

Isolation of type 1 and type 2 cloned mite allergen-specific T cells from an asthmatic child

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Allergen-specific T cells have been thought to play a central role in the pathogenesis of asthma. It has been well documented that allergen-specific T cells derived from atopic patients are predominantly of type 2 T helper cell pattern. However, allergen-specific T cells derived in nonatopic normals are of type 1 in contrast to atopic patients. The purpose of the study was to develop and characterize both mite allergen-specific TH₁ and TH₂ clones from the same asthmatic child. With exogenous supplemental cytokine, both TH₁ and TH₂ clones from the same patient have been developed and maintained in this laboratory for more than one year. All these T cell clones showed dose dependent allergen-specific proliferative response and expressed with CD4⁺, CD45RA⁺ markers. Elucidation of the origin and interaction between these two different types of T helper cells might shed light on understanding the pathogenesis of atopic diseases and the mechanisms of hyposensitization in atopic patients.

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Allergen-specific IgE and mast cells are the effector molecules and cells in triggering inflammatory responses in allergic diseases; however, the central theme of the pathogenic mechanisms involved in allergic diseases is the role of allergen-specific T cells and related cytokines (1–4). Cytokines like interleukin-4 (IL-4) are important for the immunoglobulin class switch from IgM to IgE and IL-5 is critical of eosinophilia which cause more inflammatory response during the late stage of disease (5–7). It has been well documented that both IL-4 and IL-5 are secreted by type 2 T helper (TH₂) cells. In contrast to nonatopic normals, allergen-specific T cells derived from atopic patients are predominantly of type 2 T helper cell pattern predominantly (8–12).

It has been well documented that many factors such as the microenvironmental cytokine appearing early in the immune response, the route of antigen administration, the kinds of antigen presenting cells, the ligand density on antigen presenting cells, the antigen dose, and the use of adjuvants can affect the development of T helper cells (13). The difference and interaction between type 1 and type 2 have been a major focus of research in the past several years (14, 15). Several studies suggested in vitro supple-

ment of exogenous cytokines such as IFN- γ or IL-4 in addition to IL-2 could affect the development of T cells (16, 17). In addition, most recent reports also suggested exogenous IL-12 also can affect the development of T helper cells (18).

To further understand the interaction between type 1 and type 2 allergen-specific T helper cells, several allergen-specific T cell lines or clones have been derived from an asthmatic patient. Elucidation of the origin and interaction between these two different types of T helper cells might further help to understand the pathogenesis of atopic diseases and the mechanisms of hyposensitization in atopic patients. This information might contribute to the future directions for therapy for asthmatic patients.

Materials and methods

Subjects

An 8-year-old girl was positive for IgE antibody to house dust mite (CAP, Pharmacia Diagnostic AB, Uppsala, Sweden) and was diagnosed to have bronchial asthma. She was followed up regularly at Asthma Clinic of National Taiwan University Hospital.

Antigens

The allergen was prepared as described (19). Briefly, 1g of lyophilized mite body (*Dermatophagoides pteronyssinus*, Der p) was defatted with 100ml of ether, then homogenized and stirred continuously in 100ml of phosphate-buffered saline for 36 hr. After centrifugation (10,000 $\times$ g), the crude extract was filtered through 0.22 μ m membrane, lyophilized and stored at -70°C until use. Before use, the lyophilized extract was reconstituted in distilled water; also before use the protein concentration of mite antigens was determined.

Culture medium and reagents

T cells were cultured in RPMI 1640 medium (GIBCO, Gaithersburg, MD) containing 1mM glutamine, 1mM sodium pyruvate, 5×10^{-5} M 2-ME, 100U/ml penicillin, 0.1mg/ml streptomycin, and 10% FCS. For the first 10 to 14 days of culture, T cells were cultured in serum free medium (AIM-V, GIBCO, Gaithersburg, MD). Appropriate fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated mouse anti-human antibodies: anti-Leu-2a (CD8, clone SK1), anti-Leu-3a (CD4, clone SK3), anti-Leu4 (CD3, clone SK7), anti-Leu-12 (CD19, clone 4G7), anti-Leu 18 (CD 45RA, clone L48) and anti-TCR 1(α/β , clone WT31) (Becton Dickinson, Mountain View, CA) were used for phenotypic analysis of human lymphocytes.

Transformation of B cells by Epstein-Barr virus

Heparinized peripheral blood was collected and mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia Diagnostic AB, Uppsala, Sweden). Epstein-Barr virus transformation of B cells was performed by adding 1 ml of B95-8 cell culture supernatant diluted in 4 ml of RPMI 1640 supplemented with 20% FCS and 2 μ g/ml cyclosporin A (Sandoz Pharma Ltd, Basel, Switzerland) with the cell concentration of 4×10^6 cells/ml. The cells were then incubated and maintained in the laboratory. After establishment, EBV-transformed lymphoblastoid cell lines (EBV-LCL) were frozen and stored in a liquid nitrogen tank.

Establishment of mite-specific T cell clones

To establish the mite-specific T cell lines, peripheral blood mononuclear cells (PBMC) were stimulated with 50 μ g/ml mite allergens in SFM. Three days later, recombinant human IL-2 (with the concentration of 10 IU/ml was added to the culture. Ten to 14 days later, the viable cells were harvested and plated in 96-well plates with the concentration of 10^4 cells/

well with 10^5 cells/well irradiated PBLMNC (4500 rads) or EBV-LCL (10000 rads) as antigen presenting cells and 25 μ g/ml mite antigen. T cells were expanded and screened by proliferative response to mite antigen.

To develop the different types of T helper cells, different cytokines such as IFN- γ (1000 U/ml), IL-4 (5 ng/ml) and IL-2 (10 IU/ml) were added during the early culture. After two weeks' culture, T cells collected from different culture conditions were tested for allergen-specificity and cytokine production.

Subcloning of mite-specific T cells

To further subclone the established T cell lines, T cells were plated in 96-well plates with cell concentration of 0.3 or 1 cell per well. T cells were fed with APC plus antigens in medium with 10 IU/ml IL-2 every two weeks. T cells were expanded before further characterization.

Proliferative assay

To test the antigen specificity, 5×10^4 cloned T cells were stimulated with irradiated EBV-LCL (5×10^4 cells/well) or PBMC (1×10^5 cells/well) with or without antigens in 96-well plates. After incubation at 37°C for five days, each well 1 μ Ci ^3H -thymidine was added and incubated for another 16 hours. Cells were harvested onto filter paper and radioactivity was determined with β -counter (Beckman Instrument Inc., Galway, Ireland).

Cytokine mRNA detection by reverse transcriptase-polymerase chain reaction (RT-PCR) assay

Peripheral blood mononuclear cells with the concentration of 1×10^6 cells/ml were set up in 24-well plates in RPMI 1640 medium supplemented with 2% ABS and 2% TCH, 4mM L-glutamine, 25mM HEPES (pH 7.2), 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, 100 μ g/ml streptomycin and 0.25 mg/ml amphotericin in the presence of irradiated PBMCs plus mite antigen (50 μ g/ml) or concanavalin-A (10 μ g/ml, Con A, Sigma). Thirty ml of heparinized blood was centrifuged at 2500 rpm for 10 min. After removing the plasma, an equal volume of RBC lysis buffer (NH $_4$ Cl 7.47 gm/l, Tris 2.05 gm/l, pH 7.2) was added. The samples were mixed for 10 min, and then stood for 5 more min. After centrifugation, the supernatant was discarded and equal volume of RBC lysis buffer was again added. This procedure was repeated three times. Finally, there was a yield of about 1.5 ml of WBC concentrate was yielded. The samples were aliquoted and stored at -70°C before use. RNA was prepared

by acid-quanidium thiocyanate method (16). The RNA pellet was washed once in 75% alcohol, then the RNA pellet was dried in a vacuum for 5 to 15 min. The RNA pellet was washed once in DEPC-treated water. RNA in DEPC-treated water was mixed a oligo-dT primer, dNTPs and reverse transcriptase for synthesis of single-stranded cDNA. An aliquote (3 μ l) of the RT reaction was adjusted to contain 1 μ l of primers, 2 μ l of each 10 mM deoxynucleotides (dATP, dCTP, dGTP and dTTP) (Pharmacia, Inc., Picataway, N. J.), 5 μ l of 10X PCR buffer [100mM Tris-HCl pH 8.3, 500mM KCl, 25mM MgCl₂, 0.01% gelatin (W/V)] and 0.2 unit of Tag DNA polymerase (Bethesda Research Laboratory, Inc., Gaithersburg, MD). The PCR procedures included: 1) Heat to 94°C and maintain at 94°C for 45 seconds to denature; 2) Cool to 60°C and maintain at 60°C for 45 seconds to anneal DNA with primers; 3) Heat to 72°C and maintain at 72°C for 2 min to extend DNA chain. Nucleotide sequences of 5' and 3' primers used for the polymerase chain reaction amplification were as follows:

β actin 5' primer 5'-GTGGGCCGCTCTAGGCACCAA3', 3' primer 5' CTC TTT GATGTCACGCACGAT TTC3';
IL-2 5' primer, 5'-CATTGCACTAAGTCTTGCACTTGTCAC-3', 3' primer, 5'-CGTTGATATTGCTGATTAAGTCCCTG-3';
IL-3 5' primer, 5'-ATGAGCCGCCTGCCCGTCTG-3', 3' primer, 5'-GCGAGGCTCAAAGTCGTCTGTTG-3';
IL-4 5' primer, 5'-ATGGGTCTCACCTCCCAACTGCT-3', 3' primer, 5'-CGAACACTTTGAATATTTCTCTCTCAT-3';
IFN- γ 5' primer, 5'-GCATCGTTTTGGGTTCTCTTGCGCTGTACTGC-3', 3' primer, 5'-CTCCTTTTCGCTTCCCTGTTTAGTCTGCTGG-3'.

Results

Allergen-specificity of cloned T cell lines from asthmatic children

Proliferative response of peripheral blood mononuclear cells to mite allergen showed a dose-dependent response (Fig. 1). In this study, 100 μ g/ml of mite allergen was used to stimulate antigen-specific T cell clones. After two weeks of initial culture with different cytokines such as IFN- γ or IL-4, bulk T cells still possessed the ability to respond to mite antigen (Fig. 2). In addition, the T cells cultures with supplemental IFN- γ or IL-4 showed better proliferative response than that of T cells cultured with only IL-2.

Three cloned allergen-specific cell lines CDL4, CDL5 and CDL6 were established from cells cultured in medium with IL-4 and IL-2. In contrast, three cloned cell lines CDR2, CDR3 and CDR4 were established from T cells cultured in medium with IFN- γ . All these cloned six T cell lines showed selective proliferative response to mite allergen (Fig. 3). Allergen-specificity of two cell clones, CDR3 and CDL5, were further tested with different concentration of antigens, and results showed dose-dependent responses (Fig. 4).

Surface phenotypic analysis of cloned T cells

An analysis of cell surface markers of the cloned T cell lines is shown in (Table 1). All cloned T lines expressed CD3, CD4, and TCR- α/β on the cell surface. Phenotypic analysis of these T cells with

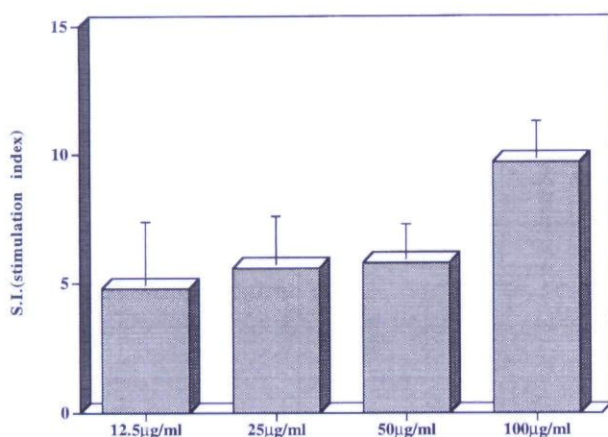


Fig. 1. Dose-dependent proliferative responses to mite allergen of peripheral blood mononuclear cells. The maximal response of T cells reached at the concentration of 100 μ g/ml of mite allergen.

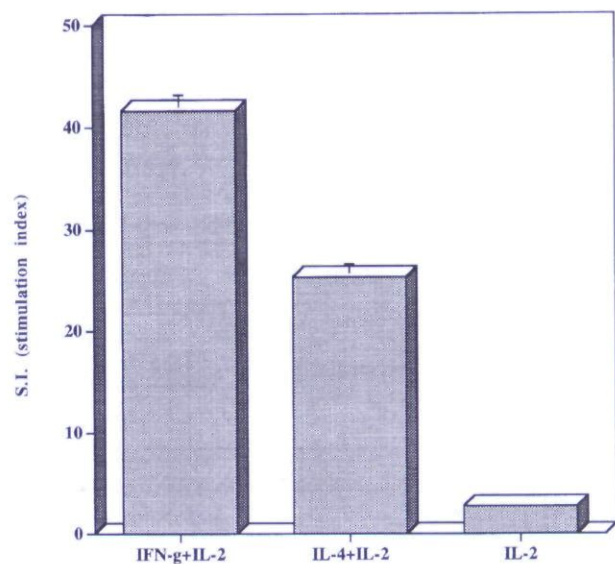


Fig. 2. Allergen-specific proliferative responses of bulk T cells after two weeks' culture in medium with different cytokines are demonstrated. The T cell proliferative response was better in T cells cultured with supplemental IFN- γ or IL-4 in addition to IL-2 compared with T cells cultured with IL-2 only.

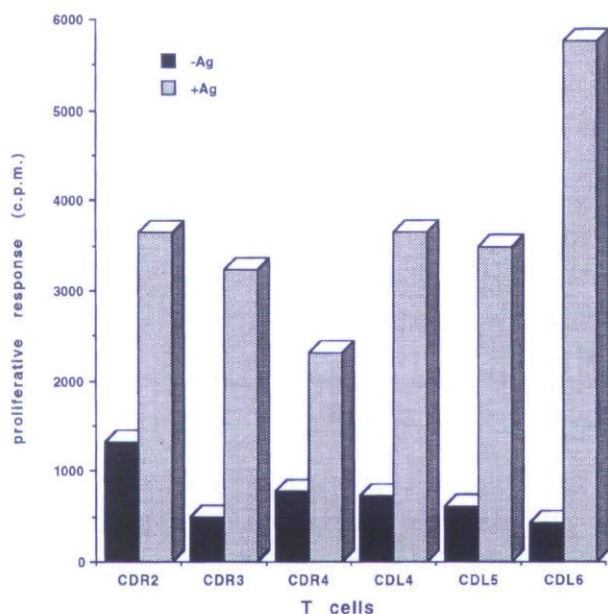


Fig. 3. After cloning with limiting dilution, a total six cloned T cell lines were generated and maintained in the laboratory for more than one year. All these T cell clones showed antigen-specific proliferative response.

CD45RA- isomers showed they are memory T cells in nature.

Cytokine production of T cell clones

After stimulation, only T cells cultured in medium containing IFN- γ could produce IFN- γ after two weeks' culture. This finding suggested *in vitro* supplement of IFN- γ could stimulate the T cells from atopic patients to produce cytokines of type 1 T helper cells after initial culture, the cytokines production pattern of these T cell clones is demonstrated in Table 2.

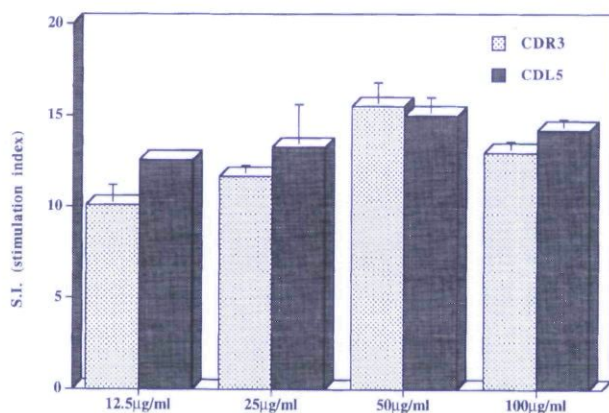


Fig. 4. Proliferative responses of CDR3 and CDL5 to different concentration of mite antigens showed as dose-dependent with the maximal proliferation at the concentration of 50 μ g/ml.

Table 1. Phenotypic analysis of cloned mite-specific T cells.

	CD3	CD4+RA	TCR $\alpha\beta$	NK	CD19	CD8
CDR2	97.64%	98.02%	97.04%	<1	<1	<1
CDR3	95.83%	97.74%	97.54%	<1	<1	<1
CDR4	99.04%	97.73%	98.02%	<1	<1	<1
CDL4	99.12%	97.86%	96.75%	<1	<1	<1
CDL5	98.43%	97.82%	97.22%	<1	<1	<1
CDL6	97.98%	98.34%	97.45%	<1	<1	<1

Discussion

Both *in vivo* and *in vitro* evidence suggested cytokines such as IL-4 are important for the immunoglobulin class switch from IgM to IgE, and IL-5 is critical for eosinophilia which cause more inflammatory response during the late stage of disease (2-4). It has been well documented that both IL-4 and IL-5 are secreted by type 2 T helper cells. Allergen-specific T cells derived from atopic patients are of type 2 T helper cell pattern predominantly; in contrast, allergen-specific T cells derived from nonatopic normals are predominantly type 1 T helper cells (5-8).

The difference and interaction between type 1 and type 2 have been a major focus of research in the past several years. Most recently, studies suggested *in vitro* supplement of exogenous cytokines such as IFN- γ or IL-4 in addition to IL-2 could affect the development of the T cells. These data suggested *in vivo* environments such as local concentration or types of cytokines might determine the fate of the T cells (9, 10). Dysregulation between TH1 and TH2 has been noted and contributed to many diseases such as acquired immunodeficiency syndrome (AIDS), systemic autoimmune diseases, chronic graft-versus-host disease and certain protozoal infections. Immunotherapy targeted on cytokines has become an important research direction recently. This mechanism has also been strongly suggested in immunotherapy for allergic diseases. The data here further demonstrated the existence of precursor T cells for both TH₁ and TH₂ allergen-specific

Table 2. Cytokine production pattern of cloned mite-specific T cells.

	CDR2	CDR3	CDR4	CDL4	CDL5	CDL6
IL-2	++ ^a	+++	++	-	-	-
IFN- γ	++	++	++	-	-	-
IL-3	++	+++	++	+	+	+
IL-4	-	-	-	++	+	++
IL-5	-	-	-	++	+	++

^aThe intensity of the cytokine mRNA was compared to that of β -actin.
+++ strong, ++ moderate, + weak, - negative.

ic T cells, which might be critical of cytokine-targeted immunotherapy.

Mite allergen has been found to be the most important aeroallergen worldwide, just as in the Taiwan area (20). Previously several mite allergen specific T cell clones have been derived from atopic and nonatopic patients in our laboratory (19). The data suggested mite specific T cell clones of atopic patients produced IL-4 predominantly, and only T cell clones of nonatopic normals secreted IFN- γ . However, polymorphism of major histocompatibility complex antigens limited further study with these T cell clones.

The data herein demonstrated allergen-specific TH₁ and TH₂ cell clones can be generated from the same patient with the supplementary cytokines in the culture. Before, most of the previous studies focussed on the effect of cytokine on the development of T helper cells. The result demonstrated MHC-matched TH₁ and TH₂ cells can be generated from the same patient. Our initial study also demonstrated different T cell antigenic determinants by MHC-matched Der p 1-specific TH₁ and TH₂ cells derived from the same asthmatic children (data not shown). With this kind of approach, we may investigate further the developmental mechanisms and signal transduction of TH₁ and TH₂ cells which might shed light on future immunotherapy for diseases caused by dysregulation of T helper cells.

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