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Anti-mannose binding lectin antibodies in sera of Japanese patients with systemic lupus erythematosus

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SUMMARY

Mannose-binding lectin (MBL) is a key element in innate immunity with functions and structure similar to that of complement C1q. It has been reported that MBL deficiency is associated with occurrence of systemic lupus erythematosus (SLE). We hypothesized that anti-MBL antibodies, if present, would affect the occurrence or disease course of SLE, by reduction of serum MBL levels, interference of MBL functions, or binding to MBL deposited on various tissues. To address this hypothesis, we measured the concentration of anti-MBL antibodies in sera of 111 Japanese SLE patients and 113 healthy volunteers by enzyme immunoassay. The titres of anti-MBL antibodies in SLE patients were significantly higher than those in healthy controls. When the mean + 2 standard deviations of controls was set as the cut off point, individuals with titres of anti-MBL antibodies above this level were significantly more frequent in SLE patients (9 patients) than in controls (2 persons). One SLE patient had an extremely high titre of this antibody. No associations of titres of anti-MBL antibodies and (i) genotypes of MBL gene, (ii) concentrations of serum MBL, or (iii) disease characteristics of SLE, were apparent. Thus, we have confirmed that anti-MBL antibodies are indeed present in sera of some patients with SLE, but the significance of these autoantibodies in the pathogenesis of SLE remains unclear.

Keywords Lupus/ systemic lupus erythematosus autoantibodies MBL C1q polymorphisms

INTRODUCTION

Both genetic and environmental factors are important in the development of systemic lupus erythematosus (SLE), a systemic autoimmune disease of unknown origin [1,2]. With respect to genetic background, deficiencies in components of the classical pathway of complements (C1q, C1r, C1s, C4 or C2) are known to be major predisposing risk factors for SLE [3–6]. In complement deficiencies, an abnormal clearance of not only immune complexes [3], but also apoptotic cells, has been suggested as contributive towards the occurrence of SLE [7]. Inappropriate levels of apoptotic nuclei are suggested to be a source of autoantigens in SLE [8].

Mannose-binding lectin (MBL) comprises a trimer of three identical polypeptides, and several trimers further combine to form a bouquet-like structure resembling C1q [9]. The MBL gene is located on the long arm of chromosome 10 at 10q11.2-q21 and contains 4 exons [10]. Several polymorphisms have been reported for the MBL gene, and a large interindividual difference in serum MBL concentration among test subjects is caused by the possession of variant alleles. Codon 52, 54 and 57 polymorphisms are all on exon 1, and the presence of any of the minority alleles results in a significant reduction of the serum MBL concentration. Furthermore, homozygosity for minority alleles results in almost complete deficiency of serum MBL [11,12]. This has been attributed to increased degradation of the mutated protein [12]. In the promoter region of the MBL gene, polymorphisms are reported at positions −550, −221 and + 4, and they also greatly influence the levels of serum MBL [13,14]. MBL mediates lectin-dependent activation of the complement pathway [9], and plays an important role in host defense against microorganisms by phagocytosis. Individuals lacking this protein could develop severe episodes of bacterial infections from early life [15–17].

Recently, several studies have suggested that MBL deficiency, or low serum MBL levels caused by polymorphisms in the structural portion or promoter region of the MBL gene, may be associated with occurrence of SLE [18–22]. Two possible explanations for the associations between MBL deficiency and the occurrence of SLE are suggested. Firstly, MBL can bind to and initiate uptake of apoptotic cells by macrophages [23], and an abnormal

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clearance of apoptotic cells caused by MBL deficiency may result in the overexpression of autoantigens. Alternatively, viral infection is believed to be one of causes of SLE [24–26], and MBL deficiency may lead to more frequent infections. On the other hand, deposits of MBL were found in glomerular tissues of SLE patients [27,28], and D-mannose and N-acetylglycosamine, both possible ligands for MBL, are present in the salivary glands of patients with Sjögren's syndrome [29]. In this situation, MBL may have a pathogenic role during the course of SLE.

It has been reported that autoantibodies to C1q are associated with hypocomplementemia and glomerulonephritis [30]. If autoantibodies to MBL, a molecule similar to C1q in structure and functions, are present in patients with SLE, they may: reduce MBL levels; interfere with MBL functions; or bind to MBL deposited to various diseases. We investigated whether anti-MBL antibodies are indeed present in sera of Japanese patients with SLE.

PATIENTS AND METHODS

Patients and controls

Samples used for the study were taken from 111 Japanese patients with SLE, at Division of Rheumatology, Department of Internal Medicine, University Hospital of Tsukuba, Japan. All patients fulfilled the 1997 American College of Rheumatology (ACR) Classification Criteria for SLE. Patients with drug-induced lupus were excluded. The study was approved by the local ethics committee, and written informed consent was obtained from all participants of this study. Medical information including clinical manifestations, and laboratory data were collected simultaneously with sampling. Samples from 113 Japanese healthy volunteers served as controls.

Detection of immunoglobulin G (IgG) binding to MBL

Sumilon S plates (Sumitomo Bakelite, Tokyo Japan) were coated overnight at 4°C with 100 μl/well of recombinant MBL [31] in a carbonate/bicarbonate-buffer (pH 9·6) at a concentration of 1 μg/ml. The plates were washed three times with tris-buffered saline (TBS, pH 7·4) containing 0·05% Tween-20 (TBS/Tw). Unoccupied binding sites were blocked by incubation with 1% bovine serum albumin (BSA) in TBS for 1 h at 37°C. One hundred μl/well of serum samples diluted to 1 : 50 in TBS/Tw containing 0·3% BSA and 1 mM EDTA were added to the wells, and the plates were incubated overnight at 4°C. EDTA was included to inhibit the Ca^{2+} dependent binding of MBL to carbohydrates present on the Fc portion of IgG. All samples were analysed in triplicates. After incubation, 100 μl/well alkaline phosphate (AP)-conjugated goat antihuman IgG, specific for Fab fragment (Sigma, St Louis, MO, USA) diluted 1 : 5000 in TBS/Tw, was added to each well. The microtiter plates were incubated for 1 h at room temperature. Subsequently, alkaline phosphate substrate (Sigma) was added to each well. The plates were incubated for 2 h at room temperature. Optical densities (OD) were measured at 405 nm. The concentration of IgG reactive with MBL is expressed in units/ml of serum (U/ml), where the concentration in a standard sample was defined as 1000 U/ml. Standard curves were generated in all assays performed.

Inhibition assays

Anti-MBL positive sera diluted to 1 : 50 were preincubated with TBS or recombinant MBL at concentrations from 0·1563 μg/ml to 10 μg/ml at room temperature for 1 h. The samples were then put onto MBL-coated plates, and IgG binding to MBL was measured as described above.

Typing of the MBL gene

Genomic DNA was purified from peripheral blood leucocytes using the DnaQuick DNA purification kit (Dainippon Pharmaceuticals, Osaka, Japan), and stored at −30°C. Typing of the MBL gene allele was performed by using the polymerase chain reaction- restriction fragment length polymorphism method according to the methods of Madsen *et al.* [11]. The wild-type allele was designated as allele A, and codon 54 substitution (glycine to aspartic acid) was designated as allele B. Previous studies have shown that codon 52 and 57 polymorphisms are not present or extremely rare in the Japanese population [32,33].

Measurement of the serum MBL concentration by enzyme immunoassay

Serum concentration of MBL was measured by a specific enzyme immunoassay utilizing two rabbit polyclonal anti-MBL antibodies as described previously [31]. All samples were stored at −80°C and no previous freeze/thaw was done.

Statistics

Mann–Whitney *U*-test, Fisher's exact test, chi-square analysis and Spearman's rank correlation test were used. *P*-values of <0.05 were considered to be statistically significant.

RESULTS

Detection of autoantibodies to MBL in patients with SLE

Titers of IgG reactive with human MBL in patients with SLE were significantly higher than those in healthy controls; $P < 0.0001$, median MBL concentration ± standard deviation (s.d.); 47·4 ± 49·3 and 30·6 ± 29·2, in SLE patients and healthy controls, respectively (Fig. 1). The assay was performed in the presence of EDTA in order to inhibit the binding between the carbohydrate recognition domain of MBL and carbohydrates on the Fc portion of IgG. Furthermore, selected samples were digested with pepsin and $F(ab')_2$ fragments were purified. $F(ab')_2$ fragments did bind to MBL coated plates, indicating that IgG–MBL interaction detected in this assay is indeed antigen-antibody binding (results not shown). We found a patient with an extremely high level of serum anti-MBL, and the titre of anti-MBL antibodies in the serum of this patient was designated 1000 U/ml. The number of subjects having a titre of more than 2 sd. above the average of healthy controls (89·5, indicated by dotted line in Fig. 1) was 9 of the patients with SLE, and 2 of the healthy controls. This difference was statistically significant ($P = 0.0341$ by Fisher's exact test).

A titration curve could be adequately drawn using serial dilutions of the standard serum (Fig. 2a). In addition, adding excess amounts of recombinant MBL to diluted standard serum inhibited the binding of IgG to solid phase MBL in a dose dependent manner (Fig. 2b).

Associations between levels of anti-MBL antibodies, and MBL gene genotypes or serum concentrations of MBL in patients with SLE

Serum MBL concentrations reflected the MBL genotype of the individual in accordance with previous reports (Fig. 3) [11,12].

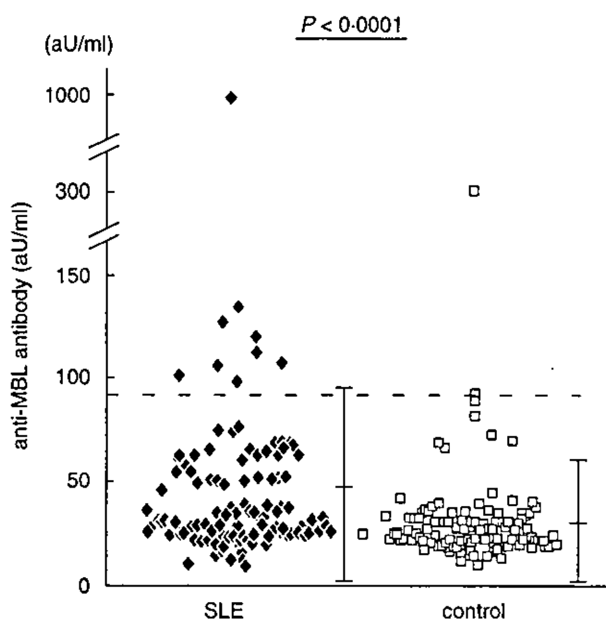

Fig. 1. Autoantibodies to mannose-binding lectin (MBL) in serum samples. Anti-MBL antibodies were measured in 111 samples from patients with systemic lupus erythematosus (SLE) and in 113 samples from healthy controls, in the presence of EDTA (1 mM). Dotted line indicates 2 standard deviation (s.d.) above average in healthy controls. *P*-value by Mann–Whitney *U*-test. aU, arbitrary units.

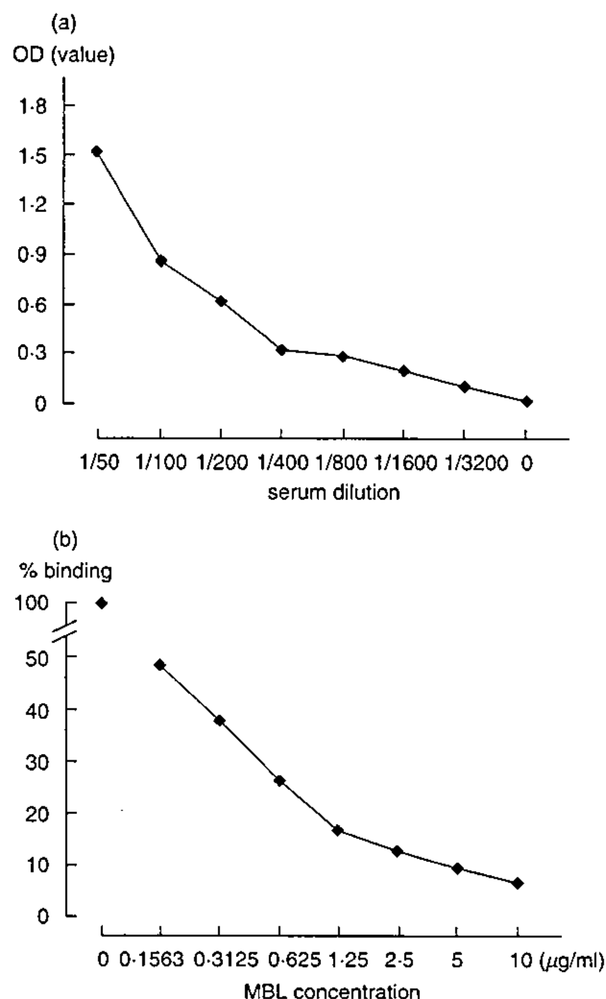

Fig. 2. Titration curve and inhibition assay for autoantibodies to mannose-binding lectin (MBL). (a) Titration curve for anti-MBL antibodies using serial dilutions of the standard serum in the presence of EDTA (1 mM). (b) Inhibition assay for anti MBL antibodies adding excess amount of recombinant MBL to diluted standard serum in the presence of EDTA (1 mM).

Serum MBL concentrations in SLE patients were not significantly different from those in healthy individuals ($P = 0{\cdot}5296$). Among individuals with the same genotype, SLE patients tended to have higher MBL concentrations than controls, but without statistical significance (AA; $P = 0{\cdot}3385$, AB; $P = 0{\cdot}5556$, BB; $P = 0{\cdot}1573$ by Mann–Whitney's *U*-test).

We next examined whether genotypes of the MBL gene in patients with SLE are associated with levels of anti-MBL antibodies (Fig. 4). Titres of anti-MBL antibodies tended to be lower in patients with allele B (AA; $60{\cdot}15 \pm 133{\cdot}3$, AB; $50{\cdot}10 \pm 26{\cdot}95$, BB; $38{\cdot}23 \pm 18{\cdot}88$), but no significant differences were observed.

Finally, we compared the serum concentrations of MBL and titres of anti-MBL antibodies in patients with SLE. We found no significant relationship between them (Fig. 5).

Relationships between the presence of anti-MBL antibodies in sera, and clinical characteristics or disease parameters of SLE

We investigated whether patients having anti-MBL antibodies at titres above 2 sd. of the average in healthy controls had some significant clinical characteristics (Table 1). No significant associations were observed. However, patients with higher serum concentration of anti-MBL antibodies tended to have a lower occurrence of anti-DNA antibodies, although statistical significance was not achieved. The incidence of infections requiring hospitalization during their course of SLE was not significantly higher in patients with higher serum concentration of anti-MBL antibodies.

We next analysed whether or not titres of anti-MBL antibodies are associated with various disease parameters of SLE in 111 SLE patients. Anti-DNA antibodies and total IgG tended to be positively related with anti-MBL antibodies, but statistical significance was not achieved. No other correlation was observed (Table 2).

DISCUSSION

In this study, we found the presence of autoantibodies against MBL in some patients with SLE. This is in accordance with the study by Seelen *et al.* [34], which was published very recently.

We confirmed that we were indeed detecting anti-MBL antibodies by; addition of EDTA in the enzyme immunoassay, thereby inhibiting the Ca^{2+} dependent binding of carbohydrate recognition domain on MBL to carbohydrates on IgG; digesting IgG with pepsin, and confirming that the binding region of IgG was on $F(ab')_2$; and detecting an inhibition of aqueous MBL to the binding of IgG to solid phase MBL. These methods and results are similar to those reported by Seelen *et al.* [34], except that we did detect dose dependent inhibition by our inhibition assay. The reason for this discrepancy is unclear, but may possibly be due to

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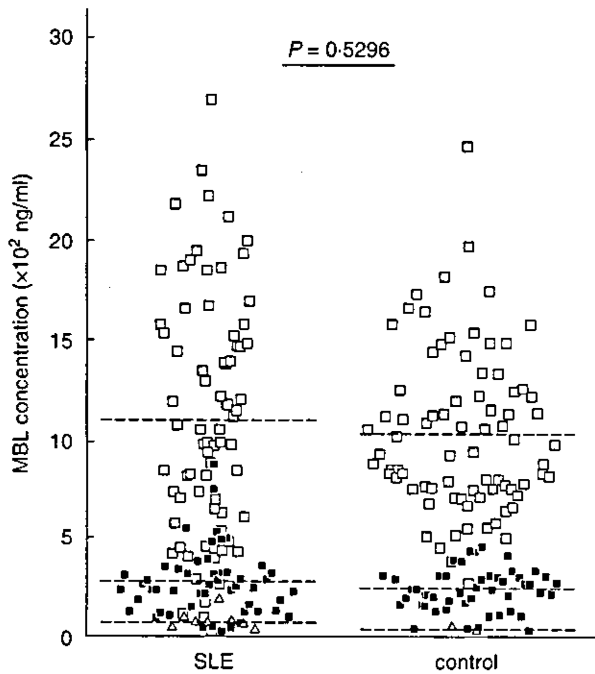

Fig. 3. Serum mannose-binding lectin (MBL) concentrations in 111 patients with systemic lupus erythematosus (SLE) and 113 healthy controls. Subjects with homozygosity for the codon 54 wild-type allele (□), subjects with heterozygosity for the codon 54 variant (■), and subjects with homozygosity for the codon 54 variant allele (Δ) are indicated in both patients with SLE and healthy controls. Dotted lines indicate average of titres of serum MBL concentrations in each genotype on both groups. *P*-value by Mann–Whitney *U*-test.

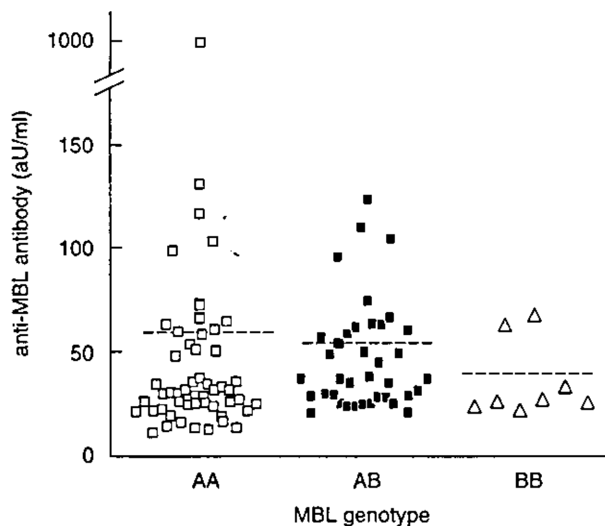

Fig. 4. Association between genotypes of the mannose-binding lectin (MBL) gene and levels of anti-MBL antibodies in patients with systemic lupus erythematosus (SLE). AA; homozygosity for the codon 54 wild-type allele, AB; heterozygosity for the codon 54 variant, BB; homozygosity for the codon 54 variant allele. Dotted lines indicate average of titres of anti-MBL antibodies in each genotype. aU, arbitrary units.

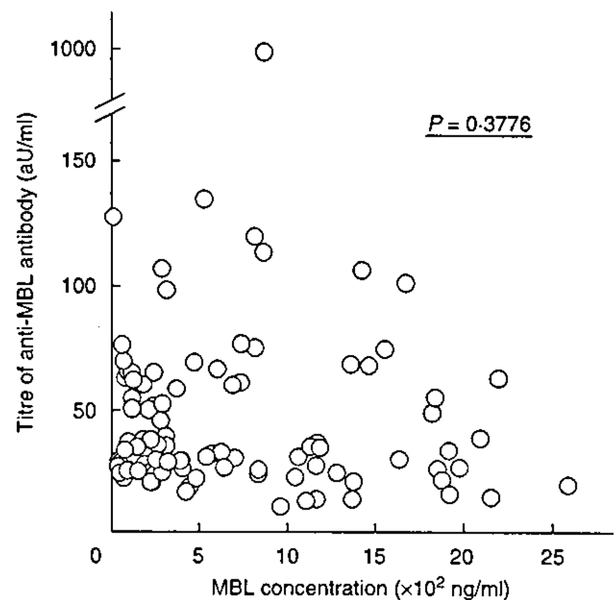

Fig. 5. Association between titres of anti mannose-binding lectin (MBL) antibodies and concentrations of MBL in systemic lupus erythematosus (SLE) patients. *P*-value by Spearman's rank correlation test. aU, arbitrary units.

Table 1. Disease characteristics of 111 patients with systemic lupus erythematosus (SLE) categorized by positivity of anti-mannose-binding lectin (MBL) antibody

	Positive (n = 9)	Negative (n = 102)	*P*-value
Malar rash	3	44	0·7309
Discoid lupus	0	13	0·5951
Photosensitivity	1	22	0·6821
Oral ulcers	2	20	0·9999
Arthritis	5	59	0·9999
Serositis	4	22	0·2099
Renal disorder	1	29	0·4399
Neurological disorder	0	9	0·9999
Haematologic disorder			
Haemolytic anemia	0	8	0·9999
leukopenia	4	52	0·7422
lymphopenia	4	48	0·9999
thrombocytopenia	1	27	0·4447
Anti-ds DNA Ab	4	74	0·1225
Anti-Sm Ab	0	8	0·9999
Antiphospholipid Ab	3	18	0·3673
ANA	8	95	0·5033
Infections requiring hospitalization	3	29	0·7155

Anti-MBL antibody positive was defined as having a titre higher than mean +2 s.d. of 113 healthy individuals. Serositis, pleuritis or pericarditis; renal disorder, proteinuria or cellular casts; neurological disorder, seizures or psychosis; Anti-ds DNA Ab, anti-double strand DNA antibody; Anti-Sm Ab, anti-Sm antibody; Antiphospholipid Ab, antiphospholipid antibody. P = AA + AB versus BB by chi-square analysis.

Table 2. Associations of titres of anti-mannose-binding lectin (MBL) antibody and various disease parameters of systemic lupus erythematosus (SLE) in 106 SLE patients

Disease parameters of SLE	*P*-value[a]
Anti-DNA antibody	0·2173
C3	0·8844
C4	0·2131
CH50	0·7919
IgG	0·0665
IgA	0·9026
IgM	0·1637

[a]Spearman's rank correlation test.

the nature of anti-MBL antibodies in individual patients, or concentrations or conformations of MBL used in the assays.

Similarities in structure and function exist between MBL and C1q, and it is known that C1q-deficient or anti-C1q antibody positive individuals have a high probability of developing SLE [5,30,35,36]. It has been reported that MBL deficiency may be associated with the occurrence of SLE [18–22], although deficiency of MBL is not an extremely high risk factor, in contrast to deficiencies of other complement molecules such as C1q. The presence of autoantibodies against MBL may cause similar pathological conditions to those found in MBL deficiency, as with the case of anti-C1q antibodies. In this context, it is noteworthy that a previous study has shown that anti-C1q antibodies do not recognize MBL [37], which suggests that anti-MBL and anti-C1q antibodies are not identical.

In accord to previous studies, serum MBL concentrations were closely associated with the MBL genotypes of the individuals studied (Fig. 3). However, in this study, no significant differences in serum MBL concentrations were observed between SLE patients and healthy controls, when individuals with the same genotype were compared. This is different from the study by Seelen *et al.* [34], where they found that serum MBL concentrations were higher in SLE patients than in controls. This difference may be due to differences in MBL genotype distributions or disease activities of SLE in the individuals studied, or other unknown factors.

We next asked whether there is any association between levels of anti-MBL antibodies and MBL genotypes. No such correlation was observed (Fig. 4). However, levels of anti-MBL antibodies in patients having genotype AB were higher than those in patients with genotype AA, if we excluded one patient with genotype AA with an extremely high level of anti-MBL antibodies (Fig. 4). In addition, some genotype BB patients had anti-MBL antibodies (Fig. 4). We went on to study the relationship between serum MBL concentration and levels of anti-MBL antibodies. There was no statistically significant relationship (Fig. 5). These findings support the notion that elevated serum MBL is not a causative factor for anti-MBL antibody production, and other factors should contribute to the production of these autoantibodies. One possible factor is the production of mutated MBL protein in genotype AB or BB individuals. Individuals with genotype AB or BB produce a mutated MBL protein which is degraded in sera, since they are unable to form a stable oligomerized structure [12,38]. These degraded MBL protein products may have a role in the occurrence of anti-MBL antibodies. However, at this point, this remains only a speculation. Other factors must be important as well, since some patients with genotype AA also have anti-MBL antibodies. Many questions need to be solved, before the mechanisms of autoantigen recognition and autoantibody production including anti-MBL antibodies could be clarified.

We examined the disease characteristics of SLE in anti-MBL antibodies positive patients (Table 1). There were no significant relationships between the possession of a significantly high titre of anti-MBL antibodies, and the characteristics or parameters of SLE. This is in accord with the report by Seelen *et al.* [34], which showed no difference between anti-MBL levels in sera of patients with active disease and inactive disease, especially concerning renal involvement. However, among patients having high titre of anti-MBL antibodies, smaller number of patients tended to have anti-DNA antibodies, and more patients (3 of 9 patients, 33%) developed intestinal pneumonitis, which usually occur in less than 10% of SLE patients [39]. Thus, we felt that some cases had somewhat atypical features of SLE. Whether this is only a coincidence or not is unclear. A study of larger number of patients should be done to clarify the clinical significance of anti-MBL antibodies in SLE.

It has been reported that individuals lacking MBL are prone to severe episodes of bacterial infections from early life [15–17]. A recent study has shown that presence of MBL minority alleles is a risk factor for infection in patients undergoing bone marrow transplantation [40]. It is also reported that the MBL deficiency, resulting from the possession of the variant alleles of the MBL gene, is a risk factor in patient receiving immunosuppressive therapy [19,20]. Although we anticipated that decreased MBL function caused by anti-MBL antibodies might lead to more frequent infections during the course of SLE, we could not find, in the present study, any significant associations between the presence of anti-MBL antibodies and the occurrence of infections requiring hospitalization after initiation of therapy of SLE. The effect of anti-MBL antibodies to increased susceptibility to infections in individuals under immunosuppressive therapy may not be as large as that caused by MBL gene polymorphisms. Since only 9 patients had significantly high titre of serum anti-MBL antibodies, a larger study is necessary to confirm this observation.

In conclusion, we detected anti-MBL antibodies in sera of patients with SLE. However, we could not find any significant relationships with MBL genotype, clinical characteristics and parameters of SLE in this study. Further studies are necessary to elucidate the actual functions of autoantibodies to MBL in the pathogenesis of SLE, and to determine the value of measuring these autoantibodies in clinical practice.

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ORIGINAL ARTICLE

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HTLV-I env protein acts as a major antigen in patients with HTLV-I-associated arthropathy

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Abstract Our objective was to investigate the pathological mechanisms of HTLV-I (human T-cell leukemia virus type I)-associated chronic arthritis (HAAP) with respect to T-cell response to HTLV-I viral proteins. We examined T-cell clonality and the antigen recognized by T cells from the inflamed synovium of patients with HAAP by using histology, a single-strand conformation polymorphism (SSCP) analysis and T cell receptor (TCR) sequencing. The SSCP analysis showed oligoclonal expansion of T cells in the synovium, suggesting an antigen-mediated stimulation. In contrast, there was less clonal expansion in peripheral blood lymphocytes (PBL). The expression of HTLV-1 env and tax mRNA was detected in the affected synovium as well as in PBL. A number of T-cell clones in the synovium recognized HTLV-I env and tax proteins. Twenty-seven (24.9%) of 109 examined T-cell clones in the joints were HTLV-I env reactive, and 7 clones (6.4%) were HTLV-I tax reactive. Junctional sequence analysis of synovial T cells showed a lack of highly conserved amino acid motifs in the complementarity-determining region 3 (CDR3) of HTLV-I env and tax reactive T cells, suggesting that these cells recognized multiple T-cell epitopes on HTLV-I antigen. These findings suggest that HTLV-I env protein acts as a major antigen and may play a role in the development of arthropathy in patients with HAAP.

Keywords Arthropathy · HTLV-I · Rheumatoid arthritis · T-cell receptor

Abbreviations HAAP: HTLV-I-associated chronic arthritis · HTLV-I: Human T-cell leukemia virus type I · MBP: Myelin basic protein · PBL: Peripheral blood lymphocytes · SSCP: Single-strand conformation polymorphism · TCR: T-cell receptor

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Introduction

Human T-cell leukemia virus type I (HTLV-I), the etiologic agent of adult T-cell leukemia (ATL) [1], is associated with several inflammatory diseases, such as chronic myelopathy (HTLV-I associated myelopathy/tropical spastic paraparesis, HAM/TSP) [2, 3], uveitis [4], Sjögren's syndrome [5] and chronic arthritis (HTLV-I associated arthropathy; HAAP) [6, 7]. More interestingly, the transgenic mouse carrying HTLV-I tax region develops synovial inflammation with articular erosions resembling rheumatoid arthritis (RA) [9, 10]. There is ample evidence from in vivo [8], in vitro [10] and epidemiological studies [21] to suggest that HTLV-I is involved in RA-like arthritis.

Previous studies from our laboratory investigating the pathogenesis of HAAP showed infection of synovial cells by HTLV-I in inflamed joints [12], and that HTLV-I tax protein acted as a transactivator and directly caused proliferation of synovium and joint destruction [13]. In the case of HAM/TSP, HTLV-I tax specific

CD8+ cytotoxic T lymphocytes (CTL) accumulate in inflamed lesions [14, 15, 16, 17, 18], and that their complementarity-determining region 3 (CDR3) sequences of the T-cell receptor (TCR) are highly conserved, suggesting that the immune response to HTLV-I viral protein may play a role in the pathogenesis of HTLV-I-associated neurologic disease [15]. However, the role of immune reactions to HTLV-1 remains unclear in HAAP. Therefore we here investigated the clonality of T cells that infiltrated the inflamed synovium and the reactivity of the expanded T-cell clones to the HTLV-1 env and tax protiens in HAAP by using a single-strand conformation polymorphism (SSCP) analysis of TCR genes and histological studies. As a result, oligoclonal accumulation of expanded T cells in the synovium through an antigen-mediated stimulus was demonstrated. Further, a considerable proportion of the expanded T-cell clones in the synovium was found to recognize the env and tax proteins, especially env. These findings provide evidence that HTLV-I env proteins act major antigens in the inflamed lesions and may play a crucial role in the development of chronic arthritis in HAAP.

Materials and methods

Patients and cell preparation

Four patients diagnosed with HAAP exhibited marked swelling of the joints associated with destruction of the bone and joints, and were positive for anti-HTLV-I antibody (Ab). Their average age, disease duration, erythrocyte segmentation rate and CRP was 56.2 years, 8.5 years, 69/mm and 1.67 mg/dl, respectively. Synovial tissues were collected from a knee joint of each patient at the time of synovectomy, with informed consent. Simultaneously, peripheral blood was also obtained from all the patients.

Typing of HLA-DR and -DQ alleles was performed by PCR combined with dot-blot hybridization using a sequence-specific oligonucleotide probe (PCR-SSOP) according to the protocol described by the Eleventh Histocompatibility Workshop [19].

Immunohistochemical study and in situ reverse transcriptase assay

Tissue samples were cut into 5–7 μm sections and mounted on to glass slides, followed by immunohistochemical examination or in situ reverse transcriptase (RT) assay as described previously [20]. For immunohistochemical examination, CD3, CD4, CD8, CD20 and CD68 monoclonal antibodies (mAb, Dako Japan, Tokyo) were used for detection of surface markers [21]. The anti-CD4 antibody and the other first antibodies were diluted at 1:20 and 1:50, respectively, for the staining, and were detected by Envision Kit/HRP (DAB) Universal (Dako). Non-specific mouse IgG was used instead of the first antibodies in negative control staining.

RT assay was performed to detect HTLV-I env and tax mRNA using primers complementary to mRNA segments. HTLV-I env primer was 5'-CTCGAGCCCTCTATACCATG-3' and HTLV-I tax primer was 5'-ATCCCGTGGAGACTCCTCAA-3'. The slides were visualized by antidigoxygenin Fab Ab (Boehringer Mannheim, Germany) followed by digoxygenin detection system (Kreatech biotechnology BV, Amsterdam, The Netherlands). Negative control studies were performed in each experiment by using a complementary primer [22] (env: 5'-CATGGTATAGAGGGCTCGAG-3'; tax: 5'-TTGAGGAGTCTCCACGGGAT-3').

Preparation of HTLV-I tax-GST and env-GST fusion proteins

Recombinant HTLV-I tax and env proteins were produced as glutathione S-transferase (GST) fusion proteins. Briefly, PCR products encoding HTLV-I tax (pX-IV region; amino acid 1–245) or env (amino acid 1–386) from MT-2 cell lines were inserted into a GST expression vector (pGEX-3X; Pharmacia, Uppsala, Sweden) and the resultant proteins were purified by affinity chromatography using glutathione agarose. The purity of proteins was examined by SDS-PAGE and Western blot using anti-HTLV-I tax mAb (a kind gift from Dr Hatanaka, Kyoto University, Japan) or anti-HTLV-I-env mAb (Fujirebio Inc., Tokyo).

Co-culture of PBL with HTLV-I proteins

To analyze the HTLV-I tax or env reactive T-cell clones in peripheral blood lymphocytes (PBL), PBL were stimulated with tax-GST or env-GST protein, as well as GST protein alone as a control. Briefly, PBL were obtained from the HAAP patients and 10^7 cells were co-cultured with tax-GST, env-GST and GST fusion proteins at a concentration of 25 μg/ml. Seven days after the co-culture, cells were collected and total RNA was extracted.

For further study to determine CD4 or CD8 T-cell subsets in HTLV-I tax- and env- reactive clones, CD4 or CD8 expressing T cells obtained from HAAP patients were negatively selected by using Dynabeads (Dynal Inc., Great Neck, NY). Separated and unseparated cells were cultured with tax-GST and env-GST fusion protein, followed by the cDNA synthesis, described as above.

Preparation of RNA and PCR/Southern blot analysis

PBL from four patients with HAAP and three healthy controls was cultured with tax and env proteins for 7 days, and total RNA (1–5 μg) was prepared with Isogen

(Nippon Gene Co., Tokyo). cDNA synthesis and PCR were conducted using methods described by Sumida et al. [23]. Briefly, first strand cDNA were synthesized in a 20-μl reaction mixture containing oligo(dT) primer using 1 μg of total RNA; 0.5 μl of the obtained cDNA solution was then used for PCR amplification of each of the tested genes. PCR was performed with primers specific for HTLV-I tax (mRNA/RPX3 and mRNA/RPX4), env (Env/SG221 and Env/SG227), β-actin, TCR BV, or TCR AV genes [8, 12, 24, 25]. Denaturing was performed at 94°C for 1.5 min, annealing at 60°C for 1.5 min, and extension at 72°C for 1.0 min, for 30 cycles on a DNA thermal cycler (Perkin-Elmer Corporation, Norwalk, CT). One-tenth of the PCR products (tax: 144 bp; env: 327 bp; β-actin: 218 bp) was subjected to 2% agarose gel electrophoresis and hybridized with digoxygenin-labeled PCR product probe encoding HTLV-I tax, env or β-actin gene, as described previously [12].

In the case of DNAse treatment, total RNA (1–5 μg) was incubated with RNAse-free DNAse (Boehringer Mannheim, Germany) in a reaction solution containing 25 mM Tris-HCl, 5 mM $MgCl_2$, and 0.1 mM EDTA (pH 7.2) for 1 h at 37°C. After DNAse treatment, RNA was prepared with Isogen again, followed by cDNA synthesis and PCR using conditions similar to those described above.

PCR-SSCP analysis for TCR BV and AV genes

PCR products encoding TCR BV and TCR AV genes were treated as described above. Amplified DNA was diluted (1:20) in a denaturing solution (95% formamide, 10 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol) and kept at 90°C for 2 min. The diluted sample (2 μl) was electrophoresed in non-denaturing 5% polyacrylamide gel containing 10% glycerol. The gels were run at a constant power of 35 W for 2 h. After electrophoresis, the DNA was transferred to immobilon-S (Millipore Intertech, Bedford, MA) and hybridized with biotinylated BC probe (5' -A(AC)AA(GC)GTG-TTCCCACCCGAGGTCGTGTT-3'), or biotinylated TCR AV probe (5'-CCAGCTGAGAGACTCTAAA-TCCAGTGACAAG-3'), streptavidin, biotinylated alkaline phosphatase, and a chemiluminescent substrate system (Plex Luminescence Kit, Millipore Intertech).

Sequencing of cDNA encoding TCR BV, HTLV-I env and tax genes

Complementary DNA encoding the TCR BV genes from HAAP synovial tissues were purified from polyacrylamide gels for SSCP and amplified by PCR using primers specific for each TCR BV gene, as described previously [23]. cDNA encoding HTLV-I env and tax genes were also amplified by PCR. Fragments with the expected DNA sizes were enriched by preparative low-melting-point agarose gel electrophoresis. The recovered DNA fragments were ligated to pCR2 vector (Invitrogen, San Diego, CA). Following transformation with the relevant construct in INVaF' competent cells, white colonies were selected, examined for correct insert by PCR using the same primers, and subjected to a large-scale plasmid purification using cesium chloride for plasmid DNA isolation. Following purification of the plasmid, automated sequencing was performed using ABI 373 (Applied Biosystem, Foster City, CA).

Statistical analysis

Data were expressed as mean ± SEM. Differences between groups were examined for statistical significance using the Mann–Whitney U test. A p value < 5% denoted a significant difference.

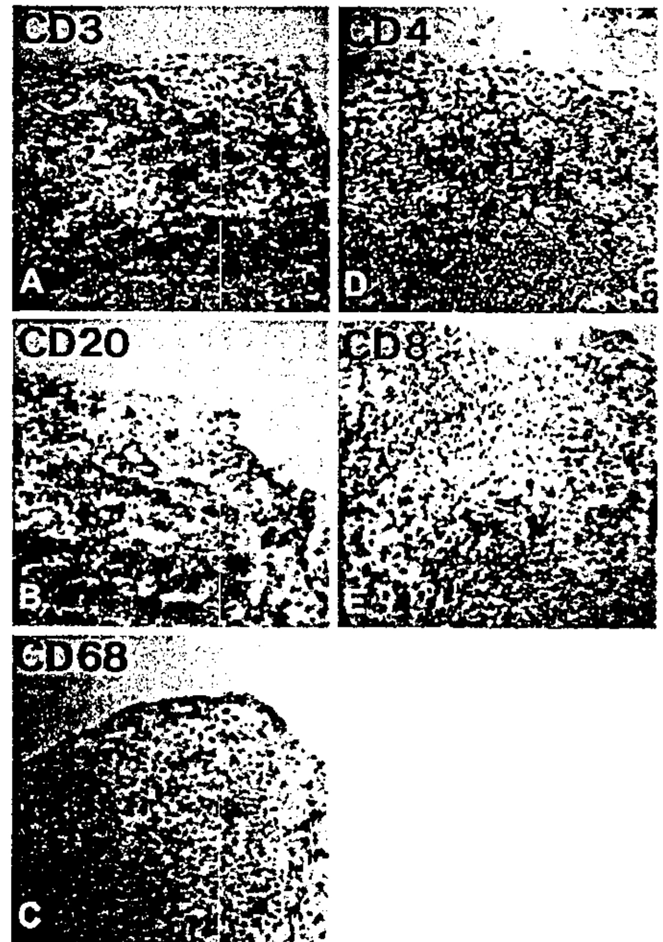

Fig. 1 Immunohistochemical examination of a synovial tissue sample from a representative patient using CD3 (**A**), CD20 (**B**), CD68 (**C**), CD4 (**D**) and CD8 (**E**) monoclonal antibodies. Note that the majority of infiltrating cells are CD4 T cells, whereas CD68 cells are present in the lining layer. The different sections represent sequential sections. (HAAP-2, magnification x200)

Results

Expression of HTLV-I env and tax mRNA in arthritic joints

Histological examination of synovial tissue samples of patients with HAAP showed hyperplasia of the lining synovial cells and marked infiltration of mononuclear cells. Immunohistochemical examination using mAbs against CD3, CD4, CD8, CD20 and CD68 showed that the infiltrating cells were mainly CD3+ T cells, and the majority of them were CD4+ T cells (Fig. 1). Although CD68+ cells were present in the lining layer, the majority of non-mononuclear cells were CD68$^-$ synoviocytes, as confirmed by morphological examination. These findings were similar to those of our previous study, demonstrating that the immunopathological features of HAAP synovium resembled those of RA [26]. To examine whether HTLV-I env and tax genes were expressed in the joints, we analyzed mRNA of HTLV-I tax and env using in situ RT assay. The results showed that HTLV-I env mRNA was expressed in a broad range of synovial cells from lining to deeper layer, whereas HTLV-I tax mRNA was detected in a few cells in the deeper layer only (Fig. 2). Ssequential analysis of immunostained sections demonstrated that HTLV-I tax and env mRNA were expressed mainly in synovial cells and in a few T cells infiltrating the synovium. In contrast, there were no signals using antisense primers for HTLV-I env and tax genes as controls (Fig. 2B, D).

We also performed a PCR-Southern blot analysis to confirm the expression of HTLV-I env and the second spliced tax mRNA. The results showed the expression of HTLV-I env and tax mRNA in the synovium as well as PBL in all cases; however, the amount of the expressed env mRNA was larger in synovium than in PBL in all patients (Fig. 3a). The expression of HTLV-I env mRNA was found in all synovia from patients with HAAP, even if mRNA was treated with RNAse-free DNAse (Fig. 3b).

HLA typing of four patients with HAAP showed that two patients (HAAP 1 and 2) used DRB1*0405 (Table 1). The other two patients (HAAP 3 and 4) possessed identical HLA-DR and DQ alleles, including DRB1*0401. DRB1*0401 and *0405 are reported to associate with RA, containing shared epitopes. The HLA type of HAAP 3 and 4 is an unusual one among Japanese (a frequency of 1/100). These results suggest a relationship between HAAP and the HLA-DR or genetic identity among Japanese in Tsushima.

Accumulation of oligoclonal T-cell clonotypes in inflammatory synovium of HAAP patients

To analyze whether accumulated T cells in inflamed joints expanded oligoclonally or polyclonally, the

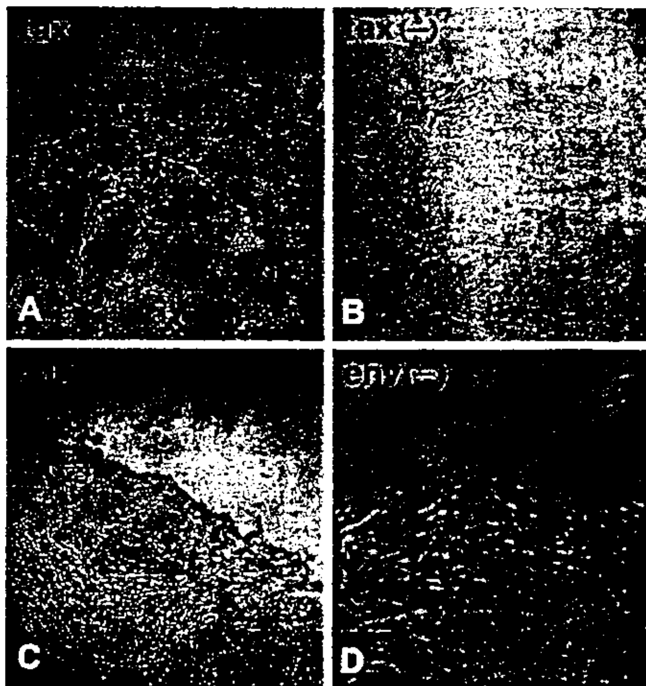

Fig. 2 In situ RT assay of sequential sections as in Figure 1 (HAAP-2) to examine the expression of HTLV-I tax (**A: tax**) and env (**C: env**) mRNA. Note the lack of signals using antisense primers for HTLV-1 tax (**B: tax-**) and env (**D: env-**) genes as controls. Note also the expression of env and tax mRNA mainly on synovial cells in the lining layer, although a few mononuclear cells in the deeper layer also expressed these mRNA (magnification X200)

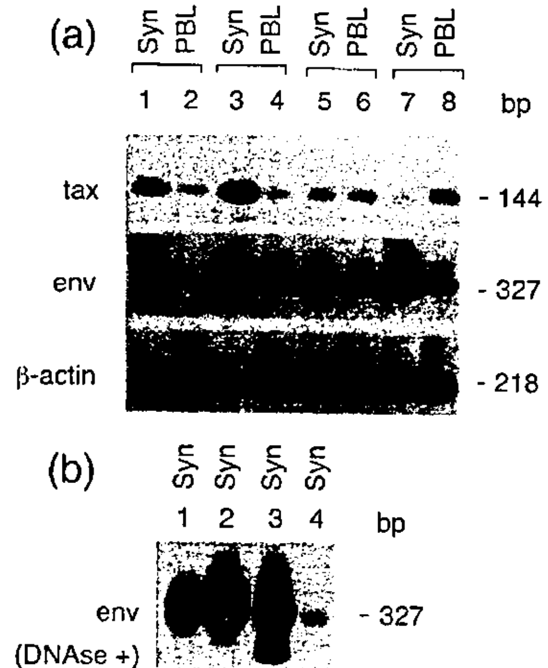

Fig. 3 (**a**) Expression of HTLV-I tax and env mRNA in the synovium and peripheral blood lymphocytes (PBL). **A**: HTLV-I tax, **B**: HTLV-I env, **C**: β-actin. Lane 1: HAAP-1 synovium, lane 2: HAAP-1 PBL, lane 3: HAAP-2 synovium, lane 4: HAAP-2 PBL, lane 5: HAAP-3 synovium, lane 6: HAAP-3 PBL, lane 7: HAAP-4 synovium, lane 8: HAAP-4 PBL. (**b**) Expression of HTLV-1 env mRNA in the synovium after DNAse treatment. Lane 1: HAAP-1 synovium, lane 2: HAAP-2 synovium, lane 3: HAAP-3 synovium, lane 4: HAAP-4 synovium

Table 1 HLA typing of patients with HAAP

Patient	HLA						
	DR					QD	
	B1		B3	B4	B5		B1
HAAP-1	0405	0901		01		0401	0303
HAAP-2	0405			01			0401
HAAP-3	0401	1502		01	0102	0301	0601
HAAP-4	0401	1502		01	0102	0301	0601

clonotype of T cells was examined by the PCR-SSCP method. Figure 4 demonstrates that T cells infiltrating into the synovium showed several distinct bands, whereas PBL from the same patient showed smear bands. The mean number of T-cell clonotypes in the arthritic joint (98.5 ± 3.0) was significantly higher than in PBL (20.0 ± 5.7, $p < 0.01$; Table 2). These findings indicate that T cells in the synovium expanded oligoclonally, suggesting an antigen-mediated stimulation.

HTLV-I env and tax reactive T cells in HAAP synovium

To investigate whether HTLV-I env and tax proteins act as antigens and are recognized by T cells infiltrating into the joints, we stimulated T cells in peripheral blood by recombinant HTLV-I env and tax proteins and compared env- and tax- reactive T cells with those obtained from the joints. As shown in Figure 5, several identical

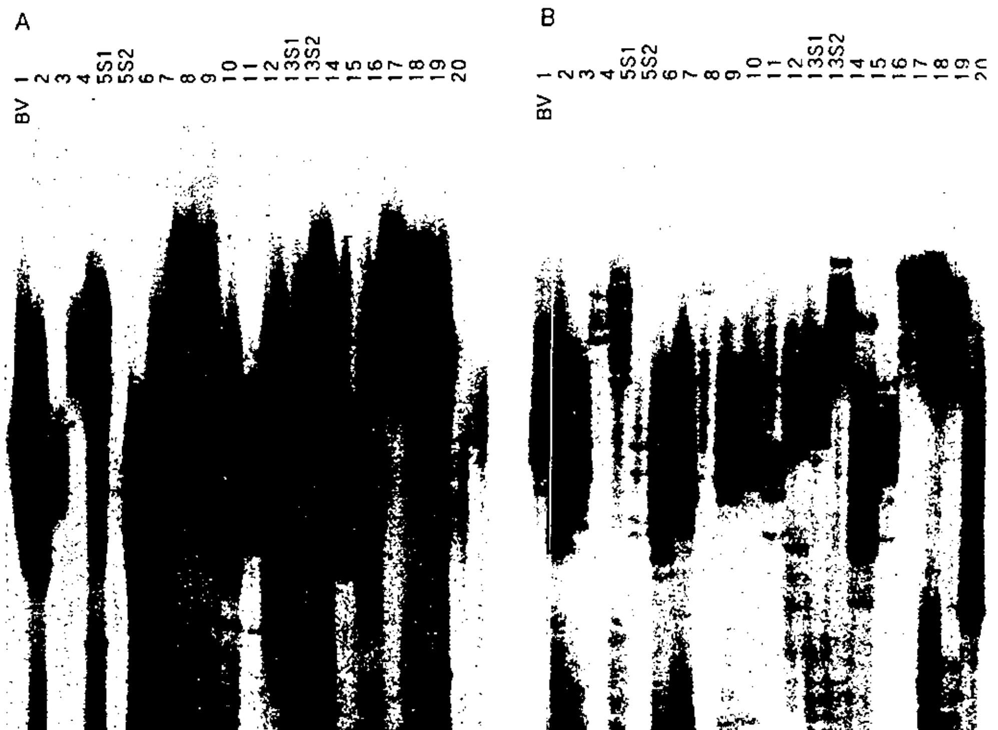

Fig. 4 Accumulation of multiple T-cell clonotypes in the synovium of patients with HAAP, as analyzed by PCR-SSCP. Representative examples of (A) PBL and (B) T cells infiltrating the synovium in HAAP-2

Table 2 Clonotype analysis of TCR BV expression in T cells of patients with HAAP[a]

Patient		1	2	3	4	5S1	5S2	6	7	8	9	10	11	12	13S1	13S1	14	15	16	17	18	19	20	total	mean ± SD
HAAP1	PBL	0	1	0	0	2		1	3	0	1	0	0	1	0	1	2	2	1	0	1	0	0	16	0.7 ± 0.2
	SIM	3	1	4	4	7	5	7	3	2	6	4	5	5	5	7	10	7	5	5	6			102	4.6 ± 0.5
HAAP2	PBL	1		1		2	1	3	2	0	1	1	2		0	0	2	0	2	0	1	0	2	21	1.0 ± 0.2
	SIM	4	10	5	5	7	7	6	5	6	6	5	2	6	2	3	3	4	3	1	4	1	4	99	4.5 ± 0.5
HAAP3	PBL	3	0	0	0	3	0	2	0	2	3	5	4	0	0			3	3	0	2	4	1	35	1.6 ± 0.4
	SIM	6	5	5	5	11	4	7	5	3	4	4	0	5	7	3	7	4	2	0	1	0	2	90	4.0 ± 06
HAAP4	PBL	0	0	0	0	2	0	1	1	0	1	0	0	0	0	0	1	0	2	0	1			8	0.4 ± 0.1
	SIM	8	8	6	1	2	7	8	10	4	10	10	2		5	5	5	4	1	0	4	1	2	103	4.7 ± 0.7

[a]cDNA obtained from PBL and SIM of HAAP patients were amplified with TCR BV primer sets. PCR products were applied to SSCP gel and electrophoresed. The number of the bands of each primer set was counted and the mean number was calculated.

PBL: peripheral blood lymphocytes; SIM: synovium infiltrated mononuclear cells.

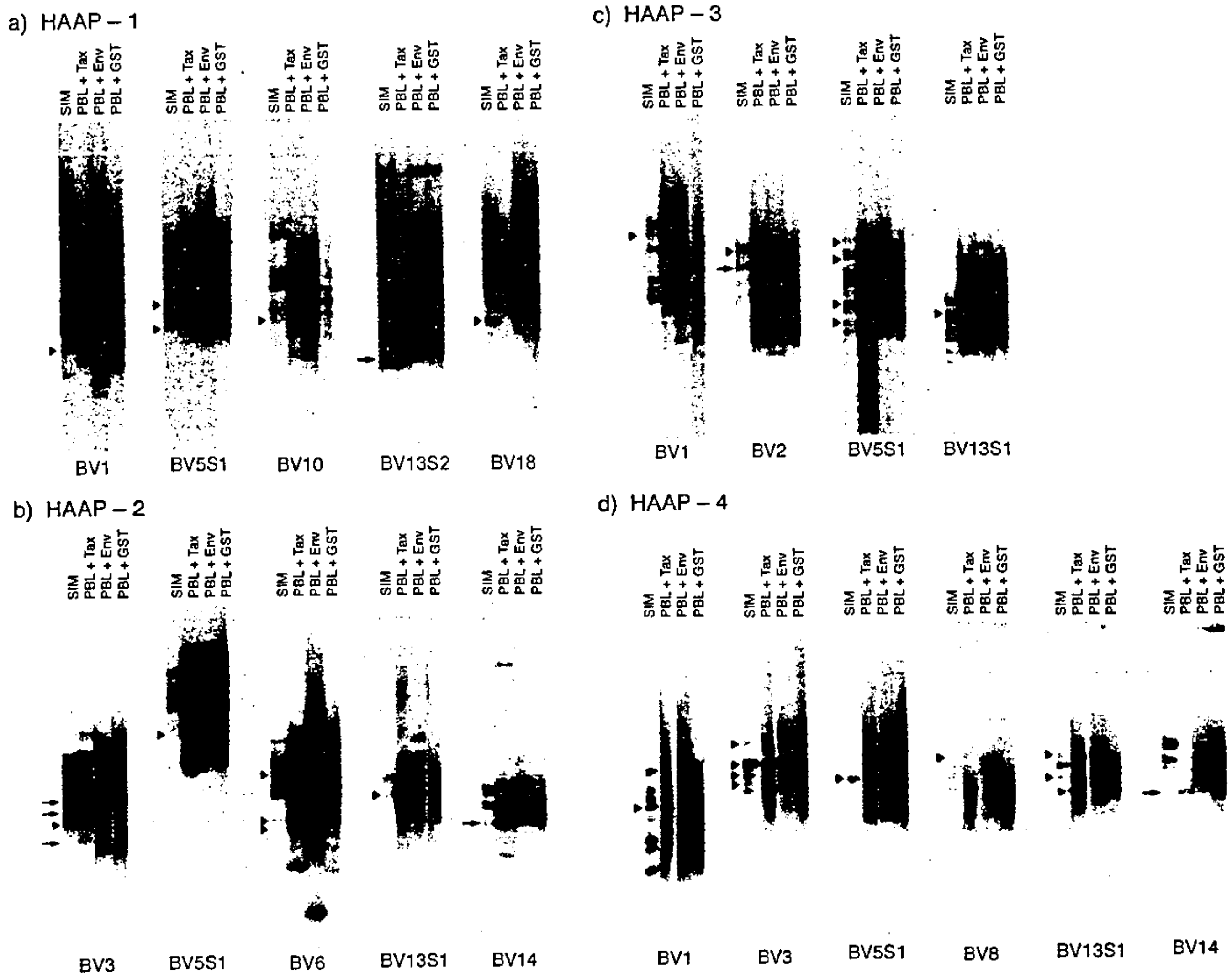

Fig. 5 Identical TCR BV genes of T cells infiltrating the synovium and env- or tax- stimulated PBL from patients with HAAP. Arrowheads or arrows indicate electrophoretic mobility bands similar to those of HTLV-I env reactive T cells or tax reactive T cells, respectively

electrophoretic mobility bands encoding TCR BV genes on SSCP gel were observed in synovial T cells and HTLV-I tax-and env-reactive T cells in PBL. No distinct bands were found in PBL stimulated with GST protein as a control. There was also no distinct band in samples from healthy control subjects after stimulation with recombinant HTLV-I tax and env proteins (data not shown). The numbers of HTLV-I tax and env reactive T cells in the synovium are summarized in Table 3. Twenty-seven of 109 (24.9%) bands in the tested joints were HTLV-I env reactive, and 7 of 109 (6.4%) bands were HTLV-I tax reactive. In addition, the presence of HTLV-1 env- and tax-reactive T cells in the joints of HAAP-1 patients was also examined using electrophoretic mobility bands encoding TCR AV genes on SSCP gel. At least 6 T-cell clones were HTLV-1 env reactive, whereas 2 clones were HTLV-1 tax reactive (data not shown).

Both CD4 and CD8 T cells are responsible for the reacting env– and tax–GST protein

Two patients with HAAP (HAAP-2 and HAAP-3) were examined for the responsible T-cell subset reacting for tax- and env-GST proteins, and 5 clones from HAAP-2 and 5 clones from HAAP-3 were able to be analyzed. As a result, 2 out of 4 env-reactive clones and the 1 tax-reactive clone appeared to be from the CD4+ subset, and the other two env-reactive clones belonged to the CD8+ subset in HAAP-2. In HAAP-3, all the 4 env-reactive clones belonged to the CD4+ subset, whereas 1 tax-reactive clone was CD8+ (Table 4).

Junctional sequence of HTLV-I env- and tax-reactive T cells

To examine the amino acid sequences of the TCR CDR3 region, DNA encoding the BV genes from HTLV-I env-reactive or tax-reactive T cells were purified from SSCP gels and their junctional sequences analyzed. As shown in Table 5, a conserved amino acid sequence,

Table 3 Number of identical TCR BV genes between SIM, env- and tax-reactive PBL of patients with HAAP[a]

HAAP1		BV1	BVSS1	BV10	BV13S2	BV18		Total
	SIM[b]	3	7	4	7	6		27
	T-S[c]	0	0	0	1	0		1
	E-S[d]	1	2	1	0	1		5
HAAP2		BV3	BVSS1	BV6	BV13S2	BV14		Total
	SIM	5	7	6	2	3		23
	T-S	3	0	0	0	1		4
	E-S	1	1	3	1	0		6
HAAP3		BV1	BV2	BVSS1	BV13S1			Total
	SIM	6	5	11	7			29
	T-S	0	1	0	0			1
	E-S	1	1	4	1			7
HAAP4		BV1	BV3	BVSS1	BV8	BV13S1	BV14	Total
	SIM	8	6	2	4	5	5	30
	T-S	0	0	0	0	0	1	1
	E-S	1	4	1	1	2	0	9

[a]Number of total clones in SIM and the clones of the same mobility with tax or env-reactive clones in PBL were counted
[b]Number of clones detected in synovium
[c]Tax-reactive clones in SIM
[d]env-reactive clones in SIM

Table 4 T cell subset analysis of HTLV-I env or tax reactive clones in SIM[a]

	Antigen	CD4 subset	CD8 subset	CD4/CD8 ratio
HAAP2	env	2	2	2/2
	tax	1	0	1/0
	total	3	2	3/2
HAAP3	env	4	0	4/0
	tax	0	1	0/1
	total	4	1	4/1

[a]PBL obtained from HAAP-2 and -3 were stimulated with env-GST or tax-GST after the depletion of CD4 subset or CD8 subset by Dynabeads. The clones reactive to either env or tax were examined in which subset they belonged to and the number of the clones were counted

NPS, was observed in the CDR3 region of BV6-carrying clones in HAAP-2 , whereas TALG was observed in the CDR3 region of BV5S1-carrying clones in HAAP-3. However, other clones did not show such conserved motifs in the CDR3 region. These findings indicate that T cells in the synovium recognize a variety of T-cell epitopes on HTLV-I env protein. We analyzed amino acid sequences of the CDR3 region of five synovial T-cell clones which showed identical electrophoretic mobility with that of peripheral HTLV-1 env- or tax-reactive T cells on SSCP. As shown in Table 6, the SSCP bands showing identical electrophoretic mobility were found to consist of identical TCR CDR3 sequences, This indicates that HTLV-1 env- or tax-reactive T cells preferentially accumulated in the synovium of patients with HAAP.

Discussion

We examined the molecular mechanisms causing arthritis in HAAP by analyzing T-cell clonality in the inflamed joints. First, we showed that the cells expressing tax and/or env mRNA were mainly synoviocytes, not T cells. This indicates the possibility that HTLV-I viral products are presented on synovial cells in the context of HLA molecules and act as autoantigens to trigger an autoimmune reaction. In fact, our SSCP analysis of TCR BV and AV genes revealed that 34 (31.2%) out of the 109 examined T-cell clones that infiltrated the HAAP synovium were reactive to HTLV-I env or tax proteins. Further, the proportion of HTLV-I env-or tax-reactive T cells in PBMC of patients with HAAP was less than 10^{-4}, because there were few clear bands in peripheral T cells of patients with HAAP, as shown by the SSCP analysis. These observations indicate that HTLV-I env- or tax-reactive T cells selectively accumulate in the inflamed synovium. Taken together, the infiltrating T cells recognizing HTLV-I env or tax proteins play a crucial role in the progression of arthritis in HAAP patients, similar to reactive arthritis and arthropathy in the HTLV-1 env-pX transgenic mouse [27]. In addition, the targets of the remaining ~70% of the infiltrating T cells remain to be determined. These T cells may have proliferated in a paracrine manner in response to IL-2 produced by the HTLV-1 reactive T cells, and may contribute to enhancement of inflammation. Alternatively, they may react to self components of the joint, such as type II collagen and proteoglycans.

We next showed that HTLV-1 env mRNA was expressed in a broad range of synovial cells from lining to deeper layers, but that tax mRNA was only expressed in the lining layer infrequently (Fig. 2). This indicates that the env protein would be presented more efficiently than the tax protein. Accordingly, the SSCP analysis revealed that 27 (24.9%), of 109 T-cell clones were HTLV-I env-reactive T cells in the synovium, whereas only 7 (6.4%) of the 109 clones were HTLV-I tax-reactive T cells in the inflamed lesions of four patients with HAAP. In addition, the infiltrating T cells were mainly CD4+ (Fig. 1), and most of the tested expanded T-cell

Table 5 Junctional sequences of TCR BV genes from T cells of patients with HAAP A) HTLV-I env reactive T cells[a]

	BV	NDN 97	BJ 106	
HAAP-1				
BV 1-1	TGTGCCAGCAGC C A S S	AGTGCTGGGACTAACGGGCAC S A G T N G H	GAGCAGTACTTC E Q Y F	BJ2S7
BV 5S1-1	TATCTTTGCGCC Y L C A	ACAAGCCGAGGACAGGCT T S R G G A	ACAGATACGCAG T D T Q	BJ2S3
BV 5S1-2	TATCTTTGCGCC Y L C A	CAATCGAGACAG Q S R Q	GAGCAGTTCTTC E Q F F	BJ2S1
BV10-1	TGTGCCAGCAGC C A S S	AGGGCTATCAGC R A I S	CAGCCCCAGCAT Q P Q H	BJ1S5
BV18-1	TTCTGTGCCAGC F C A S	GCAAGAATATCGGGGGTCCTA A R I S G V L	CAGTACTTCGGG Q Y F G	BJ2.7
HAAP-2				
BV 3-4	CTCTGTGCCAGC L C A S	AGCCCCCCGGGACTG S P P G L	CAAGAGACCCAG Q E T Q	BJ2S5
BV 5S1-1	TATCTTTGCGCC Y L C A	ACAGCTTGGGGGCAGGA T A W G A G	CAAGAGACCCAG Q E T Q	BJ2S5
BV6-1	TGTGCCAGCAGC C A S S	TTAGAGTCCGAT L E S D	GAGCAGTTCTTC E Q F F	BJ2S1
BV6-2	TGTGCCAGCAGC C A S S	TTAAATCCTAGCGGGGCCTTC L N P S[b] G A F	AATGAGCAGTTC N E Q F	BJ2S1
BV6-3	TGTGCCAGCAGC C A S S	TTTAACCCTTCCTACGAG F N P S Y E	CAGTACTTCGGG Q Y F G	BJ2S7
BV13S1-1	TGTGCCAGCAGT C A S S	TACTGGCTAGCGGCAGTC Y W L A A V	ACCGGGGAGCTG T G E L	BJ2S2
HAAP-3				
BV 1-1	TGTGCCAGCAGC C A S S	ACCGGGAGGGGGAGA T G R G R	ACTGAAGCTTTC T E A F	BJ1S1
BV 2-1	TGCAGTGCTAGT C S A S	CGGGGACTAAGTGGG R G L S G	TACAATGAGCAG Y N E Q	BJ2S1
BV 5-1	TGCGCCAGCAGC C A S S	GGGGACAACGATTTGAGGACAGCC G D N D L R T A	GGGGAGCTGTTT G E L F	BJ2S2
BV 5-2	TATCTTTGCGCC Y L C A	ACAGCATTGGGAAATGTTCAGGTGGGC T A L G N V Q V G	ACTGAAGCTTTC T E A F	BJ1S1
BV 5-3	TATCTTTGCGCC Y L C A	ACAGCATTGGGACAAGGGGACTTC T A L G Q G D F	ACAGATACGCAG T D T Q	BJ2S3
BV 5-4	TATCTTTGCGCC Y L C A	ACAGCCCGCTCGAC T A P L D	AATGAGCAGTTC N E Q F	BJ2S1
BV 13S1-3	TGTGCCAGCAGT C A S S	TACCGGCGGGGGATGGGGACC Y R R G M G T	TACGAGCAGTAC Y E Q Y	BJ2S7
HAAP-4				
BV 1-1	TATTTCTGTGCC Y F C A	AGTGGTTCGGCGGGAGGGCCTGGGACT S G S A G G P G T	GAGCAGTTCTTC E Q F F	BJ2S1
BV 3-1	TGTGCCAGCAGT C A S S	CCTAGGACGGGATTC P R T G F	ACCGGGGAGCTG T G E L	BJ2S1
BV 3-2	CTCTGTGCCAGC L C A S	AGGACGCGTAAT R T R N	GCCAAAAACATTCAG A K N I Q	BJ2S4
BV 3-3	TACCTCTGTGCC Y L C A	AGCATTTGGACTAGCGGGTACAATGAG S I W T S G Y N E	CAGTTCTTCGGG Q F F G	BJ2S1
BV 13S1-1	TTCTGTGCCAGC F C A S	AGGTACAGGGAACGA R Y R E R	AACACCATATAT N T I Y	BJ1S3
BV 13S1-2	TACTTCTGTGCC Y F C A	AGCAGTGACTCGATACTAGCTCCCCATCCATAC S S D S I L A P H P Y	GAGCAGTACTTC E Q Y F	BJ2S5

B) HTLV-I tax reactive T cells

	BV	NDN 97	BJ 106	
HAAP-1				
BV 13S1-1	TTCTGTGCCAGC F C A S	AGTTCGACAGGGGGCCGG S S T G G R	ACAGATACGCAATAT T D T Q Y	BJ2S3
HAAP-2				
BV 3-1	TGTGCC C A	ACTTCAACGAAC T S T N	ACTGAAGCTTTC T E A F	BJ1S1
BV 3-3	CTCTGTGCCAGC L C A S	AGCAACTCGGTGGAA S N S V E	GAGACCCAGTAC E T Q Y	BJ2S5
HAAP-3				
BV 2-2	TACATCTGCAGT Y I C S	GCAAAGAGACTAGCTCGA A K R L A R	GATACGCAGTAT D T Q Y	BJ2S3

[a]Junctional sequences of TCR BV in T cells reactive for HTLV-I env or tax protein. The single amino acid codes of the3, position of TCR BV, CDR3, and 5' position of the J region are given
[b]Underlining represents conserved amino acid sequences

Table 6 Junctional sequences of HTLV-I env/tax reactive T cells in the synovium of patients with HAAP[a]

		BV	NDN 97		BJ 106
HAAP-1					
Env-PBL	BV 5S 1-1	TATCTTTGCGCC	ACAAGCCGAGGACAGGCT	ACAGATACGCAG	BJ2S3
		Y L C A	T S R G G A	T D T Q	
SIM	BV 5S1-1	TATCTTTGCGCC	ACAAGCCGAGGACAGGCT	ACAGATACGCAG	BJ2S3
		Y L C A	T S R G G A	T D T Q	
Env-PBL	BV 5S1-2	TATCTTTGCGCC	CAATCGAGACAG		BJ2S1
		Y L C A	Q S R Q	GAGCAGTTCTTC	
SIM	BV 5S1-2	TATCTTTGCGCC	CBLATCGAGACAG	E Q F F	BJ2S1
		Y L C A	Q S R Q	GAGCAGTTCTTC	
				E Q F F	
Tax-PBL	BV 13S2-1	TTCTGTGCCAGC	AGTTCGACAGGGGGCCGG	ACAGATACGCAATAT	BJ2S3
		F C A S	S S T G G A	T D T Q Y	
SIM	BV 13S2-1	TTCTGTGCCAGC	AGTTCGACAGGGGGCCGG	ACAGATACGCAATAT	BJ2S3
		F C A S	S S T G G A	T D T Q Y	
HAAP-2					
Env-PBL	BV 3-4	CTCTGTGCCAGC	AGCCCCCCGGGACTG	CAAGAGACCCAG	BJ2S5
		L C A S	S P P G L	Q E T Q	
SIM	BV 3-4	CTCTGTGCCAGC	AGCCCCCCGGGACTG	CAAGAGACCCAG	BJ2S5
		L C A S	S P P G L	Q E T Q	
Tax-PBL	BV 3-1	TGTGCC	ACTTCAACGAAC	ACTGAAGCTTTC	BJ1S1
		C A	T S T N	T E A F	
SIM	BV 3-1	TGTGCC	ACTTCAACGAAC	ACTGAAGCTTTC	BJ1S1
		C A	T S T N	T E A F	

[a]Three paired bands from patient HAAP-1 and two paired bands from HAAP-2, which showed identical electrophoretic mobility on SSCP, were selected for analysis. cDNA were eluted from SSCP gels and their TCR BV junctional sequences were analyzed. The results showed that that TCR BV CDR3 sequences of T cells in synovium were identical to those of paired clones in HTLV-I env reactive or tax reactive T cells in PBL. Env-PBL, HTLV-I env reactive T cells in PBL; Tax-PBL, HTLV-I tax-reactive T cells in PBL

clones were CD4+ (Table 4). The dominance of CD4+ T cells and env-reactive T cells is in contrast with reported findings in HAM/TSP, a chronic neurological disease characterized by spastic paraparesis and urinary dysfunction. In HAM/TSP, HTLV-I-specific CD8+ CTL are present abundantly in the peripheral blood, together with accumulation of lymphocytes in spinal cord lesions [16, 18]. Further, the target of the CD8+ CTL is mainly HTLV-I tax protein [17], although some CD4+ CTL recognize HTLV-I gp46 envelope glycoprotein [28]. So far, it is unclear how some people develop HAM/TSP and others develop HAAP by infection with HTLV-1. The difference between dominantly reactive T-cell subsets and/or target proteins may contribute to the differential development of the diseases immunologically. Moreover, the nature of the recognized epitopes would influence the clinical features. For example, Hara et al. reported that the CDR3 sequence of T cells infiltrating the spinal cord of patients with HAM/TSP was highly conserved, indicating limited T-cell epitopes [15]. The conserved amino acid sequence motifs were similar to those of T cells found in brain lesions of patients with multiple sclerosis [29] and T cells specific for myelin basic protein (MBP) [30]. These findings suggest that T cells in spinal lesions of patients with HAM/TSP recognize a sequence common with MBP. More precisely, the T cells would respond to particular peptides cross-reactive between HTLV-I proviral proteins and neural cell products such as MBP. In contrast, our study revealed that synovial T cells do not show highly conserved amino acids in the CDR3 of HTLV-I env-reactive T cells, and that they had no unique sequence similar to the above-mentioned MBP-reactive T cells in patients with HAAP. This indicates that T cells in synovial lesions of patients with HAAP recognize multiple T-cell epitopes on the HTLV-I env protein.

In conclusion, T cells that infiltrated the synovium of patients with HAAP may play a major role in synovitis by recognizing HTLV-1 env proteins expressed on synovial cells. Further studies on the antigenic relation between the env protein and self components of the joint will promote our understanding of the antigenic roles of the env proteins in HAAP.

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Glucose-6-phosphate isomerase variants play a key role in the generation of anti-GPI antibodies: possible mechanism of autoantibody production

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Abstract

Glucose-6-phosphate isomerase (GPI), recognized as an autoantigen in the K/BxN arthritis model, is a ubiquitous cytoplasmic enzyme. Anti-GPI antibodies (Abs) are also detected in the serum of patients with arthritic diseases including rheumatoid arthritis (RA). So far, 24 GPI variants have been reported and most of these variants relate to non-spherocytic hemolytic disease. To understand the mechanisms of anti-GPI Ab production, cDNAs from peripheral blood mononuclear cells of subjects with or without anti-GPI Abs were cloned and sequenced. We identified 39 new GPI variants (57–1596 bp). The frequency of GPI variants in healthy control subjects (HS) with anti-GPI Abs (27/73, 31.5%) was significantly higher than that in anti-GPI Ab-negative HS (5/78, 6.4%, $p < 0.001$). The frequency of GPI variants in anti-GPI Ab-positive RA patients (22/77, 28.6%) was more significantly higher than in anti-GPI Ab-negative patients (1/63, 1.6%, $p < 0.0001$). Our results suggest that GPI variants may play a crucial role in the production of autoantibodies against ubiquitous GPI autoantigens.
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Keywords: Glucose-6-phosphate isomerase; Autoantibodies; Autoantigens; Rheumatoid arthritis

Autoantigens in chronic inflammatory arthritides such as rheumatoid arthritis (RA) are still unknown [1]. Several candidate autoantigens have been identified in RA patients, although they have not been confirmed because of the heterogeneity of the disease. The K/BxN T cell receptor transgenic mouse spontaneously develops a joint disorder with many of the clinical, histolopathological, and immunological features of RA in human [2]. The disease in the model mouse is initially provoked by T cell recognition of ubiquitously expressed self-antigen, and the consequent arthritic events in the joint are mainly driven by autoantibodies [3].

Glucose-6-phosphate isomerase (GPI), a second glycolytic enzyme, is the target of both initiating T cells and pathogenic immunoglobulins [4,5]. Several groups reported the presence of anti-GPI antibodies (Abs) in the serum of patients with severe forms of arthritic diseases including RA [6–8], suggesting their pathogenic role in human arthritides. With regard to autoantigen GPI, the gene locus is located on chromosome 19 [9], and the gene spans more than 40 kbp, including 18 exons and 17 introns [10–12]. GPI is also known as an extracelluar cytokine [autocrine motility factor (AMF), neuroleukine, or maturation factor], and probably increases the susceptibility to certain autoimmune diseases or cancer metastasis [13–16]. So far, 24 GPI variants have been identified and most of these variants are associated with a decrease in isomerase activity on

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erythrocytes, resulting in non-spherocytic hemolytic disease [17–22]. Moreover, 10 single nucleotide polymorphisms (SNPs) of GPI gene have been reported [23]. Based on the above background, we hypothesized that the amino acid mutations or deletion of GPI autoantigens results in the generation of autoantibodies against GPI, with subsequent development of arthritis.

In the present study, to clarify the relevance between GPI and anti-GPI Abs, we cloned full-length GPI complementary DNAs (cDNAs) derived from subjects with and without anti-GPI Abs, sequenced them, and compared them with intact GPI cDNA [24]. We observed numerous GPI variants in anti-GPI Ab-positive subjects compared with anti-GPI Ab-negative individuals. These findings support the notion that variant molecules of autoantigens are essential for production of autoantibodies.

Materials and methods

Subjects and cDNA synthesis. Peripheral blood mononuclear cells (PBMC) derived from 10 patients with RA (including 5 anti-GPI Ab-positive) and healthy volunteers (including 5 anti-GPI Ab-positive) were used. These subjects were defined as anti-GPI Ab-positive or -negative, after GPI enzyme-linked immunosorbent assay (ELISA) by using recombinant human GPI and rabbit muscle GPI as antigens [7]. Total RNA was prepared from PBMC by using ISOGEN method (Nippon gene, Tokyo, Japan) according to the instructions provided by the manufacturer. For first strand cDNA synthesis, 0.5 μg of total RNA with 1 μl of oligo(dT)$_{18}$ primer was incubated with 2 μg of 10 nM dNTP mix, 20 U RNase inhibitor, 4 μg of 5× reaction buffer, and 200 U M-MuLV reverse transcriptase (RT) (Revert Aid First Strand cDNA Synthesis Kit, Fermentas, Hanover, MD) in a total volume of 20 ml for 1 h at 42 °C.

RT-PCR and GPI cDNA cloning. Primer sets for full-length GPI cDNA including first codon ATG and stop codon TAA were 5′-TCAGTGTACCTTCTAGTCCCG-3′ and 5′-GTCCAAGCCCACA ACCAGA-3′ (Table 1) [17]. Full-length GPI cDNAs were prepared with 1 μl first strand cDNA, 15 pmol primer sets, 2 μg of 25 mM $MgSO_4$, 5 μg of 2 mM dNTP mixture, 5 μg of 10× reaction buffer, and 2.5 U KOD *Taq* polymerase (Toyobo, Osaka, Japan) in total 50 μl reaction volume by using PCR Gene Amp system 9700 (Applied Biosystems, Foster City, CA). The reaction mixture was incubated at 94 °C for 2 min and amplified for 30 cycles at 94 °C for 15 s, 59 °C for 30 s, and 72 °C for 2 min. Next, 5 ml of mixture was loaded onto 1% agarose gel to check for amplification. The amplified cDNAs were subcloned into pCR-Blunt II TOPO Vector by Zero blunt TOPO PCR Cloning kit (Invitrogen, San Diego, CA). To confirm the insert, over 20 clones per subject were checked by colony PCR using M13 reverse primer and T7 primer on the vector following 1% agarose gel electrophoresis. The clone with a shorter fragment than the intact length (2-kbp) was sequenced by using approximately primers (Table 1) with BigDye terminator system (AB) after purification of the plasmid (GFX Micro Plasmid Prep Kit, Amersham Life Science, Buckinghamshire, UK). The sequence of insert cDNA was compared and confirmed with intact GPI mRNA [24].

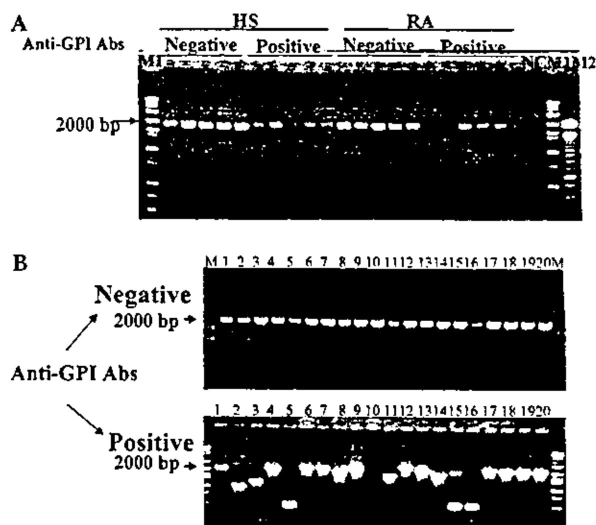

Fig. 1. (A) RT-PCR for full-length GPI cDNA. One microliter of synthesized cDNA template was used in each 50 μl PCR system. Three microliters of PCR products was loaded in 1.0% agarose gel. NC, negative control. M1, wide Range DNA Ladder (50–10,000 bp) (TAKARA); M2, 100 bp DNA Ladder (Invitrogen). Five samples were subcloned into each group, respectively. (The left one was not done in RA positive). (B) Colony PCR for insert check. M13 RV and T7 primer on the pCRII vector were used in each 10 μl PCR system. The band at 2000 bp showed an intact size of full-length GPI cDNA. Three microliters of PCR products was loaded in 1.0% garose gel. M1. Wide Range DNA Ladder (50–10,000 bp) (TAKARA). In case of anti-GPI Ab-positive, clone Nos. 2, 3, 5, 8, 9, 11, 13, 14, 15, and 16 were sequenced. Clone Nos. 11, 14, and 15 were found deletion variants after sequencing analysis. About 20 colony PCR for insert check was done in each group, respectively, to total 291.

Table 1
Oligonucleotides used in this study

No.	Sequence	Position in NM_000175
GPI-5′	5′-TCAGTGACCTTCTTAGTCCCG-3′	81–101
GPI-5′-1	5′-CGGACGTCATCAACATTGGCAT-3′	552–573
GPI-5′-2	5′-GAGTGGTTTCTCCAGGCGGCCA-3′	782–803
GPI-5′-3	5′-GTATCTGGTACATCAACTGC-3′	1074–1093
GPI-5′-4	5′-ATCGACGGAGGAGGCCCGAA-3′	1423–1442
GPI-3′	5′-GTCCAAGCCCACAACCAGA-3′	1901–1882
GPI-3′-1	5′-CTGGACTCTTGCCCCGCAGCC-3′	1472–1452
GPI-3′-2	5′-GGTGCAGGTACTGGTCATA-3′	1142–1123
GPI-3′-3	5′-CAGGGCAACAAAGTGCTTCG-3′	840–821
GPI-3′-4	5′-CCATGAGGGGTCCCAGGTC-3′	602–584

The set of GPI-5′and GPI-3′ was used for RT-PCR. Others were used for sequencing.

Results and discussion

To examine whether variant forms of GPI exist in PBMC of anti-GPI Ab-positive individuals, DNAs encoding the GPI gene were amplified by RT-PCR with primers specific for GPI (Table 1). As shown in Fig. 1A, the amount of DNA encoding intact GPI gene at 1820 bp in anti-GPI Ab-positive (+) subjects was less than in anti-GPI Ab-negative (−) subjects. This tendency was almost equally found in both RA patients and healthy subjects (HS). Amplified GPI DNAs were cloned and several different GPI genes were more frequently detected in anti-GPI Abs (+) than anti-GPI Abs (−) HS and RA patients (Fig. 1B). Sequence analysis of different length clones showed many GPI variants (Fig. 2); 39 GPI variants were identified in 291 clones. The frequency of GPI variants in anti-GPI (+) HS (23/73, 31.5%) was significantly higher compared to those in anti-GPI (−) HS (5/78, 6.4%, $p < 0.001$, Table 2). Furthermore, GPI variants in anti-GPI (+) RA (22/77, 28.6%) were also significantly more frequent than in anti-GPI (−) RA (1/63, 1.6%, $p < 0.0001$, Table 2). There was significant relationship between the presence of GPI variants and anti-GPI Abs ($p < 0.0001$). However, the proportion of GPI variants in RA did not correlate with that in HS (Table 2). These findings suggest that the newly presented B cell epitopes on altered GPI molecules are recognized as antigens and that

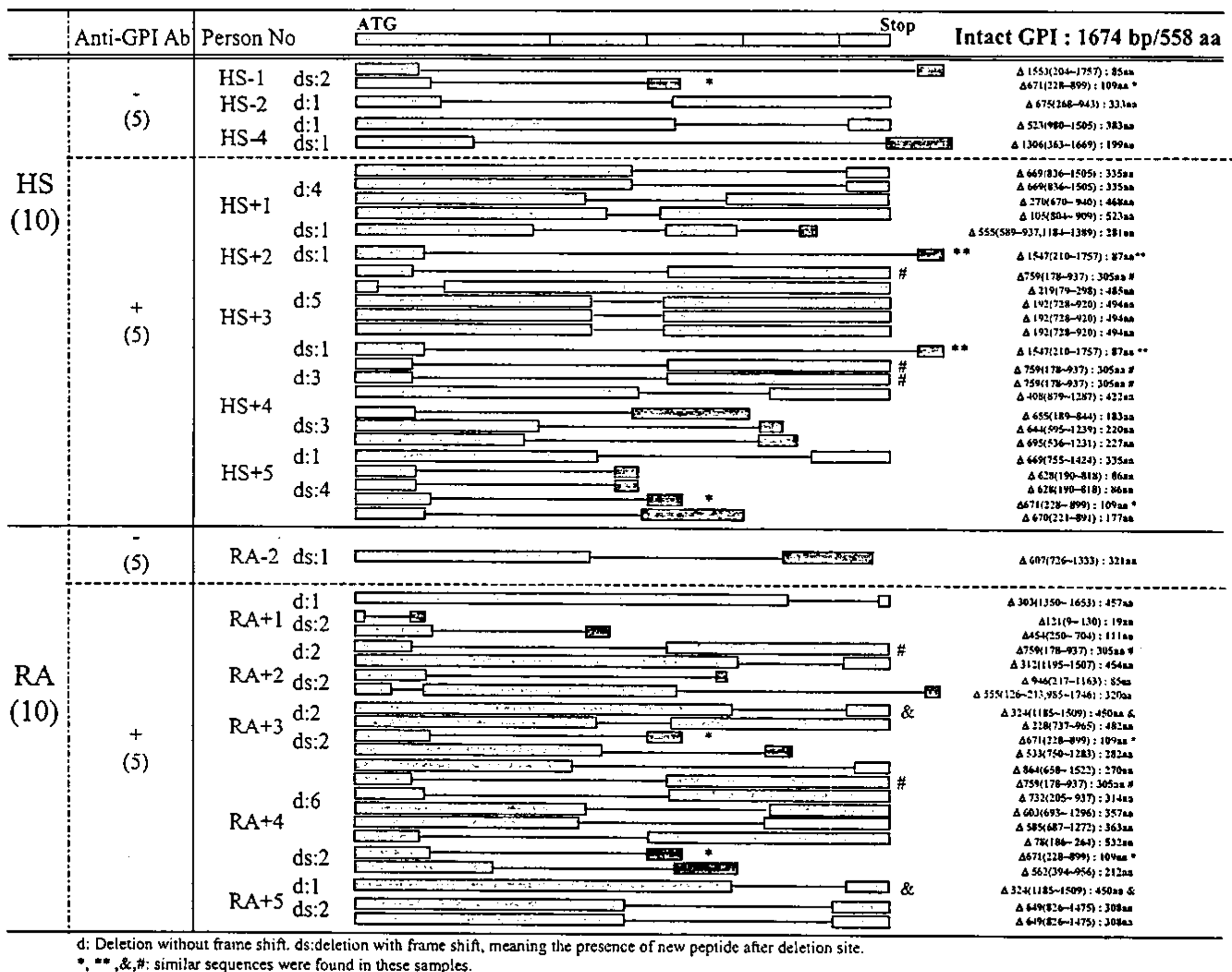

Fig. 2. Mapping of GPI deletions. Sequencing of GPI deletions was confirmed by comparison with intact GPI mRNA [14] after sequencing analysis of the plasmid having different length cDNAs. Thirty-nine kinds of GPI variants (totally 51 clones) were identified. In the most upper column, green bar showed 1674 bp/558 aa length of intact GPI/aa sequence from start codon (ATG) to stop codon. In the following column, green bar showed the part of intact GPI/aa sequence. Binding line between green bar and dark bar showed the part of deletion. Dark bar showed the part of new peptide which occurred by flame shift resulting in deletion. d, Deletion with intact peptide (without flame shift); ds, deletion with new peptide (with flame shift) including new stop codon; Δ1553(204–1757): 85aa, 1553 bp deletion between 204 and 1757 bp from start codon on intact GPI mRNA and it results in 85 aa length protein; *, **, &, and #, indicating the same sequencing. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)