

Interaction among human leucocyte antigen–peptide–T cell receptor complexes in cow's milk allergy: the significance of human leucocyte antigen and T cell receptor–complementarity determining region 3 loops

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Summary

Background Allergic individuals respond to only a few specific antigens, therefore allergic diseases are characterized by antigen specificity. Clarification of the mechanism of antigen specificity will lead to progress in the therapy of allergic diseases.

Objectives The purpose of this study is to determine the specific association among T cell epitopes, antigen-presenting molecules and T cell receptor (TCR), and to determine the TCR usage in the pathogenesis of allergies using antigen-specific T cell clones (TCCs). The results can clarify the mechanism of the antigen specificity of allergic diseases, and provide new therapeutic possibilities using analogue peptides.

Methods Short-term T cell clones specific to β -lactoglobulin (BLG) were established from peripheral blood mononuclear cells (PBMCs) collected from five patients allergic to cow's milk. We then identified the T cell epitopes and antigen-presenting molecules, and examined TCR usage. We also determined the sequence of the TCR-complementarity-determining region 3 (CDR3).

Results Six TCCs established from the five patients recognized three different peptides, and BLGp97–117 was recognized by four of the six TCCs. BLGp101–112 (KYLLFCMENSAE) was the core sequence in the fragment. Sequence analysis of TCR by the RT-PCR method revealed a marked heterogeneity in TCR usage, and similar amino acid sequences were recognized in the CDR3 region. Four of the six TCCs recognized BLG in association with human leucocyte antigen (HLA)-DRB1*0405 as antigen-presenting molecules.

Conclusion We proposed the motif of the interaction between the HLA-DRB1*0405 allele and antigen peptide, and suggested that HLA-DRB1*0405 is an immunoregulatory gene product for T cell responses to BLG.

Keywords β -lactoglobulin, T cell receptor, peripheral blood mononuclear cells, antigen-presenting cells, complementarity determining region 3, lymphocyte proliferative responses, T cell clone, human leucocyte antigen

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Introduction

Antigen specificity is one of the most important features in allergic diseases. At the molecular level, interaction among the human leucocyte antigen (HLA), a peptide and the T cell receptor (TCR) is the first step to antigen recognition, and these molecules determine the onset of the disease. Exogenous antigens are engulfed and processed by endosomal proteases into peptides in antigen-presenting cells (APCs), which are then presented to T cells by the appropriate HLA class II molecules present on the cell surface. The CD4⁺ T helper cells are activated upon recognition of these peptide–HLA complexes by TCR molecules and exert effector functions through various biological activities of the secreted lymphokines. T cell epitopes

of several major allergens have been reported [1–10]. Although little information has been reported on T cell epitopes that recognize food antigens, we identified T cell epitopes and antigen-presenting molecules recognized by T cells from patients with cow's milk allergy [11]. The T cell epitopes presented by HLA are recognized by TCR, then complementarity-determining region 3 (CDR3) interacts directly with the peptide as determined by structural analysis [12]. In the case of birch pollen allergy, studies of Bet v1-specific T cell clones (TCCs) revealed a marked heterogeneity in TCR V α and V β genes usage [13,14]. In another paper, it was reported that the peribronchial lymph node of ovalbumin-sensitized mice has an increased number of OVA-reactive T cells expressing V β 8.1/8.2 [15]. To identify T cell epitopes of β -lactoglobulin (BLG), antigen-presenting HLA class II molecules, TCR usage, and CDR3 sequence, we established six BLG-specific T cell clones from five patients with cow's milk allergy.

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Materials and methods

Subjects

Five patients with cow's milk allergy were studied, and the diagnosis of allergy to cow's milk was based on clinical symptoms, challenge test and positive CAP system results for reactivity to cow's milk and BLG, or an increased antigen-specific lymphocyte proliferative response (LPR). The control group is composed of 33 individuals who are not allergic to cow's milk.

BLG-specific lymphocyte proliferative responses

BLG-specific LPR were determined as described previously [16]. Blood samples were mixed with preservative-free heparin and were separated by gradient centrifugation using Ficoll-Paque (Pharmacia, Uppsala, Sweden). Peripheral blood mononuclear cells (PBMCs) were harvested from the interface and washed three times in PBS. PBMCs were cultured at $10^6/\text{mL}$ of the RPMI1640 culture medium supplemented with 10% pooled AB⁺ serum, 10 mM N-2-hydroxyethyl-piperazine-N'-ethanesulphonic acid, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Cultures were grown in triplicate at 0.2 mL per well in round-bottomed microtest plates (A/S Nunc, Roskilde, Denmark) with or without various concentrations of a crude BLG antigen (Sigma Chemical Co., St Louis, MO, USA) at 37°C in a humidified atmosphere of 5% CO₂ for 4 days. The BLG protein was used at a final concentration of 25 µg/mL. The amount of DNA synthesized was measured by adding 0.5 µCi [³H] thymidine (specific activity: 43 Ci/mmol, Amersham, Tokyo, Japan) per well of the microtest plates 4 h before harvesting using glass fibre filters. The amount of [³H] incorporated was measured by liquid scintillation counting, and the results were expressed as the mean of triplicate cultures. To compare responses between individuals, we expressed the results as the stimulation index (SI):

$$\text{SI} = \frac{\text{mean CPM in cultures stimulated with BLG}}{\text{mean CPM in unstimulated cultures}}$$

The specificity of proliferative responses of PBMCs to each BLG peptide was previously confirmed in children with atopic dermatitis [17].

Synthesis of BLG-derived peptides

Peptides were synthesized corresponding to the amino acid sequence of BLG reported previously [18], using a solid-phase simultaneous multiple peptide synthesizer PSSM-8 (Shimadzu, Kyoto, Japan), based on the Fmoc strategy. Peptides of 12–21 amino acid residues overlapped each other by 11 residues as described previously [11], and corresponded to the entire sequence of BLG (genetic variant of A). All peptides were purified by C18 reverse-phase HPLC (Millipore Waters, Milford, MA, USA).

Generation of antigen-specific T cell lines and clones

T cell lines were generated by stimulating PBMCs ($1.5 \times 10^5/\text{well}$) with a mixture of 22 synthetic BLG-derived peptides at a final concentration of 0.1 µM each in the RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and 10% pooled, heat-inactivated normal human male plasma in 96-well flat-bottomed culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ, USA). After 7–10 days,

irradiated (3000 cGy) autologous PBMCs ($1.5 \times 10^5/\text{well}$) pulsed with the BLG-derived peptide mixture (1 µM each for 5 h), human rIL-2 (50 U/mL), and human rIL-4 (10 U/mL) were added only to culture wells containing T cell blasts, which were maintained for another 7 days. Aliquots of cultures were combined as a bulk T cell line for each patient, and reactivity against the overlapping peptides was determined. Cloning was carried out using Terasaki plates (Nunc), by a limiting dilution at 0.3 cell per well in the presence of irradiated (3000 cGy) autologous PBMCs ($3 \times 10^4/\text{well}$) pulsed with the BLG-derived peptide mixture (1 µM), human rIL-2 (50 U/mL), and human rIL-4 (10 U/mL) in the same medium described above. Growing microcultures (approximately 5% of all culture wells) were then expanded at weekly intervals, first in a 96-well plate, and then in a 24-well plate by feeding irradiated feeder cells ($1.5 \times 10^6/\text{well}$) pulsed with the BLG-derived peptide mixture (1 µM) in the presence of rIL-2 and rIL-4.

Antigen-induced proliferative responses of T cells

Antigen-induced proliferation of the T cell lines was assayed by culturing the T cells ($3 \times 10^4/\text{well}$) in 96-well flat-bottomed culture plates in the presence of the soluble BLG-derived peptide mixture (1 µM each), appropriate overlapping peptides (1 µM), or BLG protein (25 or 50 µg/mL) and 3000 cGy-irradiated autologous PBMCs ($1.5 \times 10^5/\text{well}$). Cells were cultured for 72 h, in the presence of 1 µCi/well of [³H] TdR during the final 16-h period, and the incorporated radioactivity was measured by liquid scintillation counting. To determine restriction molecules for antigen presentation, the T cell lines were cultured with irradiated autologous PBMCs, with or without anti-HLA class II monoclonal antibodies (mAbs) HU-4 (anti-HLA-DRB1 + DRB5 IgG2a, monomorphic) [19,20], L243 (anti-HLA-DRB1 + DRB4 IgG2a, monomorphic, reactivity to DRB3 and DRB5 yet to be determined) [21], HU-11 (anti-HLA-DQ4 + DQ5 + DQ6 IgG2a) [22], HU-18 (anti-HLA-DQ7 + DQ8 + DQ9 IgG2a) [23], HU-46 (anti-HLA-DQ4 IgG2a) [22], and B7/21 (anti-HLA-DP IgG1, monomorphic) [24]. The percentage inhibition was calculated using the following formula: $[1 - (\text{value obtained with a peptide and a mAb} - \text{that with the medium alone}) / (\text{value obtained with a peptide and without a mAb} - \text{that with the medium alone})] \times 100$. Other experiments were performed using PBMCs of two donors: one haplotype-shared donor and one donor with irrelevant HLA haplotypes as APCs. In this experiment, allogeneic PBMCs ($3 \times 10^5/\text{well}$) were cultured in plates with peptides (1 µM each) in the culture medium for 2 h at 37°C, then, excess peptides and non-adherent cells were removed by gently washing the plates three times with the RPMI 1640 medium containing 3% human serum. The remaining adherent cells were irradiated at 3000 cGy and used as APCs.

HLA typing

HLA class II (*DR* and *DQ*) alleles were determined by hybridization of HLA-*DR* and *-DQ* genes amplified by polymerase chain reaction using sequence-specific oligonucleotide probes distributed during the 11th International Histocompatibility Workshop, as described elsewhere [1]. The nomenclature of the HLA-*DR* and *-DQ* alleles was according to the WHO Nomenclature Committee for factors of the HLA system [25].

Epitope mapping of BLG-specific TCCs

To determine the T cell activation-inducing epitopes that are recognized by TCCs, the T cells were screened for reactivity with 22 short overlapping peptides. Next, the peptide recognized by most BLG-specific TCCs was fragmented into C1-C9 (Fig. 1a). One to nine amino acids were excised from the N and C termini of that peptide.

First-strand cDNA synthesis and PCR amplification

The total RNA of TCCs was isolated from 1×10^6 cells by the single-step guanidinium-phenol-chloroform extraction method [26]. Single-stranded cDNA synthesis was carried out using 1.2–1.8 µg of total RNA with oligo(dT) and avian myeloblastosis virus reverse transcriptase. TCR α and TCR β cDNAs were directly subjected to DNA amplification by polymerase chain reaction (PCR) using Amplita DNA polymerase (Perkin Elmer, Norwalk, CT, USA) with oligonucleotide primers complementary to TCR V and C region sequences [27] at a final concentration of 0.25 µM. Reaction mixtures were incubated in a DNA thermal cycler (Perkin Elmer) under the following conditions: denaturation at 93 °C for 1 min, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, with a further 5-s extension after each cycle.

Cloning and sequence analysis of TCR α and TCR β chains

The PCR reaction products were cloned into the TA cloning vector pCR IITM (Invitrogen Corporation, San Diego, CA,

USA) and sequenced by the enzymatic chain termination method [28], using a sequence version 2.0 kit (United States Biochemical Corporation, Cleveland, OH, USA). For each of the TCR α and TCR β chains studied, three independent pCR IITM cloning vectors were sequenced to identify possible sequence errors caused by nucleotide misincorporation by the Taq polymerase. Sequences were compared with gene data bank entries (GenBank, Los Alamos, NM, USA; EMBL, Heidelberg, Germany) and assigned to available published TCR V and J segments [29–32]. Virtually all TCRV transcripts have the amino acid sequence Y-(L/F)-C-A (α chain) or Y-(L/F)-C-A with two additional serine residues in most cases (β chain) at the 3' end of the V gene segment and the motif F-G-X-G-T within the J segment [33]. The CDR3 length was taken as the number of amino acids between the last germline-encoded residue of the V segment and the conserved phenylalanine residue.

Results

T cell clones

The six BLG-specific T cell clones (HA3.1, HA5.7, IR1.9, AHi5, PK9.3, YA4) were established from PBMCs of five patients with cow's milk allergy. Patient HA had shown an immediate hypersensitivity type of symptoms such as systemic urticaria, which occurred within 30 min after the antigen challenge (Table 1). Patients IR, AHi and PK had shown symptoms

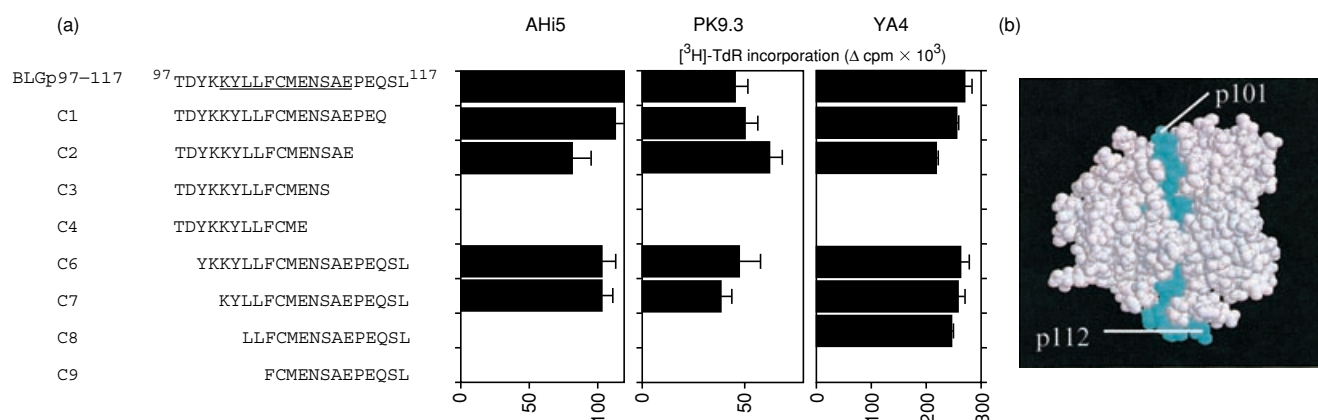


Fig. 1. Epitope mapping of BLG-specific TCCs. (a) The BLG-specific TCCs ($1\text{--}3 \times 10^4/\text{well}$) were incubated with synthetic peptides ($1 \mu\text{mol}/\text{mL}$) in the presence of autologous PBMCs ($1.5 \times 10^5/\text{well}$). Peptides truncated from the N or C terminal were used in this assay. Reactivity pattern of BLG-specific TCCs that react with BLGp17: 97–117. All TCCs that reacted with BLGp17 recognized the same region (p101–112: KYLLFCMENSAE). Values shown represent $\Delta \text{c.p.m.}$ values of triplicate cultures. Standard deviations are indicated by error bars. (b) Space filling model of BLG with the minimal T cell epitope (cyan).

Table 1. Profile of the patients

Patient	Age (years)	Sex	Symptom	Food challenge	Total IgE (IU/mL)	CAP ($\text{U}_\text{A}/\text{mL}$)		LPR* (SI)†
						Milk	BLG	
HA	1	F	Urticaria	Positive	249	2.3	1.5	ND‡
IR	3	M	Eczema	Positive	177.2	0.51	0.42	35.1
AHi	31	F	Eczema	Positive	41.4	< 0.34	< 0.34	3.3
PK	52	M	Eczema	Positive	260	< 0.34	< 0.34	2.9
YA	2	F	Eczema	Positive	1136.8	94	55.4	1.29

*Lymphocyte proliferative responses. †Stimulation index. ‡Not done.

such as systemic eczema, which occurred more than 24 h after the antigen challenge. Patient YA had shown the immediate hypersensitivity type of symptoms of systemic rash and itching, as well as the symptom of systemic eczema, which occurred more than 24 h after the antigen challenge.

HLA class II alleles involved in antigen presentation to BLG-specific TCRs

Using the mAbs, we define the restriction element of BLG-specific TCCs (Fig. 2). HLA class II (DR, DQ) alleles of all the subjects from whom we could establish BLG-specific TCCs in this study are shown in Table 2. Four (HA3.1, HA5.7, AHi5, YA4) of the six TCCs required HLA DRB1*0405 as the antigen-presenting molecule during proliferation in the presence of the antigen, and three of these TCCs recognized BLGp97–117 (Table 3).

T cell clones and epitope mapping of BLG-specific TCCs

Six TCCs derived from five individuals allergic to cow's milk were included in this study. All clones exhibited the helper T cell phenotype (CD3⁺, CD4⁺) and expressed the $\alpha\beta$ TCR (data not shown). All TCCs proliferated in the presence of only one fragment of all peptides, namely, BLGp30–47, BLGp97–117 and BLGp142–162. Four (HA5.7, AHi5, PK9.3 and YA4) of the six TCCs recognized BLGp97–117 (TDYKKYLLFC-MENSAEPEQSL). This fragment formed β sheet (Fig. 1b) [34], and was regarded as a major T cell epitope among the five patients studied. To define the minimal T cell activation-inducing epitope, we compared proliferative responses of TCCs using peptides of BLGp97–117 truncated into fragments from N and C termini (Fig. 1a). Figure 1(a) shows patterns of TCC reactivity to truncated peptides of BLGp97–117. Three TCCs (AHi5, PK9.3, YA4) which recognized BLGp97–117 did not proliferate when stimulated with C3, C4, C8 and C9, indicating that they recognized BLGp101–112 (KYLLFC-MENSAE).

TCR α and TCR β chain analysis of epitope-specific TCCs

Sequence analysis showed a marked heterogeneity of TCRV α and TCRV β gene usage by the BLG-specific TCCs like our recent report of heterogeneous usage of BLG-specific TCCs [10] (Figs 3a and b). HA5.7, AHi5, PK9.3 and YA4 were specific to the same epitope (BLGp97–117), but had different V and J gene segments. Interestingly, HA3.1 and HA5.7 were cloned from the same patient, but these clones had different V β and J β gene segments. In the α -chain, these TCCs had the same J α segment gene (J α 13), but different V α gene segments.

Comparison of CDR3 loops of TCRs

CDR3 α The six CDR3 α loops had lengths varying between nine to 13 amino acid residues, and all loops contained a positively charged amino acid (Fig. 3a). In addition, five of them shared 'QKV', 'NKF' or 'NKL'. These sequences are composed of an amide-bearing residue, a positively charged residue and a hydrophobic residue, and YA4 also have 'NDM' that is closely similar to these sequences. Moreover, the N-terminus of these characteristic amino acids of CDR3 loops is rich in glycine.

CDR3 β Six CDR3 β regions had lengths varying between eight and 15 residues, and five of them (HA3.1, HA5.3, IR1.9,

AHi5, PK9.3) contained a positively charged residue 'R', 'Q' or 'K'. All CDR3 β s contained an amide-bearing residue 'Q' or 'N', and an OH-group-bearing residue 'Y' or 'T' (Fig. 3b). HA3.1, AHi5 and PK9.3 shared 'EQY', composed of a negatively charged 'E', an amide-bearing residue and a positively charged 'Q', and a hydrophobic 'Y'. The other three CDR3 β loops did not share these sequences, but contained a similar amino acid group.

HLA-DRB1 allele in the patients with cow's milk allergy

Table 4a shows the comparison of frequency of the HLA-DRB1*0405 allele between the control group and cow's milk allergy group. The frequency in the cow's milk allergy group was higher than that in the control group, but there was no significant difference ($P=0.1087>0.05$) between the two groups. Among the patients with cow's milk allergy from whom we could establish the BLG-specific TCCs, 71.4% of the subjects had the HLA-DRB1*0405 allele (Table 4b). However, only 7.7% of the patients from whom we could not establish BLG-specific TCCs had the HLA-DRB1*0405 allele. There was a significant difference ($P=0.0065<0.05$) between the two groups, indicating that the HLA-DRB1*0405 allele is highly involved in BLG recognition.

Discussion

This study was designed to investigate the mechanism of antigen specificity in allergic diseases. We analysed the TCR usage and its CDR3 sequence in CD4⁺ T cells of patients allergic to the major cow's milk allergen BLG, in order to evaluate the potential of therapeutic strategies targeted at the allergen-specific TCR/antigen/HLA complex. We analysed the TCR α and TCR β chains that are expressed in six TCCs established from five patients allergic to cow's milk and recognize three HLA class II restricted epitopes of BLG (p30–47, p97–117, p142–162), especially HLA-DRB1*0405-BLGp97–117 complex.

Three peptides regarded as the T cell epitope formed the β sheet in the BLG molecule as revealed by crystal analysis [34]. In particular, BLGp97–117, regarded as the major epitope, is buried in the BLG molecule, and its edge is orientated toward the surface (Fig. 1b). Three TCCs recognized 12 amino acid residues 'KYLLFCMENSAE' (Fig. 1a).

Our study revealed heterogeneity in TCR usage of BLG-specific TCCs. Three TCCs (HA5.7, AHi5 and YA4) recognized the same peptide BLGp97–117 presented by the same restriction element HLA-DRB1*0405. However, these three TCCs used different TCR genes. We expected that the factor participating in antigen specificity may exist in the CDR3 loop, because there is a greater variation in the TCR-CDR3 that interacts directly with various peptides presented by HLA molecules. A marked heterogeneity in TCR usage and differences in the TCR-CDR3 length were observed (Figs 3a and b). In contrast, Wang et al. reported that the length of the CDR3 β loop is important for the recognition of the vesicular stomatitis virus peptide and its variants [35], that is, a particular substitution at residue position 6 of the VSV peptide with which the CDR3 β loops interaction requires a particular length of CDR3 β loops.

However, TCR-CDR3 loops, which are directly in contact with the antigenic peptide presented by the HLA molecule,

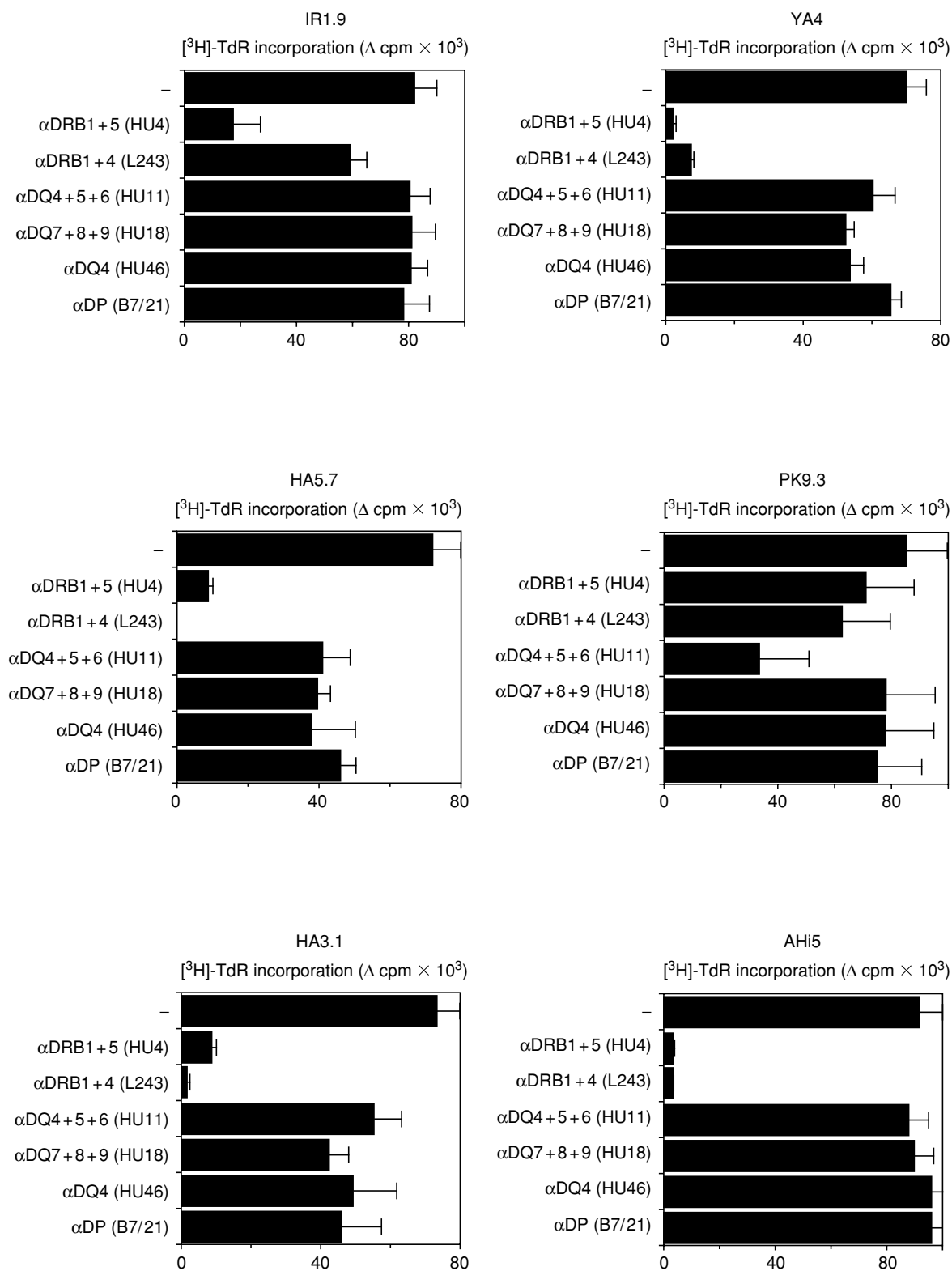


Fig. 2. Proliferation-inhibition of six TCCs using anti-HLA class II mAbs. T cells (3×10^4 /well) were incubated with HU4 (anti-HLA-DRB1 + DRB5), L243 (anti-HLA-DRB1 + DRB4), HU11 (anti-HLA-DQ4 + DQ5 + DQ6), HU18 (anti-HLA-DQ7 + DQ8 + DQ9), HU46 (anti-HLA-DQ4), B7/21 (anti-HLA-DP) and irradiated autologous PBMCs (1.5×10^5 /well), with or without BLG peptides ($1 \mu\text{M}$ each). Results are expressed as the geometric mean of triplicate determinations $\pm$ standard error. All mAbs were tested at a final dilution of 1 : 200.

Table 2. HLA class II DNA typing of the patients

Patient	DRB1*	DRB3*	DRB4*	DRB5*	DQA1*	DQB1*
HA	0405/0901	—/—	0101/0101	—/—	0301/0301	0303/0401
IR	0405/1401	—/0202	0101/—	—/—	0302/0101	0401/0503
AHi	0405/1401	—/0202	0101/—	—/—	0301/0101	0401/0503
PK	0901/0803	—/—	0101/—	—/—	0301/0302	0303/0601
YA	0405/1302	—/0301	0101/—	—/—	0301/0102	0401/0609

Table 3. Antigen-presenting molecules of BLG-specific TCCs

TCC	T cell epitope	APC
HA3.1	BLGp30–47	DRB1*0405
HA5.7	BLGp97–117	DRB1*0405
IR1.9	BLGp142–162	DRB1*1401
AHi5	BLGp97–117	DRB1*0405
PK9.3	BLGp97–117	DQB1*0601
YA4	BLGp97–117	DRB1*0405

To determine restriction molecules for antigen presentation, the TCCs were cultured with irradiated autologous PBMCs, with or without anti-HLA class II monoclonal antibodies (mAbs) HU-4, L243, HU-11, HU-18, HU-46, and B7/21. Other experiments were performed in which PBMCs from two donors, one haplotype-shared donor and one donor with irrelevant HLA haplotypes, were used as APC.

contained the characteristic amino acid residue. Analysis of the CDR3 α sequence showed that 'QKV', 'NKF' or 'NKL' composed of an amide-bearing residue, a positively charged residue and a hydrophobic residue is shared by five of six TCCs specific to BLG. There may be a specific interaction between the positively charged 'K' in these conserved residues and the negatively charged 'E' in position 6 of the T cell epitope. Moreover, the utilization of the hydrophobic residue 'V', 'F' or 'L' suggests that this residue may be involved in packing interactions with the hydrophobic residue in the T cell epitope. Moreover, we also suggest that the presence of 'G' at the N terminal of this conserved motif is required to provide maximal flexibility of the main chain in association with 'G' in the frame of the J segment. This flexibility may be necessary to allow the CDR3 loops to access a number of different conformations. Because of a lack in a side chain, 'G' may allow a closer contact between a peptide and CDR3 loops than other amino acid residues would.

Analysis of the CDR3 β sequence showed another motif 'EQY' which is composed of a negatively charged residue, an amide-bearing residue and a hydrophobic residue. This composition closely resembles that of the motif of CDR3 α loops. There may also be a specific interaction between the negatively charged 'E' in these conserved residues and the positively charged 'K' in position 1 of the T cell epitope. The utilization of the hydrophobic residue 'Y', 'F' or 'L' suggests that this residue may have been involved in packing interactions with the hydrophobic residue in the T cell epitope, the same as in the case of CDR3 α loops. Several studies demonstrated that TCRs

Table 4. HLA-DRB1 haplotypes in the patients with cow's milk allergy

(a)	Milk allergy group	Control group
<i>n</i>	18	33
non-DRB1*0405	13	29
DRB1*0405	5	4
frequency of DRB1*0405	27.8%	12.1%
	$P = 0.1516 > 0.05$	

(b)	Patient	DRB1 allele	Patient	DRB1 allele
	1 HA	0405 /0901	6 TR	0901/0101
	2 IR	0405 /1401	7 AY	0901/1403
	3 AHi	0405 /1401	8 MY	0405 /0901
	4 PK	0901/0803	9 OS	1302/1405
	5 YA	0405 /1302	10 OM	0101/1302
			11 KS	0404/1502
			12 KT	0901/1403
			13 MH	0901/1502
			14 KR	1401/1501
			15 MK	1302/1502
			16 KF	0901/1302
			17 MW	1302/1502
			18 TK	0403/0803
frequency of DRB1*0405		80.0%		7.7%
		$P = 0.0077 < 0.05$		

(a) Comparison of the frequency of HLA-DRB1*0405 between the cow's milk allergy group and the control group. There is not a significant difference between the two groups ($P = 0.1516 > 0.05$). (b) BLG-specific TCCs could be established for patients 1–5 but not for patients 6–20. HLA-DRB1*0405 is indicated in bold. There is a significant difference between both groups in the frequency of DRB1*0405 allele ($P = 0.0077 < 0.05$).

(a)

T cell clone	TCR gene segment	TCRAV	CDR3	TCRAJ
HA 3.1	V2J13	GGGGACTCAGCTATCTACTTCTGTGCA G D S A I Y F C A	GACCTCTCTGGGGTTTACCAAGATTAC D L S G G Y Q K V T	TTTGGAACTGGAACAAAGCTCCAAGTATCCCA F G T G T K L Q V I P
HA 5.7	V1J13	TCACTAGCAGCTATCTGAAGACGA S D A A E Y F C A	CACCCAGAGACCCCAATGCTTTCAATGG V G V S G G Y Q K V T	TTTGGAACTGGAACAAAGCTCCAAGTATCCCA F G T G T K L Q V I P
IR 1.9	V9.1J21	GAAGACTCAGCCTATCTGTGTGCT E D S A M Y Y C A	CCTCCATACACTTCAACAAATTTAC P P Y N F N K F Y	TTTGGAACTGGAACAAAGCTCCAAGTATCCCA F G S G T L L N V L P
AH1 5	V2J4	AGTGATTCAGCCTTACCTCTGTGCAATG S D S A T Y L C A M	AATGCTGTGCTTACAAACAGCTCATC N G G A T N K L I	TTTGGAACTGGAACAAAGCTCCAAGTATCCCA F G T G T L L A V Q P
PK 9.3	V4J10	TCTGAGCTGCGCGGTTTCCCTGAGCAGACTGCTGTACTA S E P A P G F P E R H C C V L	CTGCTCCACCGGGCGAGAGGAACTCACC L P P H G A G G G N K L T	TTTGGAGCAGGCACTCAGCTAAAGTGAATC F G T G T Q L K V E L
YA 4	V13.1J43	GACTCAGCGGTTTATTTCTGCGCT D S G V Y F C A	GGGCTGTACAATAACAATGACATGCGC G L Y N N N D M R	TTTGGAGCAGGCACTCAGCTAAAGTGAATC F G A G S R L T V K P

(b)

T cell clone	TCR gene segment	TCRBV	CDR3	TCRBJ
HA 3.1	V7.1J2.7	TGAGCCCTGTATCTCTGGCCACGAGC S A L Y L C A S S	P/N D P/N CACGTTAGGCT <u>GGAC</u> H V R V D	TTTCGGCGGGGCAACAGGCTCAGGTCACA F G P G T R L T V T
HA 5.7	V5.1J1.1	TGGCCCTTTATCTTTGGCCAGCAGC S A L Y L C A S S	TTGAAT <u>GGACAGG</u> A L N G Q G	TTTGGACAGGCAACAGGCTCAGGTTGTA F G Q G T R L T V V
IR 1.9	V13.1J1.4	TCTGTGTACTTCTGTGCGCAGCT S V Y F C A S S	GAACCC <u>GGACAGG</u> E P R T G	TTTGGCAGTGGAAACCAAGCTCTCTGTCTTG F G S G T Q L S V L
AH1 5	V13J2.7	TCTGTGTACTTCTGTGCGCATC S V Y F C A I	AGCGAGCTCGG <u>ACAAA</u> C S G R R T N	TTTCGGCGGGGCAACAGGCTCAGGTCACA F G P G T R L T V T
PK 9.3	V2J2.7	AGCAGCTTCTACATCTGCAGT S S F Y I C S	GTTCCTCTCGGG <u>CTAGCGGGGGCA</u> <u>GGAGCC</u> V A L G A S G G A G A	TTTCGGCGGGGCAACAGGCTCAGGTCACA F G P G T R L T V T
YA 4	V12.2J1.2	TCTGTGTACTTCTGTGCGCATC S V Y F C A I	AGTGATC <u>CAGT</u> S V S S	TTTCGGTTCGGGCAACAGGTTAACCGTTGTA F G S G T R L T V V

Fig. 3. Nucleotide and deduced amino acid sequences of TCR α (a) and TCR β (b) junctional regions of particular TCRs specific to BLG. For each sequence, only the 7–15 V gene amino acids are shown, followed by the presumed Ig-like loops (CDR3). The underlined CDR3 sequences are encoded by the J segment gene. The double underlined CDR3 sequences indicate characteristic amino acid sequences. CDR3 β sequences are isolated to P nucleotides, N region and D region, and the dotted lined sequences indicate D region (see text).

interact with peptide/MHC complexes in a diagonal orientation, in which the CDR3 loops of TCR α and TCR β chains bind to the N and C termini of the peptide, respectively [12,36–38]. These previous studies were performed using the class I system: results of our present study suggest that the CDR3 α loop is in

contact with 'K' in position 1 of the T cell epitope, and the CDR3 β loop is in contact with 'E' in position 8 of the T cell epitope presented by the HLA class II system.

However, these conserved amino acid sequences of CDR3 α and CDR3 β loops are encoded by the J segment gene. The

heterogeneous usage of the J segment gene is shown in Fig. 3. Based on the results of this study, we propose that the HLA-DRB1*0405 allele participates in BLG recognition. In this study, five of the six TCCs were restricted by the HLA-DR molecules which is consistent with our previous report [10]. Only PK9.3 was restricted by the HLA-DQ molecule. Moreover, four of the five TCCs restricted by the HLA-DR molecule required a particular restriction element, HLA-DRB1*0405. Therefore, we suggest that HLA-DRB1*0405 is an immune-response gene product that induces T cell responses to BLG. However, there is no correlation between HLA-DRB1*0405 and allergy to cow's milk. Therefore, HLA-DRB1*0405 does not seem the major susceptible gene to the cow's milk allergy. Indeed, we have established BLG-specific TCCs that are restricted by HLA-DRB1*0405 from healthy individuals (data not shown). However, comprehension of the interaction among components of the HLA-DRB1*0405-BLG peptide-TCR complex contributes to clarifying the artificial regulation of T cell activation.

We are going to analyse the interaction between the HLA-DRB1*0405 allele and an antigenic peptide using analogue peptides of BLGp101–112. In the future, these studies may clarify further the mechanism of antigen specificity. Considering the linkage between BLGp97–117 and HLA-DRB1*0405, we expect the therapeutical possibility using analogue peptides of BLGp97–117. These observations indicate that peptides for the desensitization of T cells need to be designed keeping in mind the different requirements of different ethnic groups.

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