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Characterisation of allergen-specific responses of IL-10-producing regulatory B cells (Br1) in Cow Milk Allergy

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ABSTRACT

CD19+CD5+ regulatory B cells regulate immune responses by producing IL-10. IL-10-producing regulatory B cell (Br1) responses by allergen stimulation were investigated in human food allergy. Six milk allergy patients and eight milk-tolerant subjects were selected according to DBPCFC. PBMCs were stimulated by casein in vitro and stained for intracellular IL-10 and apoptosis. In response to allergen stimulation, Br1 decreased from 26.2 ± 18.3 to 15.5 ± 8.9% ($p = 0.031$, $n = 6$) in the milk allergy group and increased from 15.4 ± 9.0 to 23.7 ± 11.2% ($p = 0.023$, $n = 8$) in the milk-tolerant group. Apoptotic non-IL-10-producing regulatory B cells increased from 21.8 ± 9.3 to 38.0 ± 16.1% ($p = 0.031$, $n = 6$) in the milk allergy group and unchanged from 28.8 ± 13.8 to 28.0 ± 15.0% ($p = 0.844$, $n = 8$) in the milk-tolerant group. Br1 may be involved in the immune tolerance of food allergies by producing IL-10 and simultaneously undergoing apoptosis in humans. The exact roles for Br1 in immune tolerance needs to be further investigated.

1. Introduction

Food allergies are classified as IgE-mediated type and non-IgE-mediated type [1]. Th1/Th2 imbalances are known to be the main immunopathological mechanism of non-IgE-mediated food allergies [2]. CD4+Foxp3+ regulatory T cells have been reported to play an important role in immune tolerance, including allergy tolerance [3], but their role in allergy tolerance is still controversial.

CD5 is a pan-T cell marker that is also expressed in various developmental and activation states on human B cells, and it is a well-established negative regulator of TCR and BCR signalling [4]. CD5+ B cells are a subset of B cells known as regulatory B cells that negatively regulate immune responses [5]. CD5 plays a key role in B and T cell selection, as well as in the generation and maintenance of tolerance [6].

IL-10 is a potent immunosuppressive cytokine that inhibits T-cell activation and cytokine production in vivo [7]. A portion of CD5+ regulatory B cells contain IL-10 [8]. In a preliminary study, IL-10-producing regulatory B cells (Br1) decreased in milk allergy patients [9]. IL-10-producing B cell (Br1) responses to casein stimulation were investigated and characterised in this study.

2. Materials and methods

2.1. Subjects and inclusion criteria

Subjects between 10 and 28 years of age who visited the Department of Allergy and Clinical Immunology at the Seoul Allergy Clinic between March 2010 and April 2010 and were suffering from a repeated history of late eczematous reactions or exacerbations of atopic dermatitis (AD) were screened for the current study. They received blood tests and skin prick tests as described below and fulfilled the criteria of Hanifin and Rajka [10]. Signed consent forms were obtained from either the patient or the parent. The study was approved by the Institutional Review Board of Chungnam National University Hospital, Daejeon Korea.

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Double-blind placebo-controlled food challenge (DBPCFC) tests were conducted for milk; diagnostic criteria for food allergy late eczematous reactions were well-described in previous reports and included the following: (1) late eczematous reactions including exacerbation of AD by DBPCFC, (2) exacerbation onset four hours after the food challenge, and (3) negative skin prick test reactions and food-specific IgE levels for milk [11].

2.2. Blood tests and skin prick tests

Subjects received blood tests and skin prick tests. Blood tests included a CBC with differential count for the eosinophil fraction, serum total IgE levels, and blood-specific IgE for milk (which was measured using UniCAP; Pharmacia and Upjohn Diagnostics AB, Uppsala, Sweden). Serum food-specific IgE levels less than 0.35 kU A/L were classified as undetectable. Skin prick tests were conducted on the patient's left forearm using commercial allergen extracts (Bencard, Brentford, England). Histamine hydrochloride (1 mg/ml) (Bencard) was used as a positive control and physiological saline was used as a negative control. Reactions were read after 15 min and classified as negative (0: no reaction, +: reaction greater than the control reaction but smaller than half that caused by histamine) or positive (++: half the level, +++: equal to, and ++++: twice the level caused by histamine).

2.3. DBPCFC

To diagnose milk allergy, DBPCFC was conducted according to the diagnostic methods used in previous reports [12]. Briefly, an elimination diet for all possible foods suspected of provoking allergic AD was performed.

A double-blind placebo-controlled food challenge (DBPCFC) was conducted just after administration of the elimination diet. DBPCFC was performed using a mixed cereal flour vehicle, consisting of brown rice flour, glutinous rice flour, and barley flour. Two challenges were performed, separated by five days; one was only the placebo, while the other contained the suspected food antigen in the vehicle. Randomisation and preparation of the challenges were performed by dieticians in the clinical research unit. All reactions were scored as to type, time of onset, severity and duration.

2.4. Clinical severity scoring and diagnosis of food allergy

The signs and symptoms of allergy provocation appeared as newly developed symptoms or an exacerbation of AD. Thus, clinical severity was evaluated using the SCORing Atopic Dermatitis (SCORAD) index, a system used worldwide to assess the severity of atopic eczema [13]. A diagnosis of food allergy was made if the clinical severity score increased by greater than 20% in subjects demonstrating a basal score greater than 50 points, or if the score increased by over 10 points for those with a basal score less than or equal to 50 points. Apparent new skin lesions or itching were regarded as positive reactions, which were required for the diagnosis of food allergy by food challenge.

2.5. Casein stimulation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood by density-gradient separation using Ficoll-Hypaque (Biomedicals, Aurora, OH, USA) and then resuspended at 1×10^6 cells/ml in an α-minimum essential medium (alpha-MEM; Irvine Scientific, Santa Ana, CA). PBMCs were then cultured with or without a 50 ug/ml mixture of purified alpha-, beta-, and kappa-caseins (Sigma, St. Louis, Mo) in α-MEM. The casein concentration was referred to in a previous study [14]. Cells were cultured in 24-well plates at a concentration of 1×10^6 cells/mL. As an unstimulated control, 1×10^6 cells/mL were also cultured without casein.

2.6. Cell surface staining, intracellular staining, and flow cytometric analysis

After eighteen hours of culture, cells were counted, and cultured PBMCs with or without casein were stained with specific monoclonal antibodies with allophycocyanin (APC)-labelled anti-CD19 (eBioscience, San Diego, CA, USA) and phycoerythrin. Cy7(-PE.Cy7)-labelled anti-CD5 (eBioscience, San Diego, CA) was used for B cell subset analysis. With staining for surface CD19 and CD5, the cells were co-stained with an apoptosis detection kit using FITC-conjugated annexin V (eBioscience, San Diego, CA) simultaneously.

Cells to be stained were prepared at a concentration of 1×10^6 in 100 μl of FACS staining buffer (eBioscience, San Diego, CA) in an eppendorf tube. Monoclonal antibodies were added at a concentration of 1 μg/ml according to the manufacturer's instructions. After incubation for one hour in a dark room and three washes with FACS staining buffer, cells were resuspended into 500 μl of FACS staining buffer just before flow cytometric analysis.

Just after finishing cell surface staining, permeabilization/fixation of cells was performed using a permeabilization/fixation kit (eBioscience, San Diego, CA). Intracellular IL-10 was stained using phycoerythrin-labelled anti-IL-10 rat IgG1 monoclonal antibody (eBioscience, San Diego, CA). Stained cells were acquired with a FACSCaliber device (BD, Biosciences, Milpitas, CA, USA), and the data were analysed with CellQuest software (BD, Biosciences).

Table 1
Characteristics of the milk allergy group and the milk-tolerant group.

	Milk allergy group	Milk-tolerant group	Unit
No	6	8	
M:F	3:3	5:3	
Age	19.1 ± 12.3	18.8 ± 13.7	Year
Total IgE	1123.3 ± 1523.5	1411.1 ± 1563.2	IU/L
Blood eosinophil	7.23 ± 4.86	8.25 ± 7.3	%
Blood lymphocyte	35.2 ± 13.2	34.3 ± 12.1	%
Milk-specific IgE	Undetectable	Undetectable	kUA/L
Casein-specific IgE	Undetectable	Undetectable	kUA/L
Skin prick test for milk	All negative	All negative	Grade

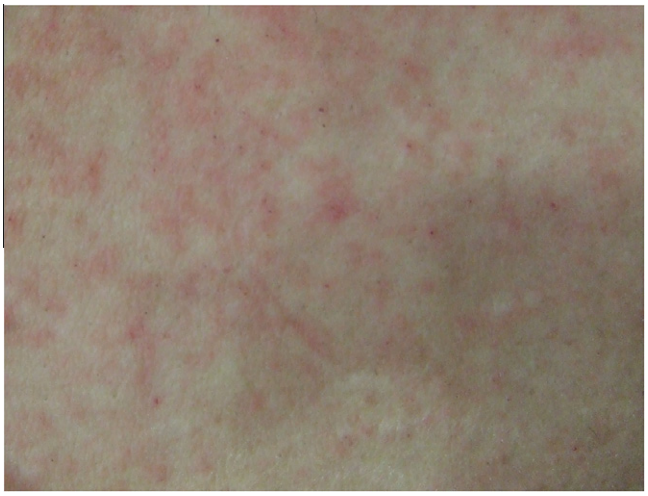

Fig. 1. Representative skin lesions of late eczematous reactions to cow's milk. Erythematous papular eruptions developed following food challenge.