Characterization of T-cell clones specific to Ovomucoid from patients with egg-white allergy

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Summary. *Background:* Allergic reactions to foods are specific problems for infants and young children. Ovomucoid (OM) is one of the major allergens found in egg-white. We previously established several T-cell clones (TCCs) specific to OM in non-polarizing conditions from 4 patients (TM and YN are immediate-type, IH and YT are non-immediate-type) with egg-white allergy. We characterized their reactive epitopes, antigen-presenting molecules (HLA class II), and usage of TCR alpha and beta genes and the CDR3 loop sequence.
Objective: The objective of this study was to characterize these seven clones (TM1.3,TM1.4,YN1.1,YN1.5,IH3.1,IH3.3 and YT6.1) for cytokine production patterns and cell-surface-marker phenotypes.
Methods: We measured the production of cytokines, namely interleukin (IL)-4, IL-5 and interferon-γ (IFN-γ) by stimulation with ovomucoid peptides and stained intracellular IL-4 and IFN-γ, and determined cell-surface markers using anti-interleukin-12 receptor (IL-12R) ß1, anti-IL-12Rß2 and anti-interleukin-18 receptor α (IL-18Rα).
Results: Most TCCs secreted both IL-4 and IFN-γ in response to the OM peptide mixture, but the secretion patterns were variable; an IFN-γ dominant pattern was seen in IH3.1 and YT6.1, an IFN-γ>IL-4 pattern in TM1.3 and TM1.4, an IL-4> IFN-γ pattern in YN1.5. In intracellular IFN-γ and IL-4 staining, IFN-γ single-positive cells were predominant in TM1.3, TM1.4, IH3.1 and YT6.1 and IFN-γ and IL-4 double-positive cells were predominant in YN1.1, YN1.5 and IH3.3. All TCCs were IL-12Rß1-positive, and TM1.3, IH3.1, IH3.3 and YT6.1 were both IL-12Rß2- and IL-18Rα-positive. TM1.4 and YN1.1 were both IL-12Rß2- and IL-18Rα-negative. Based on these results, TM1.3 and TM1.4, IH3.1 and YT6.1 had a predominantly Th1 character and YN1.1, YN1.5, and IH3.3 possessed a predominantly Th0 character.
Conclusions: The phenotypes of TCCs were not in accordance with their clinical manifestations. TCCs established from patients with immediate-type hypersensitivity had either the Th1 or Th0 phenotype as well as those with non-immediate-type hypersensitivity.

Key Words: AD (atopic dermatitis), IFN-γ (interferon gamma), IL-12Rß2 (interleukin-12 receptor beta 2), IL-18Rα (interleukin-18 receptor alpha), OM (ovomucoid), TCC (T cell clone), Th (helper T cell).

Abbreviations used:

AD:	atopic dermatitis	PBMC:	Peripheral blood mononuclear cell
FITC:	Fluorescein isothiocyanate	PE:	phycoerythrin
HLA:	Human leukocyte antigen	RAST:	Radioallergosorbent test
IFN-γ :	Interferon gamma	rIL:	recombinant Interleukin
IL:	Interleukin	TCC:	T cell clone
IL-12R:	IL-12 receptor	TCL :	T cell line
IL-18R:	IL-18 receptor	TCR:	T cell receptor
OM:	Ovomucoid	Th:	helper T cell

Introduction

Allergic reactions to foods are specific problems for infants and young children and present a wide spectrum of clinical reactions, including cutaneous, gastrointestinal and respiratory symptoms as well as systemic anaphylactic symptoms. It is also known that food allergies are more prevalent in children due to an immature gastrointestinal epithelial membrane barrier that allows more proteins to cross the barrier and get into circulation [1]. Among various food antigens, the hen's egg, particularly its egg-white, is one of the most common causes of food allergy in young children. One of the major components of egg-white, comprising approximately 10% of the total egg-white proteins, is ovomucoid (OM) [2], and it has been reported to play a more important role in the pathogenesis of allergic reactions to egg-white than other egg-white proteins [3,4].

Interferon-γ (IFN-γ) production characterizes the Th1 subset while interleukin (IL)- 4 production characterizes the Th2 subset [5]. The differentiation into either Th1 or Th2 cells is a crucial step that determines the direction of subsequent adaptive immune responses. Interleukin-12 receptor (IL-12R) ß2 mRNA synthesis is restricted to Th1 cells [6,7], and the mRNA expression of Interleukin-18 receptor α (IL-18Rα) was observed only in the Th1 clones [8]. We recently established OM-specific TCCs from four Japanese atopic dermatitis (AD) patients with egg-white allergy [9]. In that study, all the TCCs specific to OM exhibited the CD4+ phenotype. These clones were established in non-polarizing conditions, and it was of interest to see whether they had Th1 or Th2 characters in relation to clinical phenotypes. We determined the cytokine production patterns and cell-surface-marker phenotypes of TCCs specific to OM.

Materials and Methods

Subjects

Clinical information on the four patients whose TCCs have been established was reported previously [9]. The diagnosis of allergy to hen's egg-white was based on clinical symptoms, hen's egg-white challenge test results and CAP-RAST [10] against hen's egg-white and OM. All the patients had AD symptoms. Patient TM and YN presented immediate-type hypersensitivity symptoms, such as systemic urticaria and severe coughing, which occurred within thirty minutes after the antigen challenge, and their levels of total IgE and CAP-RAST for egg-white and OM were high. Patient IH and YT had presented non-immediate symptoms, such as systemic eczema, which occurred more than two hours after antigen challenge, and their levels of total IgE and CAP-RAST against egg-white and OM were low [11].

TCC culture

OM-specific TCC establishment was reported previously [9]. These TCCs were cultured in an RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 2 mM of L-glutamine, 100 units/ml of penicillin, 100 mg/ml of streptomycin, and 10% pooled A.B, heat-inactivated normal human male plasma in 24-well flat-bottomed culture plates (Falcon, Becton Dickinson, Lincoln Park, N.J.). The plates were incubated at 37ºC in 5% CO_2 humid air. After 7-9 days, irradiated (30 Gy) autologous peripheral blood mononuclear cells (PBMCs) (1.5×10^5/well) pulsed with OM peptide mixture (1 mM each for 5 hr), human recombinant interleukin (rIL)-2 (50 U/ml) (Genzyme, Cambridge, MA), and human rIL-4 (10 U/ml) (Biosource International, Camario, CA) were added to the culture wells. Thus, the TCCs were maintained for another 7 days.

Production of IL-4 and IFN-γ in supernatants of TCCs

The TCCs (3×10^4 cells/well in 96-well flat-bottomed culture plates) were cultured in the presence of a soluble OM peptide mixture (1μM) and irradiated autologous PBMC (1.5×10^5/well) for 56 hr. Culture supernatants of the TCCs were collected and stored in aliquots at -80ºC until the determination of lymphokine concentrations. Enzyme-linked immunosorbent assay (ELISA) kits for detecting human IL-4 (Biosource Int'l), and IFN-γ (Ohtsuka, Tokyo, Japan) were used for quantification of the lymphokines in the supernatants, according to the manufacturers' instructions.

Intracellular IFN-γ and IL-4 staining

TCCs specific to OM were cultured at a density of 2×10^6 cells /mL in an RPMI 1640 medium for 4 hr at 37ºC. During the 4-hr incubation, the cells were stimulated with a combination of 25 ng/mL of phorbol 12-myristate 13-acetate (PMA) (SIGMA) and 2μg/mL of ionomycin (SIGMA) in the presence of 10 ng/mL of Brefeldin-A (SIGMA). Then the cells were directly stained with a fluorescein isothiocyanate (FITC)-conjugated anti-CD4 monoclonal antibody (Coulter-Immunotech, Marseille, France) for 15 min at room temperature, and fixed with a FACS Lysing Solution (Becton Dickinson, Mountain View, CA) for 10 min. After washing, they were preincubated with a FACS Permeabilizing Solution (Becton Dickinson) for 10 min and after washing again, they were incubated with FASTIMMUNE IFN-γ FITC/IL-4 PE (Becton Dickinson) for 30 min at room temperature. The cells were then washed twice and resuspended in a phosphate-buffered saline. Flow cytometric analysis was performed using a FACS Calibur. The results were expressed as the percentage of each cytokine-producing cell population in a total population of CD4 cells.
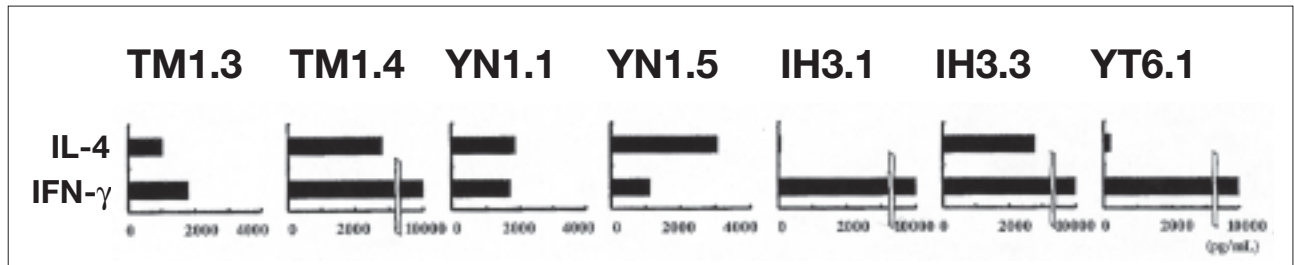
Figure 1. Cytokine production patterns of our TCCs. T-cells were cultured in the presence of an OM peptide mixture (1mM each). After incubation for 56 hours, culture supernatants were collected immediately, and the cytokines were measured by ELISA. Net cytokine concentration in culture supernatants is expressed as the mean value of duplicate cultures.
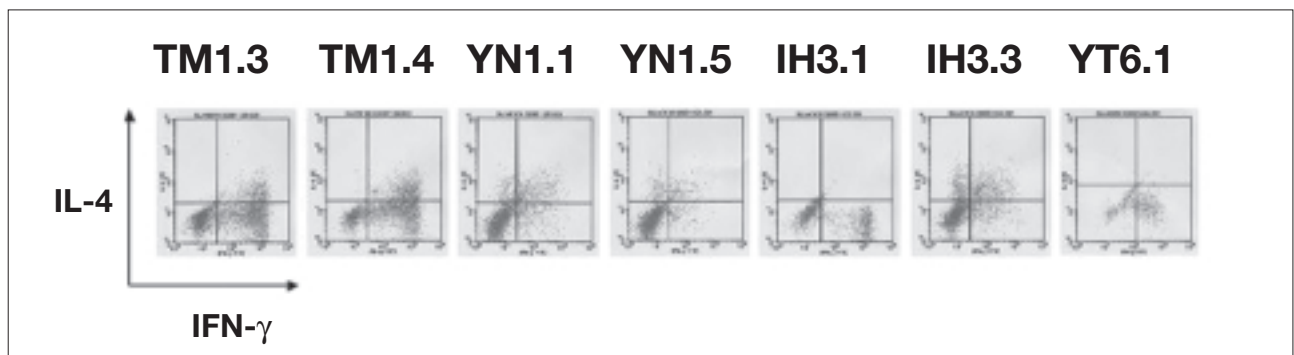

Figure 2. Intracellular IFN-γ and IL-4 staining. TCCs specific to OM were cultured at a density of 2×10^6 cells per mL in an RPMI 1640 medium with PMA and ionomycin in the presence of Brefeldin-A for 4 hr at 37ºC. After Pma stimulation, the cells were directly stained with a FITC-conjugated anti-CD4 monoclonal antibody. After permeabilization, the cells were stained with IFN-γ FITC/IL-4 PE. Flow cytometric analysis was performed using a FACS Calibur.

Cell-surface-marker phenotypes of TCCs

A FITC-labeled monoclonal anti-IL-12Rß1 antibody (PharMingen, San Diego, CA), a phycoerythrin (PE)-labeled monoclonal anti-IL-12Rß2 antibody (kindly donated by Dr. F Sinigaglia, Italy), anti-IL-18Rα, and anti-human CCR-3 (R&D Systems, McKinley Place, MN) were used to analyze the phenotype of our TCCs. FITC-labeled anti-Leu4/CD3, anti-Leu3a/CD4 (Becton Dickinson), anti-TCR-α/ß-1-WT31 (Becton Dickinson), anti-TCR-γ/δ (Endogen, Woburn, MA), and PE-labeled anti-Leu2a/CD8 (Becton Dickinson) were also used to analyze the phenotypes of our TCCs by double-color staining. The stained cells were analyzed using a FACScan instrument (Becton Dickinson). The forward scatter threshold was set to exclude debris only from the preparation.

Results

Production of IL-4 and IFN-γ in supernatants of TCCs

Cytokine secretion was investigated in the TCCs specific to OM. Since these clones were maintained in the presence of IL-2 and IL-4, they were washed with the culture medium and then cultured with the OM peptide mixture (1 μM) and irradiated autologous PBMC, but in the absence of IL-2 and IL-4, for 56 hr. Cell proliferation was similar among these clones when examined using a 3H-thymidine uptake (data not shown). Most TCCs secreted both IL-4 and IFN-γ in response to the peptide mixture, as well as OM crude protein (data not shown), but the secretion patterns were variable; an IFN-γ dominant pattern (IFN-γ >> IL-4) was seen in IH3.1 and YT6.1, an IFN-γ >IL-4 pattern in TM1.3,TM1.4 and IH3.3, IL-4> IFN-γ pattern in YN1.5, as shown in Figure 1.

Intracellular IL-4 and IFN-γ staining

Since the patterns of IL-4 and IFN-γ secretion were variable among the TCCs, intracellular IL-4 and IFN-γ staining was analyzed. As shown in Figure 2, intracellular IFN-γ single-positive cells were predominant in TM1.3, TM1.4, IH3.1 and YT6.1 and IFN-γ and IL-4 double-positive cells were predominant in YN1.1, YN1.5 and IH3.3.

Together with cytokine secretion data, TM1.3, TM1.4, IH3.1, and YT6.1 had a Th1-dominant phenotype and YN1.1, YN1.5, and IH3.3 had a Th0-dominant phenotype.
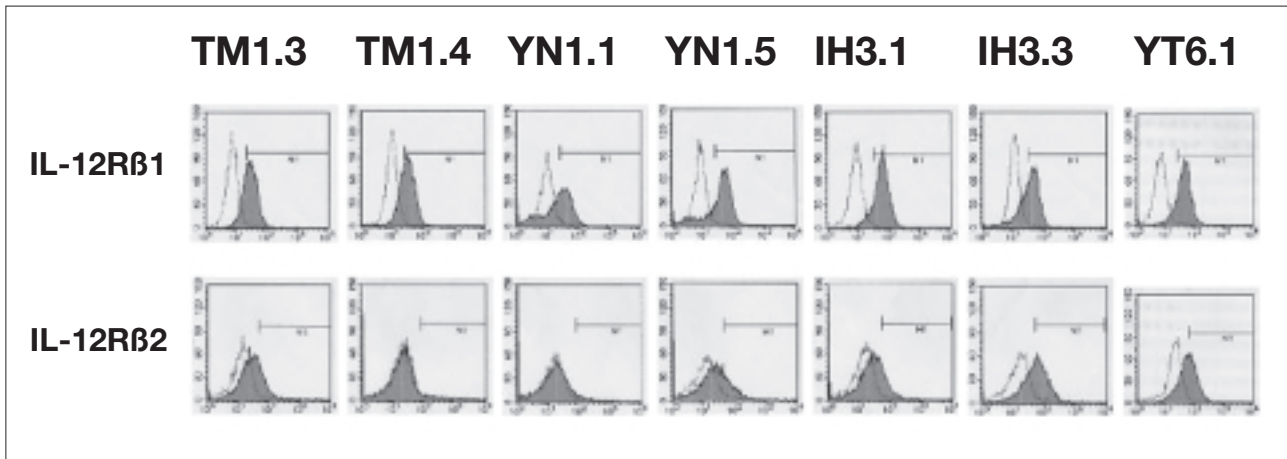
Figure 3. Flow cytometric analysis of our TCCs. FITC-labeled monoclonal antibody, anti-IL-12R ß1, and a PE-labeled monoclonal antibody, anti-IL-12R ß2, were used. Stained cells were analyzed using a FACScan instrument.

Cell–surface–marker phenotypes of TCCs.

All TCCs specific to OM exhibited CD3$^+$, CD4$^+$, CD8$^-$, αβTCR$^+$ and γδTCR$^-$ phenotypes (data not shown). Moreover, all TCCs were IL-12Rß1-positive (Fig.3). TM1.3, YN1.5, IH3.1, IH3.3 and YT6.1 were IL-12Rß2-positive and TM1.4 and YN1.1 were IL-12Rß2-negative (Fig.3). IL18Rα was co-expressed with IL-12Rß2 in these clones (data not shown).

Discussion

Murine helper T-cells are divided into two subsets, Th1 and Th2 cells. The former produce IL-2 and IFN-γ and the latter produce IL-4 and IL-5 [12]. Such dichotomy is also evident in human cells, albeit to a less polarized extent than in murine T-cells [13]. Th1 cells cause non-immediate-type hypersensitivity reactions whereas Th2 cells promote IgE production leading to immediate-type hypersensitivity.

In our previous study, it was shown that the proliferative responses of PBMCs to Ovalbumin (OVA) in children with AD who are sensitive to hen's eggs were significantly higher than those of healthy children and hen-egg-sensitive children with immediate symptoms. However, in patients with AD there were no significant correlations between the proliferative response to PBMCs and the RAST values [14]. We also reported on the high sensitivity and specificity of proliferative responses of lymphocytes to OVA for the detection of hen-egg allergy in patients with AD [15]. These studies indicate that the molecular basis of non-immediate-type hypersensitivity, as seen in AD patients who are sensitive to hen's eggs is different from that of immediate-type hypersensitivity to hen's eggs.

Chicken OM has been reported to be the most important allergenic protein in egg-white [2,3] and consists of three tandem homologous domains [16]. Hence, we previously established several OM-specific TCCs in non-polarizing conditions from 4 patients with egg-white allergy to investigate the molecular basis of hen-egg allergy [9]. In this paper, we characterized these TCCs with regard to helper T-cell phenotypes. Patients TM and YN presented immediate-type hypersensitivity symptoms, and their levels of total IgE and CAP-RAST for egg-white and OM were high. On the other hand, patients IH and YT presented non-immediate symptoms, and their levels of total IgE and CAP-RAST for egg-white and OM were low. These clinical manifestations suggested that Th1 and Th2 cells mainly contribute to the pathogenesis of hen-egg allergy in the former two patients and the latter two patients, respectively. All of the twenty-four Der pI-specific TCCs from a patient with severe atopic disease were reported to have the Th2-type character [17]. This may indicate that imbalance between Th1 and Th2 in a patient's condition influences the subtypes of these TCCs. Hence we expected that Th1 clones would be established from IH and YT and that Th2 clones would be from TM and YN.

In the present study, we analyzed IFN-γ and IL-4 production patterns by stimulation with OM, intracellular IFN-γ and IL-4 staining in 7 T-cells clones.

The results were further confirmed by the cell-surface marker of Th1 cells. TM1.3, TM1.4, IH3.1, and YT6.1 had a Th1-dominant phenotype and YN1.1, YN1.5, and IH3.3 had a Th0-dominant phenotype. IH3.1 and YT6.1 had a Th1-dominant phenotype in accordance with the fact that they were established from patients who presented non-immediate-type hypersensitivity. However, TM1.3 and TM1.4 were established from patient TM who presented immediate-type hypersensitivity symptoms, but both had a Th1-dominant phenotype. YN1.1 and YN1.5 were from patient YN who

also presented immediate-type hypersensitivity symptoms but both had a Th0 phenotype. Our results indicate that the cell phenotype of TCCs does not always reflect clinical manifestations corresponding to immediate or non-immediate hypersensitivity. The phenotypes of TCCs could not be simply predicted, so that establishment of further TCCs and their characterizations are now under way.

Acknowledgements

References

1. Hefle SL. The chemistry and biology of food allergens. *Food Technol* 1996, 50: 86-92.
2. Rhodes MB, Bennet N, Feeney RE. The trypsin and chymotrypsin inhibitors from avian egg whites. *J Biol Chem* 1960, 235: 1686-1693.
3. Bernhisel-Broadbent J, Dintzis HM, Dintzis RZ, Sampson HA. Allergenicity and antigenicity of chicken egg ovomucoid (Gal dIII) compared with ovalbumin (Gal dI) in children with egg allergy and in mice. *J Allergy Clin Immunol* 1994, 93: 1047-1059.
4. Urisu A, Ando H, Morita Y, Wada E, Yasaki T, Yamada K, Komada K, Torii S, Goto M, Wakamatsu T. Allergenic activity of heated and ovomucoid-depleted egg white. *J Allergy Clin Immunol* 1997, 100: 171-176.
5. Mosmann TR, Coffmann RL. Heterogeneity of cytokine secretion patterns and functions of helper T cells. *Adv Immunol* 1989, 46: 111-147.
6. Rogge L, Barberis-Maino L, Biffi M, Passini N, Presky DH, Gubler U, Sinigaglia F. Selective expression of an interleukin-12 receptor component by human T helper 1 cells. *J Exp Med* 1997, 185: 825-831.
7. Szabo SJ, Dighe AS, Gubler U, Murphy KM. Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J Exp Med* 1997, 185: 817-824.
8. Torigoe K, Ushio S, Okura T, Kobayashi S, Taniai M, Kunikata T, Sanou O, Kojima H, Fujii M, Ohta T, Ikeda M, Ikegami H, Kurimoto M. Purification and characterization of the human interleukin-18 receptor. *J Biol Chem* 1997, 272: 25737-25742.
9. Suzuki K, Inoue R, Sakaguchi H, Aoki M, Kato Z, Kaneko H, Matsushita S, Kondo N. The correlation between ovomucoid-derived peptides, HLA class II molecules and TCR-CDR3 compositions in patients with egg-white allergy. *Clin Exp Allergy* 2002, 31:1223-1230.
10. Bousquet J, Chanez P, Chanal I, Michel FB. Comparison between RAST and Pharmacia CAP system: a new automated specific IgE assay. *J Allergy Clin Immunol* 1990, 85: 1039-1043.
11. Kondo N, Fukutomi O, Agata H, Motoyoshi F, Shinoda S, Kobayashi Y, Kuwabara N, Kameyama T, Orii T. The role of T lymphocytes in patients with food-sensitive atopic dermatitis. *J Allergy Clin Immunol*. 1993,91(2):658-668.
12. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol*. 1989,7:145-173.
13. Tsicopoulos A, Hamid Q, Haczku A, Jacobson MR, Durham SR, North J, Barkans J, Corrigan CJ, Meng Q, Moqbel R, Kay AB. Kinetics of cell infiltration and cytokine messenger RNA expression after intradermal challenge with allergen and tuberculin in the same atopic individuals. *J Allergy Clin Immunol*. 1994, 94(4):764-772.
14. Kondo N, Agata H, Fuk utomi O, Motoyoshi F, Orii T. Lymphocyte responses to food antigens in patients with atopic dermatitis who are sensitive to foods. *J Allergy Clin Immunol*. 1990,86(2):253-260.
15. Shinbara M, Kondo N, Agata H, Fukutomi O, Kuwabara N, Kobayashi Y, Miura M, Orii T. Interferon-gamma and interleukin-4 production of ovalbumin-stimulated lymphocytes in egg-sensitive children. *Ann Allergy Asthma Immunol*. 1996,77(1):60-66.
16. Kato I, Schrode J, Kohr WJ, Laskowski M Jr. Chicken ovomucoid: determination of its amino acid sequence, determination of the trypsin reactive site, and preparation of all three of its domains. *Biochemistry* 1987, 26: 193-201.
17. Parronchi P, Manetti R, Simonelli C, Rugiu FS, Piccinni MP, Maggi E, Romagnani S. Cytokine production by allergen (Der pI)-specific CD4+ T cell clones derived from a patient with severe atopic disease. *Int J Clin Lab Res*. 1991,21(2):186-189.

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