.8歳，平均罹病期間15.4±4.8(10～20)年である．対象患者にLcS400億個を含む乳製品(ヤクルト400®)を1日2本，4週間飲用

表2 *Lactobacillus casei* Shirota 株投与前後のリンパ球サブセット，NK 細胞活性および HTLV-I プロウイルス量の変化

サブセット	投与前		投与後		p^*	
	絶対数 $\times 10^2/mm^3$	頻度 %	絶対数 $\times 10^2/mm^3$	頻度 %	絶対数	頻度
$CD4^+$細胞	5.79±4.54	26.38±12.71	5.96±3.16	27.13±11.09	0.674	0.575
$CD8^{high+}$細胞	3.89±1.61	20.47±6.93	4.72±2.02	22.60±9.01	0.208	0.327
$CD8^{high+}$ナイーブ細胞	1.13±1.71	5.24±7.11	0.93±1.06	4.25±4.08	1.000	0.674
$CD8^{high+}$メモリー細胞	4.84±2.60	24.05±6.44	5.25±2.13	24.47±7.37	0.401	0.889
$CD8^{high+}$エフェクター細胞	7.56±4.46	37.35±10.81	7.57±2.56	36.10±9.93	1.000	0.674
$CD8^{high+}$エフェクター・メモリー細胞	5.99±2.36	33.36±14.54	7.21±3.47	34.07±13.78	0.263	0.889
$CXCR3^+$細胞	4.38±2.38	21.07±4.28	3.93±1.16	18.78±6.57	0.735	0.398
CD4 陽性 $CXCR3^+$細胞	2.03±2.13	8.26±5.06	1.60±0.75	7.46±3.28	0.866	0.612
$\gamma\delta T^+$細胞	0.40±0.25	2.28±1.46	0.47±0.34	2.35±1.83	0.208	0.674
$NKG2A^+$細胞	0.72±0.43	3.68±1.84	0.74±0.37	3.84±2.39	0.674	0.889
$CD16^+CD56^+/CD3^-$細胞	3.14±1.32	18.63±11.33	3.38±1.73	16.13±8.81	0.575	0.635
NK 細胞活性(%)	26.54±16.13		39.43±15.48		0.015	
HTLV-1 プロウイルス量	867.38±874.62		641.75±343.12		0.401	

HAM 患者 10 症例(NK 細胞活性のみ 9 症例)のまとめ(mean±SD)
*：ウィルコクソン符号付順位和検定

してもらい，投与前後で臨床および検査所見を比較検討した．全体の臨床評価は納の運動障害度スコア(0～13 段階)，排尿障害スコア(頻尿，残尿，尿失禁それぞれ 0～3 の合計)，modified Ashworth scale(MAS)による痙性スコア(0～4)[11]，10m 歩行時間，日常生活動作，下肢の筋力などを用いた．運動障害スコアは 0：正常，1：走るスピードが遅い，2：歩行異常，3：駈足不能，4：階段昇降に手すり要，5：片手による伝い歩き，6：片手による伝い歩き不能，7：両手による伝い歩き 5m 以上 10m 以内なら可，8：両手による伝い歩き 5m 以内なら可，9：4 つばい移動可，10：4 つばい移動不能，いざり等移動可，11：自力で移動不能，寝返り可，12：寝返り不能，13：足の指も動かせない，の 13 段階で評価した．排尿障害スコアは，頻尿，残尿，尿失禁を 0：正常，1：わずかに存在，2：明らかに存在，3：著明に存在，として評価し，特に残尿については 2：圧迫排尿状態，3：自己導尿とした．検査は，HTLV-I プロウイルス量，抗 HTLV-I 抗体価(PA 法)，リンパ球サブセット($CD4^+$，$CD8^+$，$CD56^+CD16^+/CD3^-$：NK 細胞，NKG2A，CXCR3，$\gamma\delta T$ 細胞の頻度および絶対数)，NK 細胞活性を投与前後で比較した．

結　果

LcS 4 週間の投与により，検討した HAM10 例全例において排尿障害スコアの改善($p=0.0085$)が，9 例で下肢痙性の改善が認められ，半数の 5 例で運動障害度が改善した．全体の評価は 10 例中 7 例で有効から著効を示した．また，10 例中 9 例で排便障害が改善した(表 1，図 1，図 2)．飲用前後で抗 HTLV-I 抗体価，HTLV-I プロウイルス DNA 量，リンパ球サブセットに有意な変化はなかったが，NK 細胞活性が有意に増加($p=0.015$)し(表 2)，これは投与前の NK 細胞活性が低い症例ほど，増加幅が大きい傾向であった．

考　案

今回の検討で LcS を HAM 患者に 4 週間投与することで，HAM の特徴的な臨床症状である排尿障害，運動障害，下肢の痙性を有意に改善できることが明らかになった．検査所見で LcS 治療後 HAM 患者の NK 細胞活性は増加したのに対

し，リンパ球サブセット同様 HTLV-I プロウイルス量には有意な変化がみられなかった．以前，われわれは天然型インターフェロン-α製剤による治療後に $CD8^{high+}$ 細胞内のメモリー細胞(CD45 $RA^{-}CD27^{+}$)，Th1 細胞の指標である $CXCR3^{+}$ 細胞数や HTLV-I プロウイルス量が有意に減少したことを報告した[12]．Th1 タイプの T 細胞やプロウイルス量の減少がインターフェロン-α の治療効果に関連した可能性があるのに対し，LcS 投与後の HAM の臨床症状改善には NK 細胞活性の回復が関連している可能性がある．今回の報告は，以前 Nagao ら[13]が健常人を対象に LcS 400 億個を含む乳飲料を投与した後，末梢血中の NK 細胞数，$CD4^{+}$T 細胞数，$CD8^{+}$T 細胞数，マイトジェンに対する T 細胞反応が変化しなかった一方で，NK 細胞活性のみが増加したという報告と矛盾しない結果である．また，治療前の NK 細胞活性が低い人ほど投与後よく増加し，同時に臨床症状が改善していたことも，HAM の臨床改善に NK 細胞活性の回復が密接に関与していることを示唆している．この LcS の治療効果は治療前後における少量(維持量)のステロイド投与の有無とは関係なかった．一般的に LcS のようなプロバイオティクスは，腸内菌叢のバランスを変え，有害な菌の増殖を抑え，食物の消化を促進することで免疫機能を高め，生体防御を高めていることが知られており，実際に LcS が NK 細胞や NK 細胞活性[14]，細胞障害性 T 細胞[15]を活性化することで，単純ヘルペスウイルス[16]やインフルエンザウイルス[17]の増殖を抑制することが報告されている．

LcS 投与後の NK 細胞活性増強の機構は不明であるが，いくつかの研究では，特に初期免疫に関与する免疫細胞の食細胞活性に対する効果が指摘されている．つまり，LcS に刺激された食細胞が，抗原提示やサイトカイン合成により獲得免疫を効率よく誘導させる役割を担っている可能性がある．実際に動物実験では，LcS がパイエル板の M 細胞に取り込まれ，腸内リンパ組織に分解されて，食細胞の刺激，サイトカイン合成，IgA 分泌，NK 細胞活性の増加などを引き起こすことが報告されている[18]．HAM 患者では NK 抑制性受容体である NKG2A 陽性細胞やγδT 細胞の末梢血単球における発現頻度が低いことが報告されているが[19]，これらの細胞群は LcS 投与前後では有意な変動を示さなかったことより，NK 細胞活性増加の調整が，NK 細胞における活性化受容体や抑制性受容体の発現レベルの変化以外の機構で行われている可能性が考えられた[20]．今後，LcS の HTLV-I に対する作用機序を検討する必要がある．

おわりに

今回のわれわれの検討で，1 日 800 億個の LcS の投与が HAM 患者の臨床症状改善に対して有効であり，さらに安価で安全であるため，HAM の維持療法として有望である可能性が示唆された．LcS が HTLV-1 キャリアーから HAM，および成人 T 細胞性白血病(ATL)の発症の予防に役立つかどうかについても検証したい．

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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)] × 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.

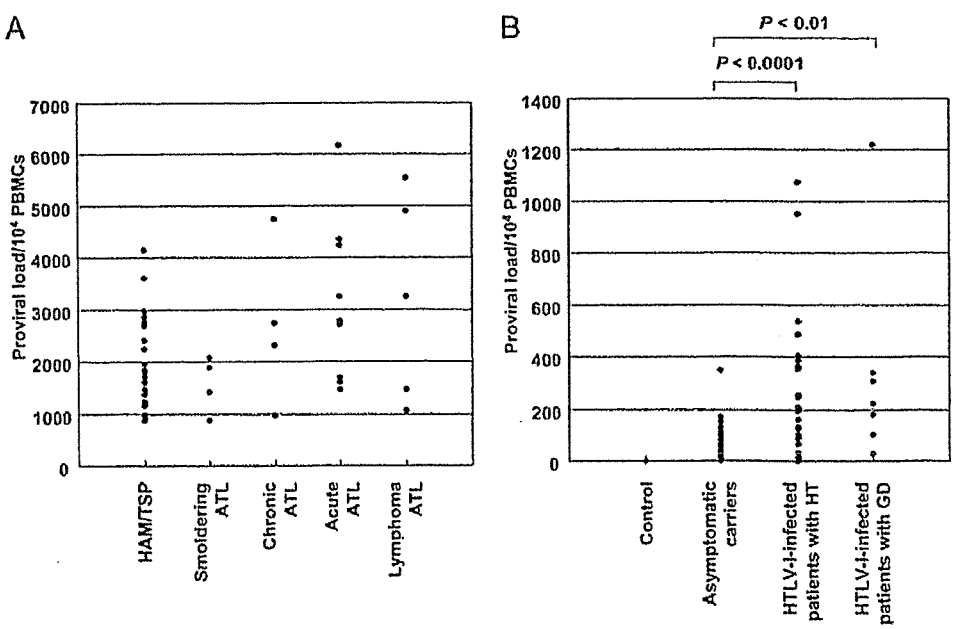

FIG. 1. HTLV-I proviral load in the peripheral blood of HAM/TSP and ATL (A) and healthy individuals without HTLV-I, asymptomatic carriers, and HTLV-I-infected patients with HT or GD (B). Data are HTLV-I copy number per 10^4 PBMCs.

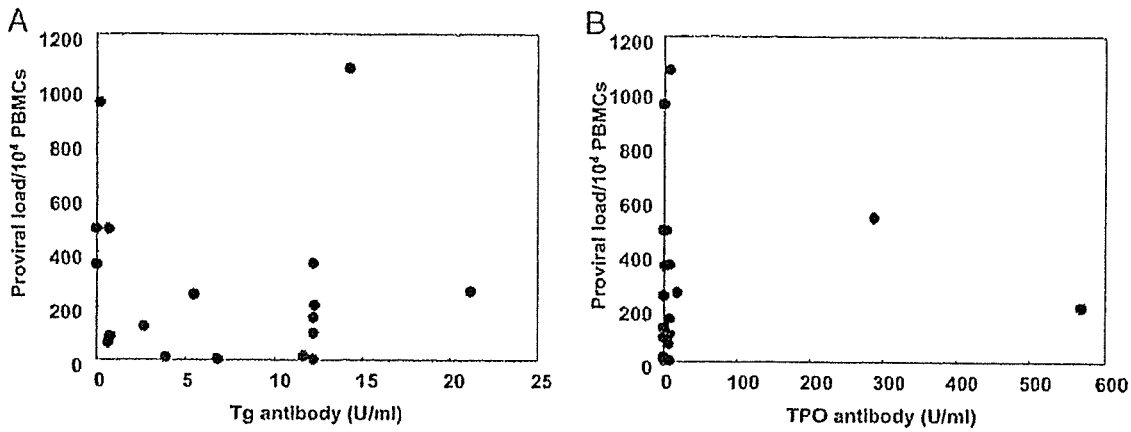

FIG. 2. Correlation between HTLV-I proviral load and antibody titer of Tg (A) or TPO (B) in HTLV-I-infected patients with HT. There was no significant correlation between copy number of HTLV-I proviral DNA and antibody titer of either Tg ($P = 0.6535$) or TPO ($P = 0.4703$).

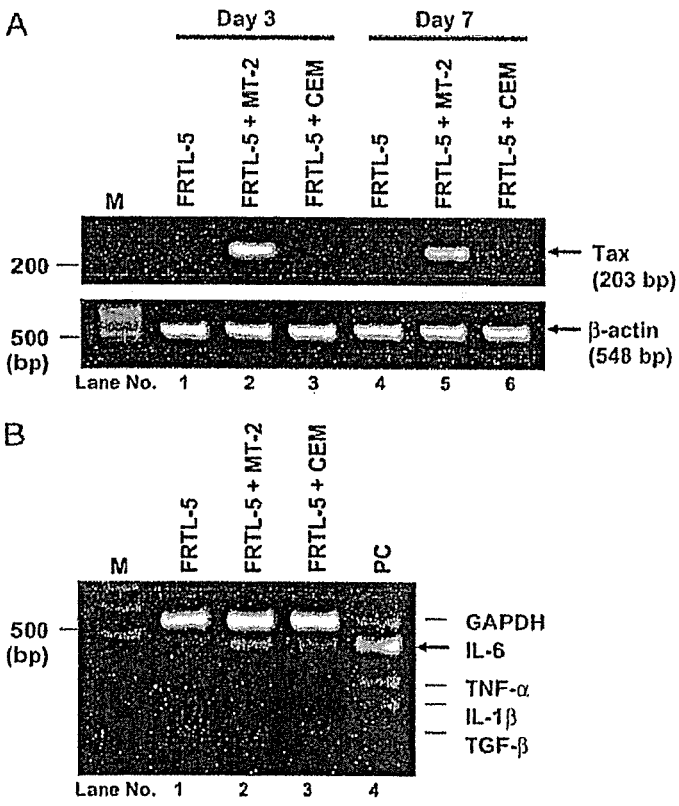

FIG. 3. HTLV-I can infect FRTL-5 cells and induce gene expression of IL-6. A, Detection of HTLV-I Tax mRNA in FRTL-5 cells by RT-PCR. FRTL-5 cells were cocultured with MMC-treated MT-2 or CCRF-CEM cells. At 3 and 7 d after cocultivation, FRTL-5 cells were harvested and then Tax mRNA expression was analyzed. 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).

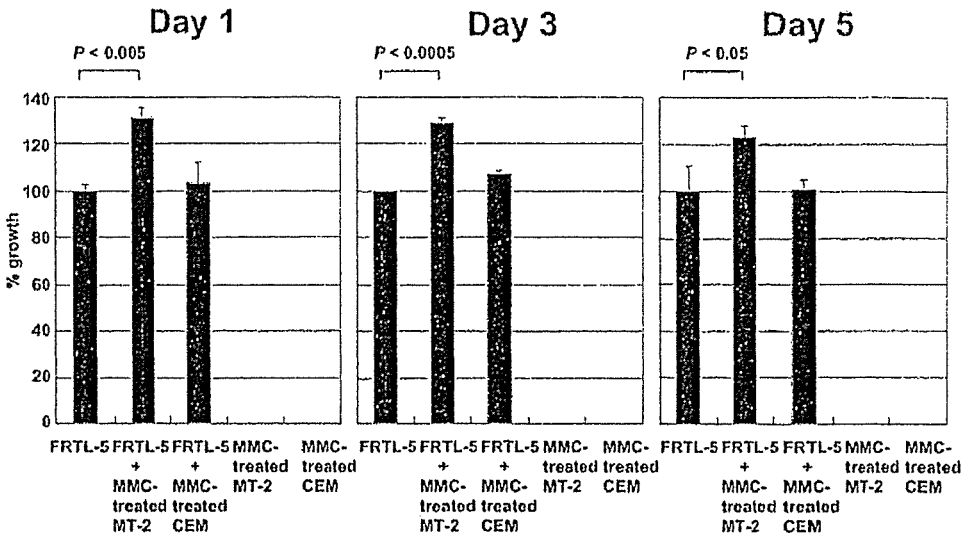

FIG. 4. Proliferation of FRTL-5 cells cocultured with MT-2 or CCRF-CEM cells. FRTL-5 cells were cultured in the presence or absence of MMC-treated MT-2 or CCRF-CEM cells for the indicated time periods. Four hours before terminating the culture, WST-8 was added to each well, and absorbance at 450 nm was 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.

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Short communication

Chronic progressive sensory ataxic neuropathy associated with limited systemic sclerosis

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Abstract

We report the case of a 33-year-old woman with limited systemic sclerosis and chronic progressive sensory ataxic neuropathy. Sural nerve biopsy showed loss of myelinated fibers mostly those of large diameter, axonal degeneration and infiltration of macrophages, but no signs of vasculitis. Physical examination, laboratory testing, neurophysiological and neuroradiological examinations suggested that the dorsal root was primarily affected in this patient. Cytokine analysis by multiplex bead array assay revealed that IL-1β and GM-CSF were increased both in serum and CSF. Although her symptoms did not respond to corticosteroid therapy, intravenous immunoglobulin (IVIg) therapy resulted in marked improvement. IVIg could be effective in case of immune-mediated reversible neuronal dysfunction associated with collagen disease without vasculitis.

Keywords: Chronic progressive sensory ataxic neuropathy; Limited systemic sclerosis; Intravenous immunoglobulin (IVIg); IL-1β; GM-CSF

1. Introduction

Neuropathies are a common neurologic manifestation of diffuse connective tissue disease, and are mainly due to ischemia caused by systemic vasculitis [1]. Systemic sclerosis (SSc) is also a connective tissue disease characterized by abundant fibrosis of the skin, blood vessels, and visceral organs [2]. Although clinical peripheral nervous system disease is regarded as uncommon in SSc [3], recent studies demonstrated that neurological involvement in SSc is more frequent than has been assumed [4]. The most commonly observed neurologic manifestations in SSc are mononeuritis multiplex, trigeminal neuropathy and entrapment neuropathies, such as carpal tunnel syndrome [3]. However, since limited SSc is relatively stable and localized, as opposed to generalized SSc, neurological complications associated with it are rarely reported. Here we report the case of a 33-year-old-woman with limited SSc, complicated by chronic progressive sensory ataxic neuropathy (CSAN) characterized by insidious onset and slowly progressive sensory impairment. Although her symptoms did not respond to corticosteroid therapy, marked improvement was achieved by intravenous immunoglobulin (IVIg) therapy. The pathogenic background and underlying mechanism for efficacious IVIg therapy in this case are discussed.

2. Patient and methods

2.1. Case history

A 33-year-old Japanese woman was admitted to our department for 9 months after the onset of finger swelling with early morning stiffness and Raynaud's phenomenon.

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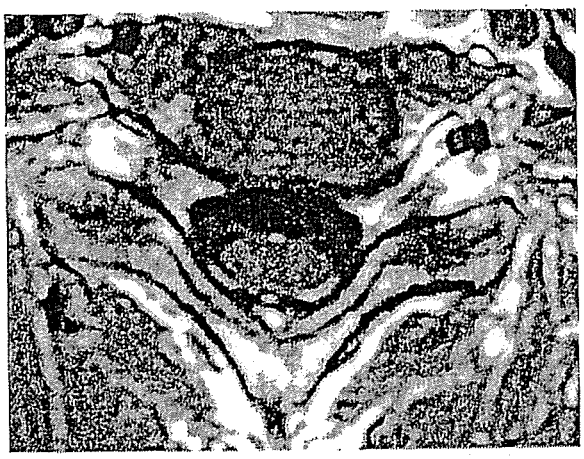

Fig. 1. *Gadolinium-diethylenetriaminepentaacetate (Gd-DTPA) enhanced T2-weighted magnetic resonance imaging at C6–7 levels*. High intensity signals in C6 and C7 dorsal roots were observed on Gd-DTPA enhanced T2-weighted imaging (axial view), indicating on-going inflammation.

Eight months after the onset, she gradually developed varying degrees of numbness and sensory deficit in the extremities, face, and trunk in an asymmetrical distribution, as well as mild muscle weakness and difficulty in swallowing. Her parents were healthy and unrelated. Although there were no neurological disorders in her family, her younger brother underwent right leg amputation because of thromboangiitis obliterans (Buerger's disease). She has a medical history of atopic dermatitis. Personal history includes 11 years of cigarette smoking averaging 30 pieces per day, occasional alcoholic beverage drinking and no toxic substance use. On admission, she presented with dysphagia and finger swelling with Raynaud's phenomenon. Neurological examination revealed facial numbness and hypoesthesia in the right V1 and V2 distributions of the trigeminal nerve, and decreased temperature sensitivity in the left upper and right lower limbs. Decreased superficial sensation was also seen in the distal parts of both upper and left lower limbs. There was absence of deep tendon reflexes and severe impairment of vibratory and position sense in the distal limbs, but Romberg and Babinski signs were not demonstrated. A systemic survey did not detect any malignancy. Blood test disclosed only mild inflammatory signs (ESR: 22 mm/h, IgM: 305 mg/dl, CRP: <0.05). Her serum was positive for anti-centromere antibodies (21.4 U/ml, normal range <10.0) and antinuclear antibody (ANA) (1:640 with a speckled pattern), but negative for anti-SS-A, anti-SS-B and anti-Scl-70 antibodies. CSF analysis showed a protein level of 81.5 mg/dl (IgG: 9.9 mg/dl, IgG index: 0.94) and a white cell count of 2/μL with 100% lymphocytes. Upper gastrointestinal imaging revealed lower esophageal and gastric hypomotility. Biopsy of the minor salivary gland showed sialadenitis with infiltration of mononuclear cells. However, salivary and lacrimal secretion tests as well as salivary gland scintigraphy showed no apparent abnormality. Skin biopsy demonstrated thickening of the dermis. Based on these findings, we diagnosed her as having limited SSc.

2.2. Neurophysiological and neuroradiological examinations

Standard nerve conduction study of the upper and lower limbs (median, ulnar, tibial and sural nerves) was performed. Although motor conduction velocity (MCV), compound muscle action potential (CMAP) and latency studies were normal, sensory action potentials (SNAP) were markedly decreased. Somatosensory evoked potentials (SEPs) showed delayed cortical N20 and P40 potentials but normal inter-peak latency between N20 and P40 (N20–P40), suggesting a lesion of the dorsal root and/or peripheral nerves. In accordance with the elevated CSF protein level, cervical magnetic resonance imaging (MRI) demonstrated gadolinium-diethylenetriaminepentaacetate (Gd-DTPA) enhancement of the C6 and C7 dorsal roots, indicating on-going inflammation (Fig. 1).

2.3. Sural nerve biopsy and anti-neural antibodies

Sural nerve biopsy showed marked decrease in myelinated fibers, mostly those of large diameter, as well as axonal degeneration and infiltration of macrophages, but no signs of vasculitis (Fig. 2). Teased fiber morphology classification according to Dyck et al. [5] was as follows: A+B 18%, C 0%, D 0%, E 77%, F 5% (A: normal; B: myelin irregularity; C: demyelination; D: demyelination and remyelination; E: axonal degeneration; F: remyelination; A and B, considered to be normal, are expressed as A+B). All the anti-neuronal antibodies and anti-ganglioside antibodies examined i.e. both IgG and IgM anti GM1, GQ1b, GD1b antibodies, IgM anti SGPG antibody and anti-Yo, Hu, Ri, CRMR-5, Tr, Ma-2, and amphiphysin antibodies were not detected in her serum (data not shown).

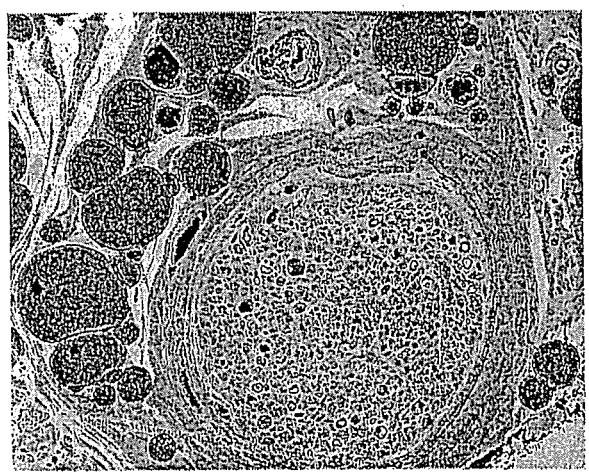

Fig. 2. *Sural nerve pathology of the patient*. Biopsy specimen of the left sural nerve showed marked decrease in myelinated fibers, mostly those of large diameter, as well as axonal degeneration and infiltration of macrophages. However, there were no signs of vasculitis. (Hematoxylin–eosin staining, magnification ×50).

2.4. Cytokine measurement by multiplex bead array assays

Cytokines in serum and CSF were analyzed in duplicate by using Multiplex bead kits (BioSource International, Inc. Camarillo, CA, USA) and Luminex® system (Luminex Corporation, Austin, TX, USA) according to the manufacturers' specifications. Analyzed cytokines were as follows: interferon-γ (IFN-γ), interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF and tumor necrosis factor-α (TNF-α).

2.5. Treatment and outcome

After admission, an initial treatment of high-dose methylprednisolone (1000 mg per day) was administered for 3 days, followed by the conventional dosage of oral prednisone (1 mg/kg/day). Although she felt mild improvement with regards to cold sensitivity in the distal limbs, she developed an apparent Romberg sign, ataxic gait and athetosis of the hands after this treatment. We, therefore administered high-dose (400 mg/kg/day for 5 days) intravenous gammaglobulin (IVIg) therapy. After starting IVIg, she showed noticeable improvement in neurological symptoms, especially the ataxic gait, as well as improvement in morning stiffness and reduction of CSF protein level.

3. Discussion

It is well known that connective tissue diseases are common causes of peripheral neuropathy in a number of patients, mainly due to ischemia secondary to systemic vasculitis [1]. In SSc, the most frequently observed clinical peripheral nervous system disease is trigeminal neuropathy (19%) [6]. Other peripheral neuropathies are also observed although as minor complications (5.6%) [3]. It has been suggested that SSc accounts for only about 1% of all vasculitic neuropathies due to connective tissue diseases [7]. Limited systemic sclerosis is typically preceded by Raynaud's phenomenon, and involves cutaneous sclerosis distal to the elbows with gastrointestinal and pulmonary fibrosis, and anti-centromere antibody positivity [8]. Our case showed all of these findings except for pulmonary fibrosis. The diagnosis of cutaneous limited fibrosis was also supported by skin biopsy. Although Sjögren's syndrome is known to occasionally present with sensory neuropathy with prominent ataxia reflecting kinesthetic sensory impairment [9,10], our case did not fulfill the diagnostic criteria of Sjögren's syndrome [11].

The unique clinical characteristic of our case was severe and progressive sensory impairment, with ataxia as the dominant clinical feature. Neurophysiological findings supported the sensory involvement with preservation of motor nerves. Since electrodiagnostic findings in patients with vasculitic neuropathy such as polyarteritis nodosa (PN) show sensorimotor axonal neuropathy, mainly due to ischemic mononeuropathy or to multiple mononeuropathies, rather than a distal symmetric neuropathy, our case was clearly different from such conditions. Indeed, sural nerve biopsy of this patient showed no vasculitis, although loss of myelinated fibers mostly those of large diameter, axonal degeneration and infiltration of macrophages were observed. Blood test disclosed only mild inflammatory signs, and neither systemic inflammation nor autoantibodies associated with vasculitis, such as antineutrophil cytoplasmic antibodies (ANCA), were observed. However, an elevated protein level without pleocytosis, and high IL-1β and GM-CSF in the CSF of this patient (Table 1) indicated on-going inflammation consistent with cervical MRI findings that demonstrated Gd-DTPA enhancement of the C6 and C7 dorsal roots. Clinical symptoms such as ataxia also suggested that the site of pathology was presumably the dorsal root ganglion (DRG) and dorsal roots. Interestingly, cytokine analysis using Multiplex bead kits and Luminex® system revealed increased IL-4 levels, which is probably associated with her atopic dermatitis, only in her serum but not in CSF. This indicates that IL-4 did not play a dominant role in her CSAN (Table 1). Although she did not respond to corticosteroid therapy, IVIg caused marked improvement in neurological symptoms, especially the ataxic gait, as well as improvement in morning stiffness, and reduction of CSF protein level.

Although its precise mechanisms of action are not entirely known, IVIg has been reported to be effective in many autoimmune neuromuscular diseases including complications of connective diseases [12]. IVIg may have immunomodulatory effects via autoantibodies, complements, Fc receptors, cytokines, adhesion molecules and T-cells in addition to antigen-specific effects in this case [13]. Indeed, we detected increased amounts of IL-1β, IL-2, IL-4, and TNF-α in the serum, and IL-1β and GM-CSF in the CSF of this patient. Since IVIg treatment modulates the production of IL-2, TNF- α and GM-CSF by peripheral blood mononuclear cells and attenuates the intensity of inflammation [14], IVIg could be effective in improving reversible neuronal dysfunction mediated by these cytokines.

Table 1
Cytokine levels in CSF and serum of this case measured by a multiplexed fluorescent bead-based immunoassay

Cytokine (pg/ml)	Serum	CSF
IL-1β	**694.49***	**265.62**
IL-2	**978.57**	33.47
IL-4	**902.13**	53.85
IL-6	N.D.	39.10
IL-8	23.55	18.12
IL-10	13.25	26.40
GM-CSF	88.41	**212.14**
TNF-α	**389.5**	N.D.
IFN-γ	N.D.	39.57

*Values higher than mean+2SD of 32 patients with non-inflammatory neurological diseases (10 motor neuron disease and 22 spinocerebellar ataxia) are shown in bold type. N.D.: not detected.

In conclusion, our case suggested that CSAN could be associated with limited SSc. Both the neurophysiological examination and spinal cord MRI were useful in supporting the clinical diagnosis. In addition, our data suggested the possibility of IVIg as a potentially efficacious therapy for CSAN patients complicated by collagen disease without vasculitis, who did not respond to corticosteroid therapy.

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ORIGINAL PAPER

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Genetic variability in the extracellular matrix protein as a determinant of risk for developing HTLV-I-associated neurological disease

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Abstract Aggrecan, which is a well-known proteoglycan in joint cartilage, also exists in the spinal cord and plays an important role in maintaining water content in the extracellular matrix structure. In this study, we first examined the variable number of tandem repeat (VNTR) polymorphism of the *aggrecan* gene in 227 HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients, in 217 HTLV-I-infected healthy carriers (HCs), and in 85 normal controls. The VNTR allele 28 (1,630 bp) was more frequently observed in HAM/TSP patients than in HCs (χ^2=12.02, p=0.0005, odds ratio 1.79, 95% C.I. 1.29–2.50) and in controls (χ^2=13.43, p=0.0002, odds ratio 2.54, 95% C.I. 1.52–4.25), although this allele was not related to disease progression or to HTLV-I provirus load. We also found that the aggrecan concentration in cerebrospinal fluid (CSF) from rapidly progressive HAM/TSP patients was significantly higher than in slowly progressive patients (corrected p=0.0145) but not in infected non-inflammatory neurological other disease controls (OND) (corrected p=0.078). We then analyzed this aggrecan VNTR polymorphism in the different set of patients with HAM/TSP (n=58) and healthy carriers (n=70). This analysis, again, revealed that allele 28 was detected more frequently in HAM/TSP group than in HCs (χ^2=11.03, p=0.0009, odd ratio 3.04, 95% C.I. 1.55–5.97). The reproducibility of our study was regarded as a second- or third-class association by comparing combined p values and the Better Associations for Disease and GEnes (BADGE) system. Our results suggest that aggrecan polymorphism can be a novel genetic risk factor for developing HAM/TSP.

Keywords Aggrecan · Extracellular matrix · HTLV-I · VNTR · HAM/TSP

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Introduction

HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a chronic progressive inflammatory disease of the spinal cord, which occurs in only 1–2% of HTLV-I-infected individuals (Gessain et al. 1985; Osame et al. 1986). As we have previously reported, that the main HTLV-I-harboring cells in the spinal cord of HAM/TSP patients are not neuronal cells but $CD4^+$ T cells (Moritoyo et al. 1996), a T-cell-mediated immunologic process initiated by HTLV-I infection can be a possible pathological process of HAM/TSP. Although the factors that cause different manifestations of HTLV-I infection are not fully understood, our recent population association studies of more than 200 cases each of HAM/TSP and HTLV-I-infected healthy carriers (HCs) in Kagoshima, an endemic area of HTLV-I infection in Japan, have revealed several important risk factors (Jeffery et al. 1999, 2000; Nagai et al. 1998; Vine et al. 2002). One of the major risk factors for developing HAM/TSP is the provirus load. The median provirus load was approximately 16 times higher in HAM/TSP patients than in HCs, and a high provirus load is also associated with an increased risk for

progression to HAM/TSP (Nagai et al. 1998). We have also reported that *HLA-A*02* and *Cw*08* genes were associated with a lower HTLV-I provirus load and protection from HAM/TSP, whereas *HLA-DRB1*0101* and *B*5401* were associated with susceptibility to HAM/TSP (Jeffery et al. 1999, 2000). Moreover, we have revealed non-HLA genetic risk factors such as TNF−α, SDF-1, and IL-15 (Vine et al. 2002), as well as the association between *HTLV-I Tax* gene variation and the risk for HAM/TSP (Furukawa et al. 2000). From these observations, we now can identify approximately 88% of cases of HAM/TSP in the Kagoshima cohort.

Our detailed clinical analysis of 213 patients with HAM/TSP has revealed that 17% showed arthropathy (Nishioka et al. 1989) characterized by erythema, swelling, and severe pain on moving which mainly occur in large joints (Nakagawa et al. 1995). As the recent study by Levin et al. identified an autoantibody against heterogeneous nuclear ribonuclear protein-A1 (hnRNP-A1) which cross-reacts with HTLV-I Tax protein in IgG isolated from HAM/TSP patients (Levin et al. 2002), it is possible that host recognition of 'self' molecules that mimic HTLV-I contributes to the tissue damage seen in HAM/TSP and its accompanying arthropathy. If this is the case, an immune reaction against a protein that exists in both the spinal cord and joint may be a good candidate for autoantigen.

Human aggrecan is a major extracellular matrix protein expressed in both joint cartilage and the spinal cord, and consists of a core protein and attached glycosaminoglycan (GAG) side chains (Asher et al. 1995; Doege et al. 1991; Milev et al. 1998; Moon et al. 2003; Takahashi-Iwanaga et al. 1998; Watanabe et al. 1998). The reported functions of aggrecan are, first, to maintain the high water content in the extracellular matrix, and second, to act as a barrier against cell migration and a guide for axonal growth in the central nervous system (CNS) along with other chondroitin sulfate (CS) proteoglycans such as phosphocan, neurocan, and versican (Adams et al. 1993; Ang et al. 1999; Asher et al. 2000; Grumet et al. 1993; Moon et al. 2003; Oohira et al. 2000; Perris and Perissinotto 2000). Some reports provide evidence that aggrecan is produced by astrocytes in the perineurial region of the CNS (Matthews et al. 2002; Takahashi-Iwanaga et al. 1998).

Interest in aggrecan function has been increasing as a result of recent research on autoimmune and inflammatory arthritis (Glant et al. 1998; Poole 1998; Zhang et al. 1998b). There are reports showing that aggrecan may act as an immunogenic epitope of T and B cells both in vivo and in vitro. Once the G1 domain has been removed from the core protein of aggrecan by the enzyme aggrecanase (Feng et al. 1998; Zhang et al. 1998a), the molecule discloses a T-cell epitope. It has also been reported that a decrease of CS content elicits a T-cell immune response, whereas a decrease of keratan sulfate (KS) content elicits a B-cell response (Glant et al. 1998).

Based on these findings, we wished to consider the possibility that genetically determined characteristics of extracellular matrix proteins and their degradation are related to the pathogenesis of HAM/TSP. To test this possibility, we analyzed the variable number of tandem repeat (VNTR) polymorphism that was recently identified in the second exon of the *aggrecan* gene, and which encodes a CS attachment site (Doege et al. 1997), in 227 HAM/TSP patients, 217 HCs, and 85 normal controls, and in 58 HAM/TSP patients and 70 HCs. We also examined the protein level of *aggrecan* in both serum and CSF from HTLV-I-infected individuals.

Finally, we have employed a special criterion proposed as the Better Association for Disease and GEnes (BADGE) system (Manly 2005) to assure the reproducibility of our genetic association study. This is because some genetic association studies have problems on reproducibility. In fact, several studies have shown poor reproducibility (Becker et al. 2004; Cardon and Bell 2001; Colhoun et al. 2003; Hirschhorn et al. 2002; Ioannidis et al. 2001; Redden and Allison 2003). This novel system is simple to use and is useful when one needs to estimate reproducibility in the absence of direct experimental replication.

Materials and methods

Study population

The genomic DNA sequences of the *aggrecan* gene were compared among 227 HAM/TSP patients, randomly selected 217 HCs, and 85 normal controls. All cases, HCs, and controls were Japanese and resided in Kagoshima Prefecture, which is an endemic area of HTLV-I infection in Japan. All HCs and normal controls were blood donors and were not related to the patients. The diagnosis of HAM/TSP was made according to the World Health Organization diagnostic criteria (Osame 1990). Sex and ages of subjects were as follows: HAM/TSP group, 69 males and 158 females, 23–76 (mean 57) years old; HC group, 101 males and 116 females, 20–74 (mean 50) years old; control group, 35 males and 50 females, 35–55 (mean 48) years old. The second set of DNA samples were derived from 58 patients with HAM/TSP and 70 HCs from our area. Sex and ages of subjects of this second group were as follows: HAM/TSP group, 20 males and 38 females, 40–65 (mean 50) years old; HC group, 30 males and 40 females, 35–50 (mean 42) years old.

To measure the level of aggrecan in serum and CSF, we used serum samples from 33 HAM/TSP patients and from 11 HCs and CSF samples from 52 HAM/TSP patients, CSF samples from 18 HTLV-I-infected non-inflammatory other disease controls (OND) (five motor neuron disease, four spinocereberal degenaration, two Parkinson's disease, two quadriceps myopathy, one thyroid dysfunction, one essential tremor, one hemifacial spasm, one arrhythmia, and one leg fracture). There was no paired sample of serum and CSF.

We defined rapidly progressive HAM/TSP patients as those who became unable to walk within 3 years after onset of the disease. Sex and ages of rapidly progressive HAM/TSP patients were seven males and 11 females, 48–65 (mean 55) years old, and those of chronic HAM/TSP patients were 11 males and 23 females, 40–64 (mean 54) years old. All samples were taken under written informed consent. The