

Identification of β -lactoglobulin-derived peptides and class II HLA molecules recognized by T cells from patients with milk allergy

R. INOUE*†, S. MATSUSHITA†, H. KANEKO*, S. SHINODA*, H. SAKAGUCHI*, Y. NISHIMURA† and N. KONDO*

*Department of Paediatrics, Gifu University School of Medicine, †Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences, Gifu, Japan

Summary

Background Cow's milk allergy impairs the health and development of many infants since it deprives them of adequate nutrition. Cow's milk fractions contain many allergens, and β -lactoglobulin (BLG) is one of the major allergens.

Objective The purpose of this study was to determine T cell epitopes, antigen-presenting molecules and cytokine production by T cells in relation to BLG. The results can provide new therapeutic possibilities of using analogue peptides of BLG for infants with cow's milk allergy.

Methods Using a mixture of a panel of overlapping synthetic peptides that cover the entire BLG molecule, we established polyclonal BLG-specific short-term T cell lines and clones from peripheral blood mononuclear cells of four patients with allergy to cow's milk carrying most of the common human leucocyte antigen (HLA) haplotypes seen in the Japanese population. We then identified the T cell epitopes and antigen-presenting molecules, and measured the production of cytokines interleukin (IL)-4, IL-5 and interferon- γ in the culture supernatants.

Results The T cell lines established from the four patients responded to seven different peptides. Three of the peptides stimulated the T cells of two donors, regardless of the HLA types. The patterns of inhibition of the proliferative responses of the cell lines by anti-HLA class II antibodies were heterogeneous; three were mainly inhibited by anti-HLA-DR mAbs, and the other was inhibited by anti-HLA-DQ mAbs. High levels of IL-5 were produced by these T cell lines.

Conclusions Patients' T cells recognized BLG in association with a variety of HLA-DR or -DQ as antigen-presenting molecules. Although some peptides did have a more potent T cell stimulatory activity than others, the T cell receptor ligands formed with the BLG molecule are heterogeneous. Peptides for the desensitization of T cells of the patients with cow's milk allergy need to be designed keeping in mind the different requirements in different ethnic groups.

Keywords: β -lactoglobulin, T cell epitope, HLA, peptide, IL-5

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Introduction

Exogenous antigens are engulfed and processed by endosomal proteases into peptides in antigen-presenting cells (APC), which are then presented to T cells by the appropriate major histocompatibility complex (MHC) class

Correspondence: Ryosuke Inoue, Department of Paediatrics, Gifu University School of Medicine, Tsukasa-machi 40, Gifu 500, Japan.
E-mail: rinoue@cc.gifu-u.ac.jp

II molecules present on the cell surface. The CD4⁺ helper T cells (Th) are activated upon recognition of these peptide-MHC complexes by T cell receptor (TCR) molecules and exert effector functions through various biological activities of the secreted lymphokines. The T cell epitopes of several major allergens have been reported [1–10]. However, little has been reported on T cell epitopes recognize food antigens. Two reports identified several T cell epitopes recognized by ovalbumin (OA)-specific T cell lines [8,9]. They also reported that OA-specific T cell lines produced significant amounts of interleukin (IL)-5. Another study suggested a central pathogenic role for IL-5 in food allergy-related symptoms, using ovomucoid (OM)-specific T cell lines and clones [10].

Cow's milk allergy is a common allergic disease in infants; its reported prevalence ranges between 2% and 7.5% [11–13]. Cow's milk has been implicated in gastrointestinal, cutaneous and respiratory hypersensitivity reactions [14]. β -lactoglobulin (BLG) is found in the milk of many animals including bovine species, sheep, deer, horse, dog, pig, dolphin and humankind. It is remarkably acid-stable, resisting denaturation even at pH 2, and remains intact until after passage through the stomach, which may explain its being the most potent allergen in cow's milk [15]. Indeed, it induced allergic reactions such as urticaria, diarrhoea or vomiting in five of six patients with gastrointestinal milk allergy [16]. Recently, the T cell recognition sites on BLG were analysed in mice [17], but they have so far not been reported in humans. We established BLG-specific T cell lines and clones from four human leucocyte antigen (HLA)-typed Japanese patients with cow's milk allergy to identify T cell epitopes on BLG, antigen-presenting HLA class II molecules, and the patterns of cytokine production.

Materials and methods

Peripheral blood mononuclear cells (PBMC) donors

The diagnosis of allergy to cow's milk was based on clinical symptoms and CAP-RAST [18] results to cow's milk (Table 1). Patient MT had shown immediate hypersensitivity-type of symptoms such as systemic urticaria and severe cough, which occurred within 30 min after antigen challenge; patients IR and TK showed delayed hypersensitivity-type of symptoms such as systemic eczema, which occurred more than 24 h after antigen challenge; and patient AH showed both the immediate hypersensitivity-type of symptoms of systemic rash, itching and delayed hypersensitivity-type of symptoms of diarrhoea and eczema. The selected donors covered frequently observed HLA haplotypes in the Japanese population [19]. Thus, individuals allergic to cow's milk with HLA-DRB1*0901 (30.3% of the general Japanese population), DRB1*0405 (27.8%), and DRB1*0803 (17.4%) haplotypes

Table 1. Clinical features of subjects studied

Patients	Age	Sex	Symptom	Total IgE (IU*/mL)	Specific IgE† (U‡/mL)
1. IR	3 years	m	Eczema	177.2	0.51
2. AH	5 months	F	Eczema Diarrhoea	427.6	> 100
3. TK	2.5 years	M	Eczema	93.5	1.05
4. MT	2.5 years	M	Eczema Wheeze	370.9	68.6

*International Unit. †Cow's milk-specific IgE. ‡Unit of allergen-specific IgE.

were selected as PBMC donors. Subjects negative for these haplotypes account for about 34% of the Japanese population.

Synthesis of BLG-derived peptides

Peptides were synthesized according to the amino acid sequence of BLG reported previously [20], using a solid-phase simultaneous multiple peptide synthesizer PSSM-8 (Shimadzu, Kyoto, Japan), based on Fmoc strategy. Peptides of 12–21 residues were overlapped by 11 residues: corresponding to the entire sequences of BLG. All peptides were purified by C18 reverse-phase high-performance liquid chromatography (HPLC) (Millipore, Waters, Milford, MA, USA). The primary structures of peptides used in this study are shown in Fig. 1.

Generation of antigen-specific T cell lines and clones

BLG-specific T cell lines were established from PBMC from four patients with milk allergy. T cell lines were generated by stimulating PBMC (1.5×10^5 /well) with a mixture of 22 synthetic BLG-derived peptides at a final concentration of 0.1 μ M each, in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 2 mM L-glutamine, 100 units/mL of penicillin, 100 μ g/mL of 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 7–9 days, irradiated (3000 cGy) autologous PBMC (1.5×10^5 /well) pulsed with BLG peptide mixture (1 μ M each for 5 h), human rIL-2 (50 U/mL, Genzyme, Cambridge, MA, USA) and human rIL-4 (10 U/mL, Biosource International, Camarillo, CA, USA) were added only to culture wells that were carrying T cell blasts and were thus maintained for another 7 days. Aliquots of growing cultures were combined as a bulk T cell line for each patient, and reactivity against the overlapping peptides was determined. Cloning was done in Terasaki plates (Nunc, Roskilde,



Fig. 1. Schematic representation of the synthetic peptides. Broken lines show synthetic peptides composed of 12–21 residues overlapping each other by 11 residues. Numbers refer to the location of the residues in the primary structure of BLG.

Denmark), by limiting dilution at 0.3 cells per well in the presence of irradiated (3000 cGy) autologous PBMC (3×10^4 /well) pulsed with BLG peptide mixture (1 μ M), 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 (1.5×10^6 /well) pulsed with BLG peptide mixture (1 μ M), in the presence of rIL-2 and rIL-4.

Antigen-induced proliferative responses of the T cells

Antigen-induced proliferation of the T cell lines was assayed by culturing the T cells (3×10^4 /well) in 96-well flat-bottomed culture plates in the presence of the soluble BLG peptide mixture (1 μ M each), respective overlapping peptides (1 μ M) or BLG protein (25 or 50 μ g/mL) and 3000 cGy-irradiated autologous PBMC (1.5×10^5 /well). Cells were cultured for 72 h, in the presence of 1 μ Ci/well of [^{3} H]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 PBMC, with or without anti-HLA class II monoclonal antibodies (mAbs) HU-4 (anti-HLA-DRB1 + DRB5 IgG2a, monomorphic) [21,22], L243 (anti-HLA-DRB1 + DRB4 IgG2a, monomorphic, reactivity to DRB3 and DRB5 yet to be determined) [23], HU-11 (anti-HLA-DQ4 + DQ5 + DQ6 IgG2a) [24], HU-18 (anti-HLA-DQ7 + DQ8 + DQ9 IgG2a) [25], HU-46 (anti-HLA-DQ4 IgG2a) [24], and B7/21 (anti-HLA-DP IgG1, monomorphic) [26]. Percentage inhibition was calculated using the following formula: $\{1 - (\text{value obtained with a}$

peptide and a mAb – that with medium alone)/(value obtained with a peptide and without a mAb – that with medium alone)} $\times 100$. Other experiments were done in which PBMC of two donors with one haplotype-shared and one donor with irrelevant HLA haplotypes were used as APC. In this experiment, allogeneic PBMC (3×10^5 /well) were cultured in plates with peptides (1 μ M 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 RPMI 1640 media containing 3% human serum. The remaining adherent cells were irradiated at 3000 cGy and used as APC.

HLA typing

HLA class II (DR, DQ) alleles were determined by hybridization of HLA-DR, DQ genes amplified by polymerase chain reaction with sequence-specific oligonucleotide probes distributed in the 11th International Histocompatibility Workshop, as described elsewhere [1]. The nomenclature of the HLA-DR, DQ alleles was according to the WHO Nomenclature Committee for factors of the HLA system [27].

Quantification of IL-4, IL-5 and IFN- γ in supernatants of the T cell lines and clones

Culture supernatants of the T cell lines and clones stimulated by APC plus Ag for the determination of proliferative responses were collected immediately before the addition of [^{3} H]TdR and stored in aliquots at -80° C until determinations of lymphokine concentrations. The ELISA kits for detecting human IL-4 (Biosource

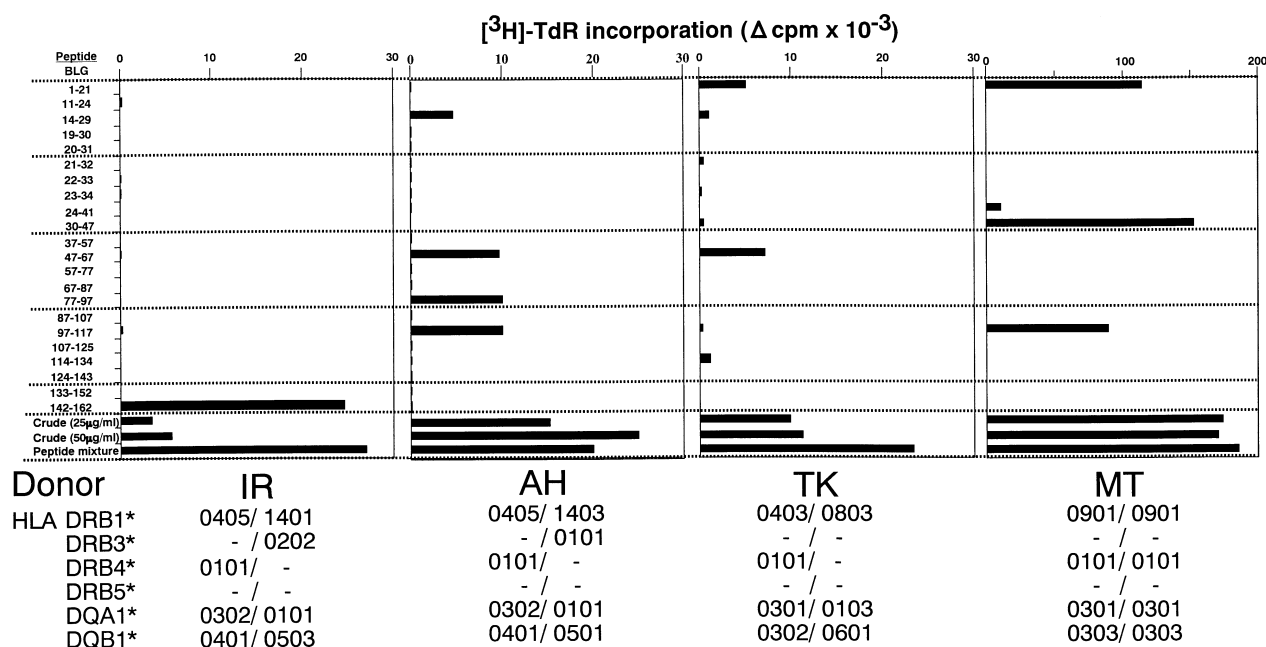


Fig. 2. Proliferative responses of four short-term T cell lines to 11- to 12-residue-overlapped synthetic peptides derived from BLG. Peptides are designated by indicating the amino acid residue numbers of the amino- and carboxyl-termini. Values shown are the mean cpm of duplicate cultures after subtraction of the mean cpm obtained from cultures without peptides. The standard error (SE) of duplicate cultures was < 20%. Cultures without peptides showed 133–794 cpm in all the lines.

International), IL-5 (Quantikine, R and D System, Minneapolis, MN, USA), and IFN- γ (Endogen, Cambridge, MA, USA) were used for quantification of lymphokines in the supernatants, according to manufacturer's instructions.

Flow cytometric analysis

Fluorescein isothiocyanate (FITC)-labeled monoclonal antibody anti-T cell receptor TCR-1a/b WT31, anti-TCR-gd I, anti-HLA DR were used to analyse the phenotype of the T cells. FITC-labeled anti-Leu4/CD3, anti-Leu3a/CD4, phycoerythrin (PE)-labeled anti-Leu2a/CD8, and anti-Leu-12/CD19 were also used to analyse the phenotype of the T cells by double colour staining. T cells were washed with PBS, and 105 cells were incubated with FITC-conjugated monoclonal antibodies (Becton Dickinson, Mountain View, CA, USA) specific to TCR ab, gd, HLA-DR, or double-stained with CD4 (Leu-3a, FITC-conjugated)/CD8 (Leu2a, PE-conjugated) or CD3 (Leu-4, FITC-conjugated)/CD19 (Leu-12, PE-conjugated). Stained cells were analysed using a FACScan instrument (Becton Dickinson). Forward scatter threshold was set to exclude only debris in the preparation.

Results and discussion

BLG epitopes recognized by polyclonal BLG-reactive T cell lines

Short-term BLG-specific T cell lines were established from

the PBMCs of four atopic donors with cow's milk allergy. These donors carried common HLA haplotypes seen in the Japanese population. The cell lines were tested for their proliferative response to a panel of overlapping synthetic peptides corresponding to the amino acid sequence of BLG. As shown in Fig. 2, seven major T cell epitopes were identified by these four donors (BLG peptides 1–21, 14–29, 30–47, 47–67, 77–97, 97–117, 142–162). T cell lines established from donors AH and MT showed relatively more heterogeneity regarding the specificity. The three peptides, BLG p1–21, 47–67 and 97–117, were recognized by the two different T cell lines regardless of the HLA phenotype. All these lines showed a proliferative response ($SI \geq 13.5$) when tested for reactivity to crude BLG protein. Thus, these T cell lines recognized not only the synthetic peptides, but also naturally processed BLG peptide fragments.

Antigen-presenting HLA molecules

Proliferation-inhibition experiments using anti-HLA class II mAbs were carried out to determine antigen-presenting molecules, as shown in Fig. 3. The T cell lines IR, AH and TK were inhibited mainly by HU4 (anti-DRB1 + 5 mAb; 96.9%, 12.2% and 94.4% inhibition, respectively) and/or L243 (anti-DRB1 + 4 mAb; 93.4%, 56.2% and 98.1% inhibition, respectively). Unlike these three lines, the MT

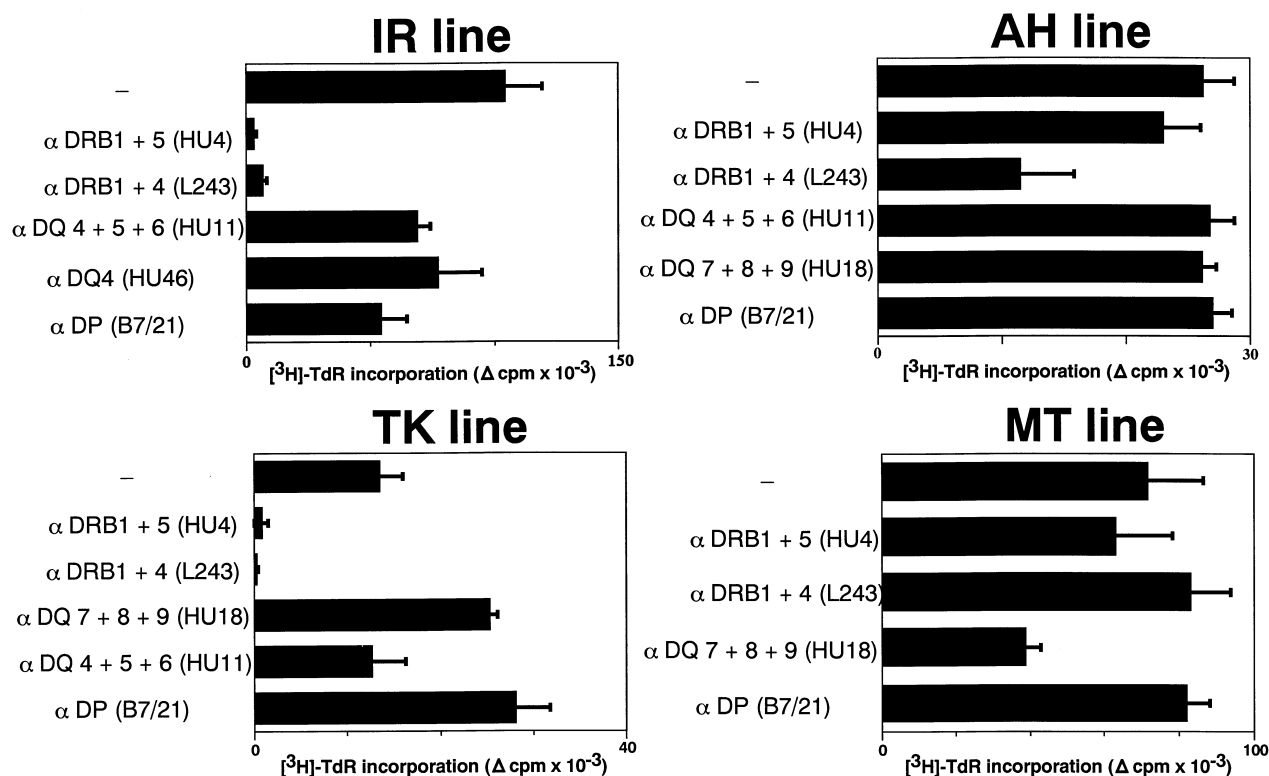


Fig. 3. Proliferation-inhibition of three bulk T cell lines using anti-HLA class II mAbs. T cells (3×10^4 /well) were incubated with HU4 (anti-HLA-DRB1 + DRB5), L243 (anti-HLA-DRB1 + DRB4), HU11 (anti-HLA-DQ4 + DQ5 + DQ6), HU18 (anti-HLA-DQ7 + DQ8 + DQ9), HU46 (anti-HLA-DQ4) and B7/21 (anti-HLA-DP) and 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.

line was not inhibited by mAbs to HLA-DR molecules, whereas the addition of anti-DQ mAb HU18 (anti-DQ7 + 8 + 9) inhibited the proliferative response of the cell line established from donor MT (46.3% inhibition). It is conceivable that DR molecules are the major antigen-presenting molecules in donors IR, AH and TK, while DQ molecules are the major antigen-presenting molecules in donor MT.

The T cell line IR, the proliferative response of which was inhibited by anti-DR mAbs, was cloned by limiting dilution, and was investigated regarding T cell epitope recognition and its restriction molecule. As shown in Fig. 4, the T cell clone IR1.9 generated from the T cell line IR recognized only BLG p142–162. IR1.9 was then cultured in the presence of BLG p142–162 and autologous PBMCs with anti-HLA class II mAbs. As shown in Fig. 5, HU4 inhibited (84.3% inhibition) the peptide-driven proliferation of IR1.9 ($P < 0.001$), indicating that the DR is the antigen-presenting molecule for IR1.9. As shown in Fig. 6, IR1.9 proliferated in response to BLG p142–162 presented by allogeneic PBMCs sharing the

HLA-DRB1*1401-DRB3*0202-DQA1*0101-DQB1*0503 haplotype alone with the donor IR. In contrast, allogeneic PBMCs with another haplotype-shared, and with irrelevant haplotypes, did not present BLG p142–162 to IR1.9. These observations indicate that the T cell clone IR1.9 recognizes BLG p142–162 in the presence of the DR molecule (DRA/DRB1*1401), which is likely to be the major antigen-presenting molecule in the IR line.

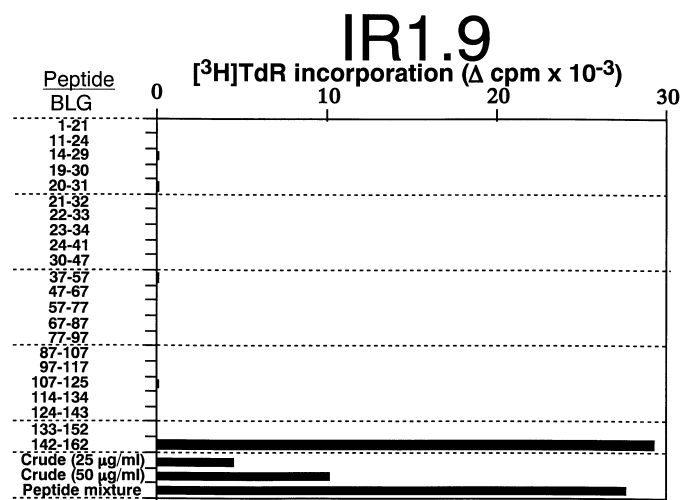
Surface-marker phenotypes

Flow cytometric analysis was performed on the T cell lines MT and IR. The MT line was CD3⁺, CD4⁺, CD8[−], CD19[−], CD25⁺, αβTCR⁺, γδTCR[−], HLA-DR⁺ and Fas⁺ phenotype. The IR line showed the same phenotype except that 54.3% of all T cells were double-positive for CD4 and CD8, and the remainder were only CD4-positive (data not shown).

Production of IL-4, IL-5 and IFN-γ by T cell lines

The cytokine secretion patterns of the T cell lines IR, AH

Fig. 4. Proliferative responses of the IR1.9 T cell clone to 11- to 12-residue-overlapped synthetic peptides derived from BLG. Peptides are designated by indicating amino acid residue numbers of the amino- and carboxyl-termini. Values shown are the mean cpm in duplicate cultures after subtraction of mean cpm obtained from culture without peptides. The standard error (SE) of duplicate cultures was < 20%.



and MT are shown in Fig. 7. High levels of IL-5 were detected in the culture supernatants of all cell lines in response to the BLG peptide mixture or crude protein, and the clone IR1.9 also produced a large amount of IL-5 (7100 pg/mL). Large amounts of interferon (IFN)- γ were also detected in these cell lines, whereas the level of IL-4 was low in the T cell line IR, which showed delayed hypersensitivity-type of symptoms.

Allergic diseases are thought to be mediated by antigen-specific IgE, and an important role has been ascribed to CD4⁺ T cells, especially to the Th2 subset. T cells seem to be the target cells for successful desensitization [28] and several studies have indicated that a shift in the production of Th2 to Th1 cytokines occurs in peptide-based immunotherapy [29,30]. *In vitro* T cell cloning studies have provided valuable information about the cytokine profile in patients with allergic diseases. A number of cytokines have been implicated in the genesis of allergic reactions,

including IL-4, IL-5, IL-6 and tumour necrosis factor, but in contrast, other cytokines such as IFN- γ may have biological actions that limit allergic responses. In another study on cow's milk hypersensitivity, the number of cells secreting IFN- γ is 10-fold larger than that secreting IL-4 in the lamina propria of the intestine, which might indicate a dominance of Th1-type responses in these patients [31].

In our cases, patient MT had immediate hypersensitivity-type of symptoms, patients TK and IR had delayed hypersensitivity-type of symptoms, and patient AH had combined symptoms. Analysis of cytokine production revealed high levels of IL-5 production by antigen-specific T cell lines; IL-4 and IFN- γ were also produced to a varying degree by these T cell lines. The differences in the ratios of IL-4 to IFN- γ might explain the differences in their clinical features. Other groups also reported, through investigation of OA- or OM-specific cell lines derived from PBMCs, that IL-5 was consistently found in these T cell

Fig. 5. Proliferation-inhibition of clone IR1.9. The clone IR1.9, generated from the IR line, was cultured in the presence of 1 μ M of the BLG peptide p142–162 and irradiated autologous PBMCs, with or without anti-HLA class II mAbs. Results are expressed as the geometric mean of triplicate determinations $\pm$ standard error. All mAbs were tested at a final dilution of 1 : 200.



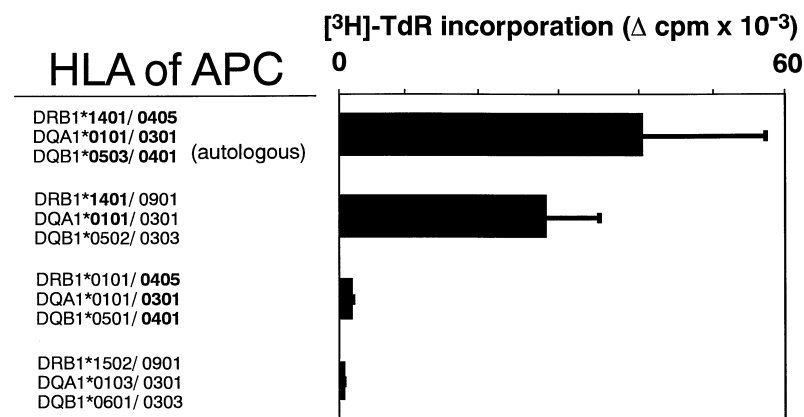


Fig. 6. Antigen presentation to IR1.9 cells by autologous and allogeneic PBMCs. Autologous or allogeneic PBMCs (3×10^5 /well) were cultured in a 96-well plate with $1 \mu\text{M}$ of the peptide p142–162 for 2 h at 37°C ; the excess peptides and non-adherent cells were removed by gently washing the plate three times with RPMI 1640 medium containing 3% human serum. The remaining adherent cells were irradiated at 3000 cGy and used as APC. T cells (3×10^4 /well) were then added to the culture plate. Shared HLA haplotypes are indicated with bold lettering. Results are expressed as the geometric mean of triplicate determinations $\pm$ standard error.

lines or clones [9,10]. IL-5 is a powerful eosinophil chemotactic and activation agent and enhances both immediate- and delayed-type hypersensitivity responses. In this regard, Holen *et al.* reported that mitogen stimulation had no detectable effect on the production of IL-4 and IL-5, and is different from allergen-mediated activation, and it is possible that PHA stimulation of T cells requires a specific costimulatory signal for IL-5 production [8]. These findings, including those in our study on IL-5 production by antigen-stimulated T cells, support the assumption of a predominant role for eosinophils in food-induced T cell-mediated allergic reactions.

We could not establish antigen-specific T cell lines or clones from three healthy controls and four patients in whom symptoms of milk allergy had improved spontaneously a few years before.

Immune regulation by the induction of oral tolerance to food antigens is thought to prevent food allergy [32]. It has been shown that induction of oral tolerance is

dependent on the age of the host [33], dose of the antigen administered [33] and the nature of the antigen. In such cases, Th anergy might be induced by the development of oral tolerance to intact antigens [31].

T cell recognition sites on BLG were analysed in mice by Kaminogawa *et al.* [17,35]. Determinant regions were indicated to lie in residues 67–75, 71–79 and 80–88 in BALB/c mice, residues 16–26, 108–122 and 122–130 in C57BL/6 mice, and residues 140–148 in C3H/He mice. Among these T cell epitopes, only residues 77–97 were commonly recognized by human T cells in the current study. Several investigators have reported a variety of T cell epitopes for each antigen [1–10], and our data also show heterogeneity of T cell epitopes on BLG. HLA types are apparently distinct in different ethnic groups, and as demonstrated in the current literature on other allergic diseases, they indeed function as BLG-presenting molecules. Lines IR and AH have similar class II molecules but recognize different epitope. The DRA/DRB4 complex

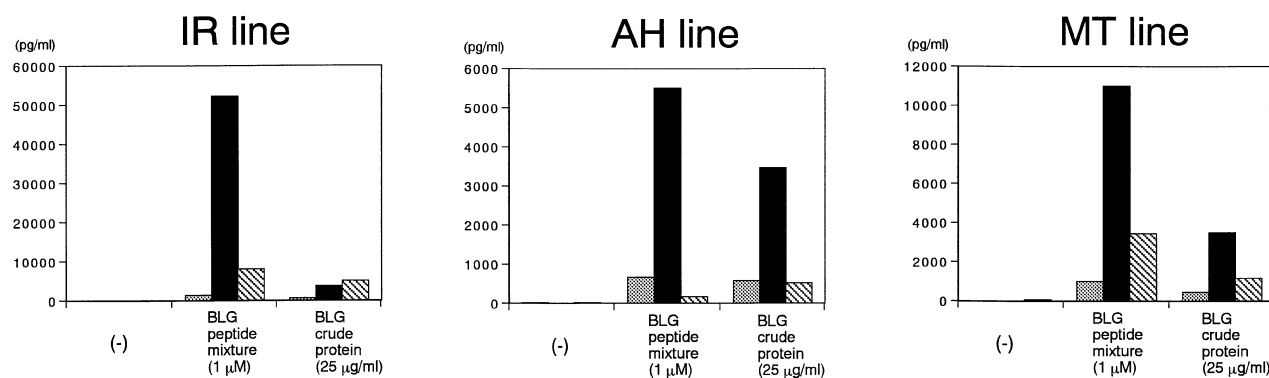


Fig. 7. Lymphokine production patterns of the T cell lines IR, AH and MT. T cells were cultured in the presence of either BLG peptide mixture ($1 \mu\text{M}$ each) or BLG crude protein ($25 \mu\text{g/mL}$). After 48 h of incubation, culture supernatants were collected immediately, and lymphokines were measured by ELISA. Net lymphokine concentrations in culture supernatant fluids are expressed by the mean value of duplicate cultures; shaded bars = IL-4; closed bars = IL-5; hatched bars = IFN- γ .

is probably involved in presenting antigenic peptides to T cells of the AH line because the mAb L243 but not HU4 inhibited the proliferation of these T cells. These observations indicate that peptides for the desensitization of T cells need to be designed keeping in mind the different requirements in different ethnic groups.

Acknowledgements

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