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Characterization of T cells specific for an epitope of human 60-kD heat shock protein (hsp) in patients with Behçet's disease (BD) in Japan

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SUMMARY

BD is prevalent in the area of the Silk Route. It has been shown that hsp are involved in the T cell activation in patients with BD in the UK, where this disease has developed sporadically. We have thus examined whether the T cell response to the hsp-derived peptides may be induced in patients with BD in Japan, an east pole of the Silk Route. As with patients in the UK, the human 60-kD hsp peptide 336–351 also yielded vigorous proliferation of T cells in Japanese patients with BD, but neither in normal subjects nor in patients with rheumatoid arthritis (RA); there was significant association between proliferation by this peptide and the presence of ocular lesion, but not any other symptoms of BD. To clarify whether the peptide stimulates T cells as a polyclonal activator, a specific antigen or a superantigen-like substance, we analysed T cell receptor (TCR) usage of responding T cells by means of MoAbs specific for TCR Vβ subfamily and polymerase chain reaction (PCR)-single-strand conformation polymorphism (SSCP)-based technique. We found that T cells with certain TCR Vβ subfamilies (including Vβ5.2–3, 8, 13.6, 18, 21.3) were increased in circulation and responded to the hsp peptide in an antigen-specific fashion. In addition, TCR Vβ gene-amplified products of freshly isolated T cells of patients with BD formed several bands in the PCR-SSCP analysis; some of them became prominent after stimulation with the peptide. This suggests that T cells in patients with this disease have already been expanded oligoclonally *in vivo*, which may be a result of stimulation by triggering antigens, including the hsp peptide. In addition, hsp peptide stimulation induced proinflammatory cytokine mRNA expression in peripheral blood mononuclear cells, including IL-8, tumour necrosis factor-alpha (TNF-α) and TNF-β in eight out of eight patients studied. Taken together, the results suggest that hsp antigen may play a role in the pathogenesis of BD, not only in the area of the Silk Route, but also outside the Silk Route area.

Keywords heat shock protein Behçet's disease T cell receptor oligoclonal expansion

INTRODUCTION

BD is a systemic inflammatory disease, characterized by recurrent signs and symptoms of oral aphthosis, genital ulcers, skin lesions, and uveitis. This disease is prevalent along the Silk Route, but is also found sporadically outside the Silk Route. The etiology of this disease is unknown, but various immune abnormalities of BD have been found [1,2]; findings indicative of this include elevated levels of circulating immunoglobulin, circulating immune complexes, and antibodies binding to oral mucosa.

We and others have found abnormalities in patients with BD similar to those observed in certain autoimmune disorders [3–11]; these include induction of lymphocyte transformation by oral mucosa, cytotoxic effect of lymphocytes on oral mucosa, DTH skin reaction to skin homogenates, histologic features characterized by an early intense lymphocytic infiltration in oral aphthous ulceration, suppressor T cell dysfunction, defective IL-2 production of mitogen-activated T cells, and the presence of *in vivo* activated B cells.

Reinvestigation of early reports of autoimmune responses to oral epithelial antigen revealed that a 65-kD band was identified with anti-65-kD hsp antibody on Western blots [12]. Originally, hsp were reported to be involved in the pathogenesis of autoimmune diseases. hsp are unique antigens with a potent immunostimulatory property, and have an extraordinarily high sequence conservation throughout eukaryotic and prokaryotic kingdoms [13]. Despite the fact that there are a number of families and functions of immunoreactive hsp [13], the 65-kD protein is an immunodominant antigen that induces circulating antibodies and T

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cell responses in human subjects [12]. Cross-reactivity between bacterial and mammalian 60/65-kD hsp is considered to be a plausible mechanism in the pathogenesis of autoimmune diseases [14–18].

It has recently been shown that selected peptides derived from the sequences of human 60-kD hsp also induced significant proliferation of T cells in patients with BD in the UK [19]. In addition, two peptides (136–150 and 336–351) of human 60-kD hsp that were most frequently recognized by T lymphocytes from patients with ocular type BD produced a high incidence of eye disease in experimental rats [20]. Thus, human hsp may act as one of the autoantigens recognized by T cells of patients with BD.

In the present study, we examined whether the same hsp-derived peptides which can stimulate T cells from BD in the UK, outside the Silk Route, are also relevant for T cell responses of patients with BD in Japan, an east pole of the Silk route. We found that a common trigger may be associated with the development of this disease, regardless of geographic location. In addition, we characterized T cells specific for the hsp-derived peptides, and analysed the recognition mechanism of the peptides by T cells of BD patients.

PATIENTS AND METHODS

Patients and controls

Twenty-eight patients with BD (14 males, 14 females) satisfied the 1987 diagnostic criterion for BD proposed by the Behçet's Disease Research Committee of Japan [21]. They also fulfilled a criterion for diagnosis of BD by the International Study Group for Behçets Disease [22]. Eighteen patients had ocular manifestations of BD, and the remaining 10 patients lacked eye symptoms. Their mean age (±s.d.) was 40·9 ± 11·1 years (range 24–59 years). Four patients did not receive any treatment, 24 patients were receiving low dose corticosteroid therapy (<10 mg/day of prednisolone) or colchicine therapy (<1 mg/day). Those who had been receiving high dose prednisolone and/or cytotoxic drugs were not enrolled in the present study.

A second group of 19 patients (three males, 16 females) with rheumatoid arthritis (RA) was studied as a disease control, and their mean age was 60·7 ± 12·7 years (range 33–77 years); all were receiving non-steroidal anti-inflammatory drugs and/or disease-modifying anti-rheumatic drugs; none was receiving intermediate to high dose corticosteroid therapy and immunosuppressive therapy.

Seventeen healthy volunteer blood donors (nine males, eight females) served as control subjects, and their mean age was 35·2 ± 7·6 years (range 22–47 years).

Patients were typed for HLA-A, B, C, DQ and DR phenotypes by a standard microcytotoxicity test.

Human studies committee's approval and individual informed consent from each patient were obtained before we conducted the present studies.

Peptides and antigens used in this study

Peptides derived from the sequence of the human 60-kD hsp 136–150, 179–197, 244–258 and 336–351 (see Table 1) that have been reported to stimulate T lymphocytes in patients with BD in the UK [19] and a control peptide derived from the sequence of the *Mycobacterium bovis* 65-kD hsp 91–105 [19] were produced by our laboratory. It has been reported that hsp 91–105 stimulates T cells of patients with recurrent oral ulcers and not BD [23].

Purified phytohaemagglutinin (PHA; Wellcome Research Labs, Beckenham, UK), purified protein derivative (PPD; Japan BCG Co., Tokyo, Japan), and Staphylococcal enterotoxin B (SEB; Toxin Technology Inc., Sarasota, FL) were purchased, and optimal culture conditions for individual reagents were determined as follows; PHA at 1 μg/ml for 3 days, PPD at 5 μg/ml for 6 days, SEB at 5 μg/ml for 4 days, and hsp-derived peptides at 50 μg/ml for 6 days (data not shown). We used these optimal culture conditions.

Table 1. T cell proliferative responses to the peptides in patients with BD with or without ocular lesions

	Mean SI (± s.e.m.)	
	With ocular lesions ($n = 18$)	Without ocular lesions ($n = 10$)
Peptide sequences from human 60-kD hsp		
136–150, NPVEIRRGVMLAVDA	3·67 ± 1·01	2·24 ± 0·31
179–197, KEIGNIISDAMKKVGRKGV	4·50 ± 1·70	2·32 ± 0·32
244–258, LLSEKKISSIQSIVP	4·18 ± 1·59	2·47 ± 0·30
336–351, QPHDLGKVGEVIVTKD	7·01 ± 1·69*	2·57 ± 0·47
Peptide sequence from mycobacterial 65-kD hsp		
91–105, ATVLAQALVREGLRN	3·09 ± 0·87	1·79 ± 0·26

T cell proliferation is high in patients with ocular lesions compared with those without ocular lesions (*$P < 0·01$; by Mann–Whitney U-test).

Cell separation and cell cultures

Peripheral blood mononuclear cells (PBMC) were separated into T cells and non-T cells by means of a sheep erythrocyte rosette technique [24]. $CD4^+$ and $CD8^+$ T cell subsets were purified from the T cells by negative selection using panning with OKT8 MoAb and OKT4 MoAb, respectively (both from Ortho Pharmaceutical Co., Raritan, NJ) [24].

PBMC (5×10^4), purified T cells supplemented with irradiated autologous non-T cells, or $CD4^+$ or $CD8^+$ T cells with the non-T cells were cultured for 6 days in culture medium consisting of RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) containing penicillin 100 U/ml, streptomycin 100 μg/ml (Life Technologies) and 10% fetal calf serum (FCS; Life Technologies). Proliferative response was measured by the incorporation of ^{3}H-TdR (1 μCi/well; Amersham International, Aylesbury, UK) during the last 16 h of the triplicate cultures. The result was expressed as a stimulation index (SI) which was calculated from the following formula: SI = mean ct/min of cultures stimulated by the peptide/mean ct/min of unstimulated cultures.

Antibodies used in this study

MoAbs used for blocking studies include anti-HLA-ABC (W6/32 (Dako Japan, Kyoto, Japan) and B-H9 (Serotec, Oxford, UK)), anti-HLA-DR (OKDR (Ortho Pharmaceutical), I2 (Coulter Immunology, Hialeah, FL), and L243 (Becton Dickinson Monoclonal Center, Mountain View, CA)), anti-CD4 (OKT4), anti-CD8 (OKT8) and anti-TCR$\alpha\beta$ (WT31; Becton Dickinson Monoclonal

Center). As negative control, we used purified murine IgG1 (MOPC21; Becton Dickinson Monoclonal Center). MoAb at a concentration of 10 μg/ml was introduced into the culture before adding the peptide.

Results were expressed as percent responses, calculated from the following formula:

$$\text{percent response} = \frac{\text{(mean ct/min of triplicate cultures stimulated by the peptide in the presence of MoAb} - \text{mean ct/min of unstimulated triplicate cultures in the presence of MoAb)}}{\text{(mean ct/min of triplicate cultures stimulated by the peptide without MoAb} - \text{mean ct/min of unstimulated triplicate cultures without MoAb)}}$$

Immunofluorescence analysis

To analyse T cell receptors (TCR) of hsp-responding T cells, a panel of FITC-conjugated anti-TCR MoAbs was used: anti-TCR Vβ5a (1C1), anti-TRC Vβ5b (W112), anti-TCR Vβ5c (LC4), anti-TCR Vβ6 (OT145), anti-TCR Vβ8 (16G8), and anti-TCR Vβ12.1 (S511) were purchased from T Cell Sciences (Cambridge, MA), and anti-TCR Vβ17.1 (E17.5F3.15.3) was obtained from Immunotech (Marseille, France). We also used unlabelled anti-TCR Vβ-specific MoAbs, anti-TCR Vβ3.1 (LE-89), anti-TCR Vβ13.6 (JU-74), anti-TCR Vβ18.1 (BA62), anti-TCR Vβ21.3 (IG125), and anti-TCR Vβ22.1 (IMMU546) (all from Immunotech). The antibodies covered about 30–60% of the total T cell population of patients with BD and normal subjects, and isotype-matched control MoAb usually stained <1% of cells [25,26].

Preliminary time course studies revealed that changes of phenotype of the T cells stimulated by the peptides were observed most clearly 9 days after culture [25], and thus PBMC stimulated for 9 days by the peptide were used for analysis of their phenotype. Stained cells were analysed by a FACScan (Becton Dickinson FACS System). Percentage of T cells bearing each TCR Vβ phenotype was expressed as percentage of total T ($CD3^+$) cells.

Polymerase chain reaction amplification, single-strand conformation polymorphism analysis and cytokine mRNA expression analysis

TCR usage of the peptide 336–351-stimulated T cells was analysed by reverse transcription-polymerase chain reaction (RT-PCR). The procedure of RT-PCR has been described in detail elsewhere [27,28]. Briefly, for TCR analysis, T cells were cultured with the peptide for 9 days. TCR β-chain was amplified using a set of Vβ gene segment-specific sense primers and a common antisense Cβ primer: the sequences of Vβ1-20 and 3′Cβ primer except Vβ6, 12, and 18 were the same as with Choi *et al.* [29]. Sequences of Vβ6, 12, and 18 were the same as with Bragado *et al.* [30], and sequences of Vβ21–24 were from Robinson [31].

For PCR-single-strand conformation polymorphism (SSCP) analysis, amplified TCR β-chain products were purified, re-suspended in denaturing solution (95% formamide, 10 mM EDTA, 0·1% bromophenol blue, 0·1% xylene cyanol), and heat-denatured. The sample was electrophoresed in non-denaturing 10% polyacrylamide gels containing 10% glycerol [32,33], developed by silver-staining or followed by DNA transfer to nylon membranes (Boehringer, Mannheim, Germany) that was visualized by hybridization using a synthetic oligonucleotide corresponding to Cβ region (CGACCTCGGGTGGGAACA), that was labelled by digoxigenin with 3′ end labelling kit (Boehringer). Hybridized oligonucleotide was detected with digoxigenin luminescent detection kit (Boehringer).

Statistical analysis

Mean SIs of irregular distribution in some subject groups were compared by the Mann–Whitney *U*-test. Paired data of mean SI were compared by the Wilcoxon signed rank test. $P < 0{\cdot}05$ was judged statistically significant.

RESULTS

Proliferative response of PBMC in patients with BD to the peptide 336–351 derived from human 60-kD hsp

It has been shown that four specific synthetic peptides derived from the human 60-kD hsp yielded a vigorous proliferation of T cells in patients with BD in the UK, whereas the control 91–105 peptide from the *M. bovis* 65-kD hsp did not [19]. These data suggest that a segment of 60-kD hsp family may be involved in the pathogenesis of BD in the UK. We first asked whether hsp is also relevant for the T cell responses of BD in Japan. The selected synthetic peptides that were exactly the same as have been analysed in the UK were used to stimulate PBMC, and the proliferative responses were estimated (Fig. 1). Among the five peptides tested in the present studies (Table 1), the peptide 336–351 provoked a significant proliferation of T cells in BD patients in Japan. The remaining three peptides, 136–150, 179–197, and 244–258, induced significant proliferation of T cells in BD patients compared with control mycobacterial hsp peptide 91–105. However, proliferative

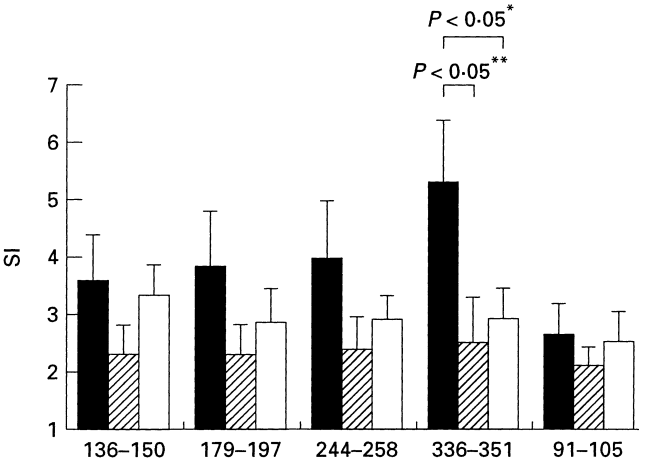


Fig. 1. Stimulation by human hsp-derived synthetic peptides of T cells in patients with BD. Lymphocytes from BD patients (■, $n = 28$), patients with rheumatoid arthritis (RA; ▨, $n = 19$) and normal subjects (□, $n = 17$) were stimulated by the synthetic peptides 136–150, 179–197, 244–258 and 336–351 of human 60-kD hsp and the control peptide 91–105 of mycobacterial 65-kD hsp. Proliferative response of T cells is expressed as a stimulation index (SI). Error bar indicates + s.e.m. Purified protein derivative (PPD) responses and phytohaemagglutinin (PHA) responses were also studied (mean SI + s.e.m.) for control antigen and mitogen; PPD responses in BD, 22·7 + 7·9; PPD responses in normals, 13·3 + 6·9; PHA responses in BD, 102·9 + 9·0; PHA responses in normals, 112·7 + 8·1. In addition, mean ct/min of T cells stimulated with the peptides in a BD patient is as follows; medium, 2389; peptide 136–150, 7797; peptide 179–197, 9973; peptide 244–258, 8916; peptide 336–351, 12 206; control peptide 91–105, 5042. Thus, peptide 336–351 yielded significant proliferation of T cells in BD patients, compared with normal subjects (*$P < 0{\cdot}05$) and patients with RA (**$P < 0{\cdot}05$) by the Mann–Whitney *U*-test.

responses to the remaining three peptides in patients with BD were not statistically different from those in normal subjects (Fig. 1).

T cells did not proliferate in response to the four peptides tested in this study in patients with RA (Fig. 1). Thus, it is suggested that although hsp was assigned some role in the pathogenesis of RA, the epitope on the hsp peptide 336–351 is important only for BD, but not for RA.

Stanford *et al.* [20] reported that two human hsp-derived peptides, 136–150 and 336–351, induce iridocyclitis in Lewis rats. Our results also suggest the importance of the hsp peptide 336–351 for development of eye symptoms, since there existed a significant association between the presence of ocular lesion and the lymphoproliferative responses by the peptide 336–351 ($P < 0{\cdot}01$), but not any other human hsp 60-derived peptides studied and a mycobacterial peptide (Table 1). Other symptoms (such as oral aphthosis, cutaneous symptoms and genital ulcer) and disease activity that was proposed by the Behçet's Disease Research Committee of Japan [34] were not related with the proliferative responses to the peptides (data not shown).

T cell subsets involved in the proliferative response of T cells to the peptide 336–351 in patient with BD

We next studied which T cell subsets are responsible for proliferation stimulated by the peptide 336–351 in BD patients. Purified $CD4^+$ and $CD8^+$ T cells from five patients with BD were stimulated with peptide 336–351 for 7 days in the presence of irradiated autologous non-T cells. Peptide 336–351 induced proliferation of exclusively $CD4^+$ ($n = 5$, SI ($\pm$ s.e.m.) $= 3{\cdot}45 \pm 0{\cdot}42$), and not $CD8^+$ ($n = 5$, SI $= 1{\cdot}19 \pm 0{\cdot}14$), T cells ($P < 0{\cdot}05$ by Wilcoxon signed rank test).

To confirm further that only $CD4^+$ T cells are involved in the proliferation of T cells when stimulated by peptide 336–351 in BD patients, we studied the inhibitory effects of a panel of MoAbs for the proliferation. To this end, unfractionated T cells were supplemented with 10% irradiated autologous non-T cells as antigen-presenting cells (APC), and were stimulated by peptide 336–351 in the presence of MoAb. Proliferative responses of the T cells to the peptide were significantly inhibited by the addition of anti-HLA-DR MoAb and anti-CD4 MoAb in patients with BD. However, addition of anti-HLA-ABC (class I) MoAb or anti-CD8 MoAb did not show any effect on T cell proliferation in BD patients (mean percent response ($\pm$s.e.m.) ($n = 4$): anti-HLA-DR, $41{\cdot}4 \pm 13\%$; anti-HLA-ABC, $96{\cdot}4 \pm 12\%$; anti-CD4, $16 \pm 4\%$; anti-CD8, $90 \pm 16\%$; control IgG1 antibody, $103 \pm 16\%$). These results further support the notion that $CD4^+$ T cells are responsible for the proliferative responses to the peptide. However, it is possible that CD8 T cells are responsible for other than proliferative responses in this disease.

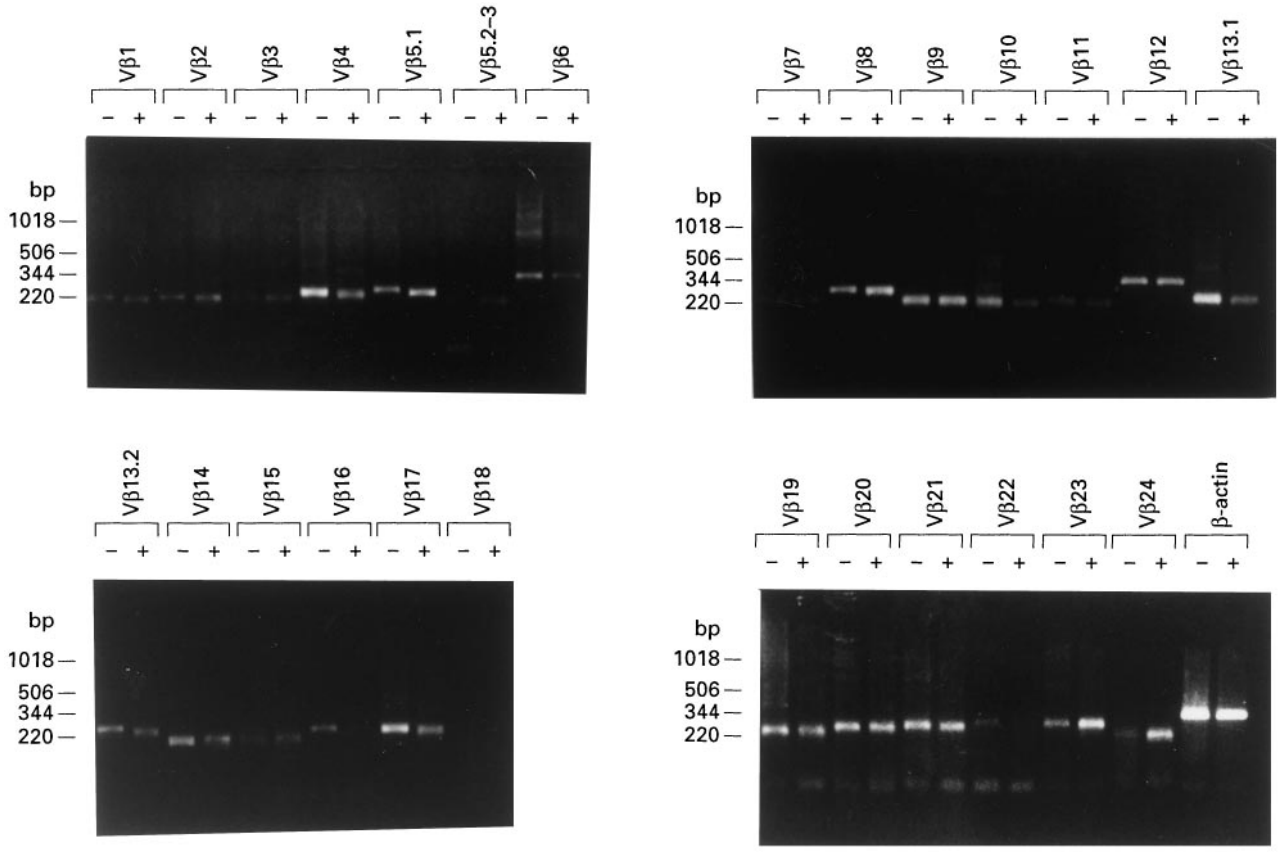


Fig. 2. T cell receptor (TCR) Vβ gene utilization of peptide 336–351-stimulated T cells in patients with BD. Peripheral blood mononuclear cells (PBMC) were stimulated by peptide 336–351 and total RNA was then extracted. cDNA was synthesized, followed by polymerase chain reaction (PCR) amplification using TCR Vβ subfamily-specific primers. TCR Vβ gene segments from freshly isolated PBMC (−) and those from peptide 336–351-stimulated PBMC for 9 days (+) were analysed by 1% agarose gel in $0{\cdot}5 \times$ TBE buffer. PBMC were obtained from patient 5, and results obtained by PCR are consistent with those by immunofluorescence analysis.

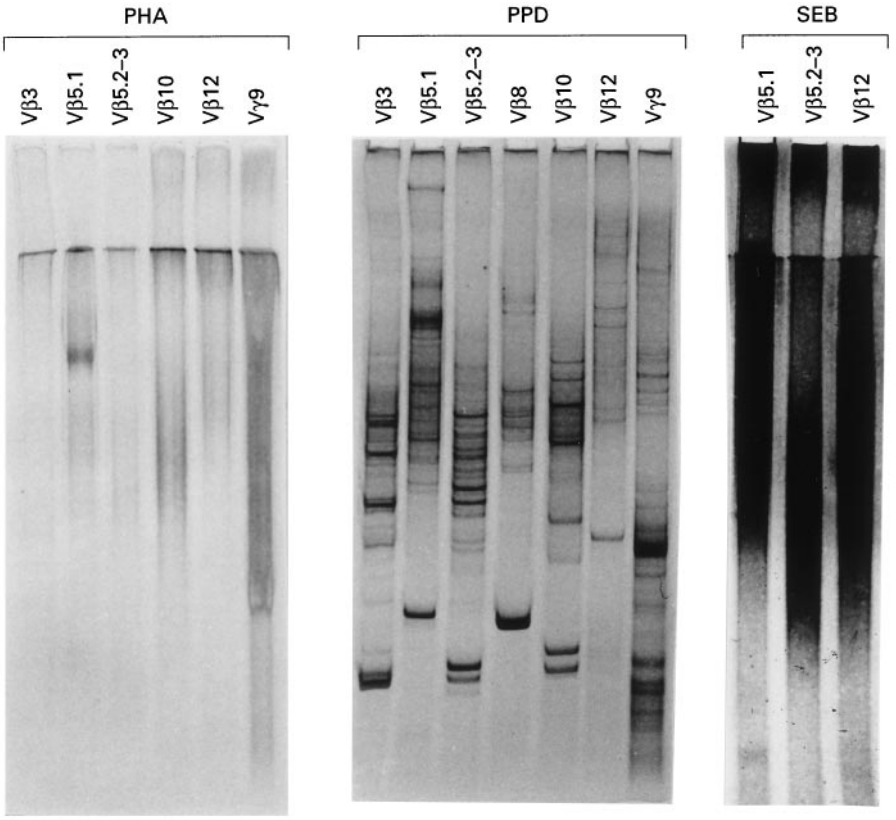


Fig. 3. Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP)-silver staining analysis for comparison of T cell receptor (TCR) clonotypes from a normal subject. Peripheral blood mononuclear cells (PBMC) were stimulated either by phytohaemagglutinin (PHA), purified protein derivative (PPD) or Staphylococcal enterotoxin B (SEB). Thereafter, total RNA was extracted and cDNA synthesized. PCR amplification was carried out using TCR Vβ subfamily-specific primers. The PCR products were heat-denatured and analysed by the SSCP method. PCR products from PHA-stimulated PBMC migrated broadly in the gel (left side), resulting in smearing of the products. In contrast, PPD stimulation of cells resulted in formation of visible bands (centre). SEB stimulation of PBMC resulted in smearing formation of relevant Vβ subfamilies (right side).

Analysis of TCR usage in peptide 336–351-stimulated T cells in patients with BD by flow cytometry

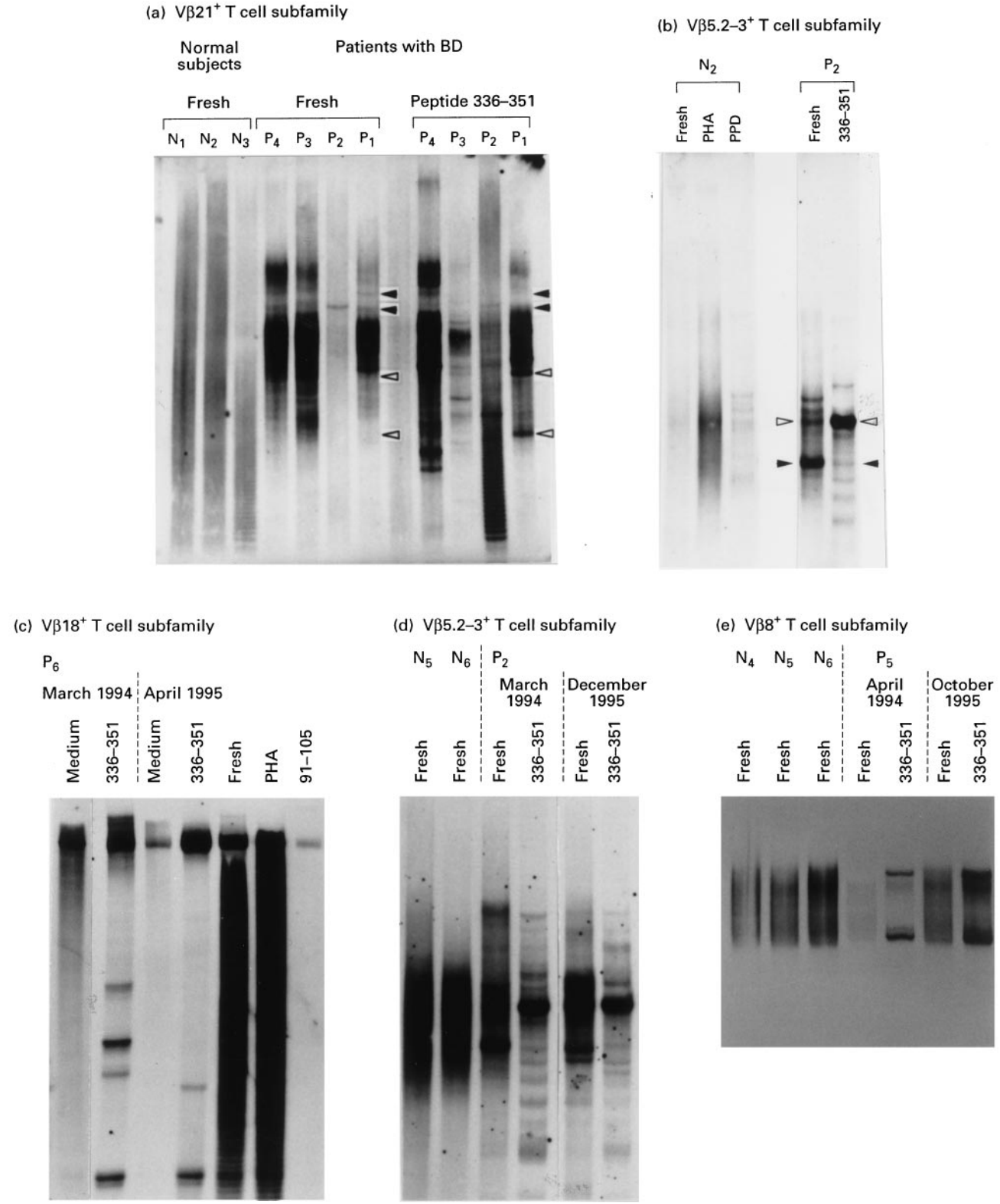
We stained unstimulated T cells (before stimulation) and peptide 336–351-stimulated T cells (after stimulation) with a panel of Vβ subfamily gene product-specific MoAbs. We found that the percentage of at least one Vβ subfamily gene product was increased more than 2·5 times in T cells stimulated by peptide 336–351 for 9 days, compared with freshly isolated T cells from the same patient in six of nine patients (data not shown). For example, T cells with TCR Vβ18.1 gene products were significantly increased in response to peptide 336–351 stimulation in three of nine patients tested (patient 1 (before 1·1%; after 13·2%), patient 3 (before 1·4%; after 4·8%) and patient 6 (before 0·5%; after 2·2%)). Similarly, T cells with TCR Vβ21.3 were increased in two patients (patient 1 (before 1·6%; after 50·3%) and patient 4 (before 2·4%; after 6·1%)). T cells bearing TCR Vβ5a, Vβ5b, Vβ13.6 or Vβ8 gene product were also increased in some patients (patient 2, Vβ5a (before 4·1%; after 40·1%) and Vβ5b (before 3·4%; after 48·1%): Vβ5.3 gene products can be stained by both Vβ5a and Vβ5b MoAbs, so that patient 2 probably included T cells with Vβ5.3 gene products; patient 3, Vβ13.6 (before 2·9%; after 14·9%); patient 5 Vβ8 (before 2·8%; after 9·4%)).

In three other patients and two normal subjects tested, a low ^{3}H-TdR incorporation of T cells was caused by peptide 336–351 stimulation, and T cells with any Vβ subfamily gene products were not increased (data not shown). Thus, we can detect the increase of T cells bearing specific Vβ subfamilies by this peptide stimulation at least in some patients with BD. It is suggested that different patients with BD have different Vβ usage, with some uniformity in three out of nine patients with Vβ18.1.

An antigen-driven, oligoclonal expansion of T cells reactive with the hsp-derived peptide in vivo *in patients with BD*

To analyse further the mechanisms responsible for T cell proliferation in response to the hsp-derived peptide in BD patients, RT-PCR technique was adopted with a set of TCR Vβ gene segment-specific primers (TCR Vβ1–24, plus Cβ) [29–31]. Ethidium bromide staining of conventional agarose gel showed that the amplified DNA, along with every respective set of primers, migrated as a single band of the expected size (Fig. 2). The results obtained by the PCR method roughly correlate with those by immunofluorescence analysis (data not shown).

In addition, it is evident that PCR products contained relevant amplified DNA, and did not include irrelevant amplified products. Since we used a set of a Vβ gene segment-specific primer and a constant Cβ primer for PCR, various diversities (D, J, and N regions) should be amplified and present within the band. To discriminate each TCR clonotype among the amplified products



(See next page for caption.)

of the same size we applied the SSCP technique, which has been reported to detect a single nucleotide mutation [32,33,35].

When PCR products from normal T cells stimulated by PHA were analysed by the SSCP method, the products migrated broadly in the gel, resulting in the smearing of the products (Fig. 3). In contrast, a specific antigen, PPD-stimulation of T cells resulted in the formation of visible bands (Fig. 3). A superantigen, SEB, provoked proliferation of T cells with a restricted set of TCR Vβ subfamilies [33,36]. Indeed, subsequent SSCP analysis of the products of SEB-stimulated T cells revealed the smearing of the products (Fig. 3). These data indicate that PHA stimulates T cells as a polyclonal activator, whereas PPD stimulates relevant T cells in an antigen-specific manner. In addition, SEB stimulates almost all T cells bearing particular Vβ subfamily gene products.

As shown above, it is possible that T cells in BD patients recognize and respond to the hsp peptide as a specified antigen, or alternatively, the hsp peptide induces proliferation of certain T cell subfamilies through a superantigen-like mechanism. In order to clarify this issue, we studied clonotypes of the T cells reactive with hsp-derived peptide in BD patients.

As a first step, we analysed clonotypes by PCR-SSCP of TCR Vβ21$^+$ T cells. After stimulation for 9 days with peptide 336–351, lower bands (two open arrowheads) became prominent, whereas upper bands (two closed arrowheads) became faint (Fig. 4a), suggesting antigen-driven stimulation, but not superantigen-like stimulation by peptide 336-351.

More interestingly, we found that PCR-amplified products of freshly isolated T cells from patients with BD formed several bands in the blot, whereas those from normal subjects migrated broadly, resulting in smearing in the blot (Fig. 4a). These results suggest that T cells from BD patients are oligoclonally expanded *in vivo*, perhaps as a result of stimulation by unknown antigens, including the 60-kD hsp peptide 336–351. We next analysed those of TCR Vβ5.2–3$^+$ T cells (Fig. 4b). The results were essentially the same as those with TCR Vβ21$^+$ T cells. These results suggest that peptide 336–351 stimulates T cells with selected Vβ subfamily gene products in an antigen-driven manner in BD patients.

A longitudinal study of TCR clonotypes that were reactive with hsp-derived peptides in BD patients

We next carried out a longitudinal study of patients to see whether disease activity affected clonotypes of antigen-specific T cell responses to the peptide. Patient 6 was studied at disease onset, and after successful treatment that caused his eye symptoms to subside. As shown in Fig. 4c, at disease onset (March 1994; SI to peptide 336–351 was 24·7) he had several clonotypes of T cells that were reactive with the peptide. After successful treatment (April 1995; SI to peptide 336–351 was 1·8), the peptide-specific T cell clones were definitively decreased. In contrast, patients 2 (SI to peptide 336–351 in March 1994 was 5·1, and that in December 1995 was 3·5) and 5 (SI to peptide 336–351 in April 1994 was 9·9, and that in October 1995 was 3·2) had recurrent eye disease, despite intensive treatment, including cyclosporin A. Nonetheless, they developed vigorous T cell responses to the peptide for every additional attack (see above individual parentheses). T cell clonotypes specific for the peptide of patients 2 and 5 were kept unchanged during periods of frequent attack (Fig. 4d,e). This finding further suggests the importance of hsp-specific T cell responses in the pathogenesis of this disease.

DISCUSSION

The etiology and pathogenesis of BD are not fully clarified. Nonetheless, abnormal immune responses were reported to be associated with the pathogenesis of this disease [1,2,37]. It has been reported that T cells in BD patients responded to mycobacterial 65-kD hsp-derived peptides and their human homologous mitochondrial 60-kD hsp-derived peptides [19].

In the present study, peptide 336–351 of human 60-kD hsp, one of the synthetic peptides we tested, yielded an enhanced lymphoproliferative response in Japanese BD patients compared with normal subjects and those with RA. It is argued that etiology, pathogenesis and clinical symptoms of patients with BD found in the Silk Route would be different from those of patients with BD outside the Silk Route. It is thus important that there is significant proliferative response to peptide 336–351 in BD patients in both areas. Therefore, we have to emphasize that BD found in both areas may belong to the same disease entity, and hsp antigen may play a role in the pathogenesis of this disease.

Epidemiological studies in our country have revealed that HLA-B51 is strongly associated with BD [38–40]. We thus examined whether B51 may be associated with proliferation to the peptides. However, T cell responses to the four peptides studied of HLA-B51$^+$ patients were comparable to those of HLA-B51$^-$ patients (data not shown). Similarly, Pervin *et al.* reported the lack of association between HLA-B51 and hsp-stimulated proliferation of T cells [19]. Rather, our previous finding clearly demonstrated that HLA-B51 is associated with aberrant regulation of neutrophil functions in patients with BD [41]. HLA-B51 may thus relate to the severity of the disease.

We found that peptide 336–351 exclusively stimulated CD4$^+$ T cells, and this response was inhibited by anti-HLA-DR and anti-CD4 MoAbs [42,43]. We also found that any HLA-DR haplotypes in BD patients were not associated with T cell proliferative responses in our studies (data not shown). Thus, there may be no strict preference of HLA-DR haplotypes with regard to antigen presentation of this peptide, and most, if not all, HLA-DR could

Fig. 4. T cell receptor (TCR) clonotype analysis of T cells that react with the hsp peptide 336–351 in patients with BD. (a) T cells from normal subjects (N_1, N_2, N_3) and those from patients with BD (P_1, P_2, P_3, P_4) were recovered immediately after isolation (fresh) or stimulated for 9 days with peptide 336–351. The cDNA was amplified by polymerase chain reaction (PCR) using Vβ21-specific primers and visualized by single-strand conformation polymorphism (SSCP)-Southern blotting analysis. After 9 days' culture with peptide 336–351 lower bands (two open arrowheads) became prominent, whereas upper bands (two closed arrowheads) became faint (P_1). (b) Similar experiments were performed using Vβ5.2–3-specific primers in the normal subject (N_2) and the patient (P_2). (c) TCR clonotypes reactive with peptide 336–351 were studied using Vβ18.1-specific primers in patient 6. TCR clonotypes reactive with the peptide were decreased after disease remission. (d, e) Similar experiments were performed using Vβ5.2–3-specific primers in patient 2 (d) and Vβ8-specific primers in patient 5 (e). TCR clonotypes reactive with the peptide were kept unchanged for every additional attack in patients 2 and 5, both of whom had refractory eye disease. Peripheral blood mononuclear cells (PBMC) were cultured with phytohaemagglutinin (PHA) for 5 days, purified protein derivative (PPD) for 9 days, and peptide 336–351 for 9 days.

present hsp-derived peptides to the T cells. It has already been reported that reactivity to the hsp peptides was not restricted by HLA class II antigen [19].

It has already been reported that $\gamma\delta$ T cells in patients with BD responded to the 65-kD hsp [44], and such $\gamma\delta$ T cells may regulate $\alpha\beta$ T cells. In the present study, we did not examine the $\gamma\delta$ T cell subset, but we found that the percentage of T cells with certain Vβ subfamily gene products was increased more than 2·5 times in hsp peptide 336–351-stimulated T cells in six of nine patients, compared with freshly isolated T cells of the same patients. In contrast, we did not detect any increase or reduction of T cells in response to peptide 336–351 stimulation in normal subjects. It is generally believed that a T cell bears a distinct clonotype of TCR, and each clonotype is responsible for an antigen-specific T cell response. Therefore, an immune response of the host to a certain antigen should be carried out by the summation of different clones. PCR-SSCP analysis enables us to estimate an accumulation of T cell clonotypes qualitatively [33,35]. The PCR products of freshly isolated PMBC from normal subjects showed smearing, but those from BD patients resulted in formation of several distinct bands in the smearing. The difference may be ascribed to the fact that T cells in BD patients would have been stimulated by the unknown antigen or possibly hsp *in vivo*, so oligoclonal expansion of selected T cells may have occurred. We also found that after 9 days culture with peptide 336–351 some bands became prominent, whereas the remaining bands became faint in BD patients. These results suggest that the peptide stimulates T cells in an antigen-driven manner, but not by superantigen-like mechanisms.

T cells proliferative responses to the peptide and clonotypes of the peptide-reactive T cells were altered after successful treatment; T cell proliferative responses and TCR clonotypes of patients with refractory disease remained unchanged for a long period of time. Thus, it is possible that not only the proliferative responses but also certain clonotypes of hsp-reactive T cells are associated with the pathogenesis of this disease.

We found that peptide 336–351-stimulated PBMC express mRNA of proinflammatory and inflammatory cytokines such as IL-8, and tumour necrosis factor (TNF) in BD patients but not in normal subjects (data not shown). These results suggest that hsp-derived peptides or hsp by itself may be responsible for provoking inflammatory reaction, either directly or indirectly, in this disease.

In conclusion, we suggest that hsp antigen may play a role, at least in part, in the pathogenesis of BD, regardless of geographic location. We believe that analysis of hsp responses by T cells is important to clarify the pathogenesis of BD, and could propose the hypothesis of positive selection in autoimmunity of this disease.

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