

Interferon- γ and interleukin-4 production of ovalbumin-stimulated lymphocytes in egg-sensitive children

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Background: The causal relation between egg allergy and cytokines derived from lymphocytes is unknown.

Objective: Interferon- γ (IFN- γ) and interleukin-4 (IL-4) production of ovalbumin-stimulated and interleukin-2 (IL-2)-stimulated peripheral blood mononuclear cells from egg-sensitive patients was investigated and compared with that of stimulated peripheral blood mononuclear cells from nonatopic healthy children.

Methods: Peripheral blood mononuclear cells from egg-sensitive patients and nonatopic healthy children were cultured with ovalbumin and IL-2 for seven days. The IFN- γ and IL-4 concentrations in culture supernatants of the peripheral blood mononuclear cells were investigated.

Results: The levels of IFN- γ production of only IL-2-stimulated or both ovalbumin-stimulated and IL-2-stimulated peripheral blood mononuclear cells from egg-sensitive patients with atopic dermatitis was significantly higher than that of healthy children and that of egg-sensitive patients with immediate allergic symptoms.

Conclusions: Increased IFN- γ production by lymphocytes after IL-2 and antigen stimulation has important implications for the mechanism of food-sensitive atopic dermatitis.

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INTRODUCTION

The role of food hypersensitivity in the pathogenesis of atopic dermatitis has been discussed. Sampson et al reported the significance of an IgE-mediated reaction^{1,2}; however, patients with atopic dermatitis caused by food allergens do not always have food-specific IgE antibodies.

Wraith et al reported that symptoms appearing more than one hour after ingestion of foods were defined as non-immediate symptoms that consisted of aggravation of eczema caused by foods, principally milk and egg. Sixty-four percent of foods causing non-immediate symptoms give a positive radioallergosorbent test (RAST)

result, while there was a 100% correlation of the RAST result with immediate symptoms.³ There is no clear correlation between the quantities of specific IgE antibodies and the nature and the severity of allergic symptoms. It was also reported that the histology of an eczematous lesion is more indicative of a type IV cell-mediated response.^{4–6}

RAST is a sensitive procedure for the diagnosis of IgE-mediated hypersensitivity, but not for other types of hypersensitivity. Numerous reports of allergen-induced proliferation of peripheral blood mononuclear cells from allergic patients have been published.^{7–11} Many of the proliferation studies have involved the use of proteins purified from inhaled allergens,^{7–9} and a few have focused on the response to food antigens.^{10,11} We previously reported that the proliferative responses of peripheral blood mononuclear cells to ovalbumin or bovine se-

rum albumin were significantly stronger in children with atopic dermatitis who are sensitive to egg or cow milk than in healthy children and egg or cow milk-sensitive children with immediate allergic symptoms.^{12,13}

Recently, Mosmann and co-workers^{14–16} found that cloned mouse T cells can be grouped into two subsets based on their differential production of certain lymphokines. These subsets have been designated Th1 and Th2 cells. Activated Th1 clones produce IL-2, IFN- γ , and lymphotoxin but not IL-4 or IL-5. In contrast, activated Th2 clones produce IL-4 and IL-5 but not IL-2, IFN- γ , or lymphotoxin. It is suggested that Th1 clones mediate cell-mediated immunity, whereas Th2 clones stimulate B cell production of IgE and mediate immediate-type hypersensitivity.¹⁵

In the present study, we analyzed the production of IFN- γ and IL-4 of ovalbumin-stimulated and IL-2-stimulated peripheral blood mononuclear cells from egg-sensitive patients with atopic dermatitis, and compared it with that of thus stimulated peripheral blood mononuclear cells from nonatopic healthy children and egg-sensitive patients with immediate allergic symptoms.

MATERIALS AND METHODS

Subjects

Children with clinical histories of allergy to egg who were referred to our institution were recruited for the study. Evidence in the children's histories that suggested hypersensitivity to egg included pruritus, erythema, urticaria, and exacerbation of eczema after the ingestion of egg protein. We obtained

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parental informed consent for 24 children whose hypersensitivity to egg was confirmed by double-blind, placebo-controlled food challenges (DBPCFC). Of the twenty-four patients, five patients had urticaria and 19 had atopic dermatitis. Ten nonatopic healthy children served as controls. Diagnosis of atopic dermatitis was defined by the criteria of Hanifin.¹⁷

DBPCFC

Double-blind, placebo-controlled food challenges were performed according to the method previously described by Bock et al.¹⁸ Foods for testing were placed in white opaque capsules (Meiji Milk Products Co., Ltd., Tokyo, Japan) with dehydrated powdered whole egg (0.4 mg per capsule) or dehydrated powdered milk (0.4 mg per capsule) or placebo (potato starch). Our first challenge dose consisted of one capsule, and the dose was doubled every hour until the patient exhibited distinct symptoms or until 16 capsules were ingested as a single challenge dose. For young children, the dehydrated powdered foods were mixed in a broth or juice. We defined the time of onset of reaction as the time from the first challenge dose to the onset of symptoms, although Bock et al defined it as being the duration from the last challenge dose. The patients were placed on diets completely eliminating egg for 2 weeks before the food challenge. The patients were instructed to stop taking all medications for seven days before and during testing. No patient had been receiving systemic steroids. The allergic symptoms and timing of onset of symptoms were recorded on a symptom score sheet in accordance with the conditions of a double-blind study.¹⁸

RAST

Radioallergosorbent test was performed as recommended by the Phad-eas RAST test kit (Pharmacia, Uppsala, Sweden).¹⁹ Egg discs were purchased from Pharmacia. RAST results were scored 0 to 4+ by comparison with serially diluted reference sera (Pharmacia) graded A to D (ie, a serum < D = 0; between D and C = 1; and

>A = 4+). A RAST score of 2+, 3+, or 4+ was recorded as positive.

Cell Separation and Cell Culture

Peripheral blood mononuclear cells were isolated from heparinized blood from control donors and patients by gradient centrifugation in Ficoll-Paque (Pharmacia)²⁰ and washed three times with phosphate-buffered saline. Peripheral blood mononuclear cells (5×10^5 /mL) were cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 15% heat-inactivated fetal calf serum. Cultures were performed in triplicate at 0.2mL per well in round-bottomed plates or culture test tubes with or without ovalbumin at 37 °C in a humidified atmosphere containing 5% CO₂ for seven days in the presence of IL-2 (125 IU/mL) (Shionogi Co., Ltd., Osaka, Japan). Ovalbumin, a food antigen, was diluted in culture medium and added to a final concentration of 5 µg/mL each. We previously reported obtaining dose-dependent curves of lymphocyte proliferative responses to ovalbumin in patients who were sensitive to egg.¹² In the study, the peripheral blood mononuclear cells were stimulated with ovalbumin at 0.25 µg/mL, 2.5 µg/mL, or 25 µg/mL, and the lymphocyte proliferative response level was almost constant at more than 2.5 µg/mL. We therefore chose a final ovalbumin concentration of 5 µg/mL.

Interferon- γ (IFN- γ) Assay and Interleukin-4 (IL-4) Assay

Culture supernatants in the culture test tubes were collected to remove the cells after seven days of culture and were stored frozen at -70 °C until assay. Interferon- γ activity was assayed by enzyme-linked immunosorbent assay (ELISA) (Centocor Inc, Malvern, PA).²¹ The interassay and intraassay coefficients of variation for the IFN- γ assay ranged from 2.9% to 13.7% (data not shown).

For IL-4 assay, we developed an improved assay, a highly sensitive chemiluminescent sandwich enzyme immunoassay.^{22,23} In brief, immunoplates (Microlite 2, Dynatech Labora-

tories Inc, Chantilly, VA) were coated with monoclonal anti-human recombinant IL-4 antibody (Genzyme, Boston, MA) at 5 µg/mL in 0.05 M bicarbonate buffer (pH9.5) (100 µL/well) and incubated overnight at 4 °C. After the coating procedure, the plates were blocked with 2% (0.05 M) bovine serum albumin bicarbonate buffer (pH 9.5) (200 µL/well) for two hours at room temperature. The plates were then washed three times with phosphate-buffered saline and a 100-µL aliquot of test sample was added to each well. The plates were incubated for 16 hours at 20 °C and then washed three times with phosphate-buffered saline-Tween (0.05% vol/vol Tween 20). After adding 100 µL/well of rabbit polyclonal anti-human IL-4 antibody solution (Genzyme, Boston, MA) at 3 µg/mL in 1% bovine serum albumin-phosphate-buffered saline-Tween (0.05% vol/vol Tween 20), the plates were again incubated for three and one-half hours at 20 °C and washed three times with phosphate-buffered saline-Tween (0.05% vol/vol Tween 20). One hundred microliters per well of alkaline phosphatase conjugated goat F(ab)₂' anti-rabbit IgG solution (Tago Inc., Burlingame, CA) diluted generally at 1/25000 in Tris-Tween was added to the plates, which were then incubated for one hour at 20 °C, and then washed four times with Tris-buffered saline-Tween (0.055 vol/vol Tween 20). One hundred microliters per well of the chemiluminescent substrate solution, 0.4 mM 3-(2'-spiroadamantane)-4 methoxy-4-(3"-phosphoryloxy) phenyl-1, 2-dioxetane (AMPPD) reagent with Emerald Amplifier in 100mM diethanolamine buffer containing 1 mM MgCl₂ (pH 9.8) was added and the plates were again incubated at 20 °C for 60 minutes. AMPPD and Emerald Amplifier plus the substrate in the chemiluminescent assay were supplied by Tropix Inc (Bedford, MA). The chemiluminescent signal was measured after 60 minutes of incubation using a Dynatech ML 1000 luminometer. The interassay and intraassay coefficients of variation for the IL-4 as-

say ranged from 2.4% to 7.6% (data not shown). The assay had a detection limit of 0.1 IU/mL for IFN- γ and 0.03 pg/mL for IL-4.

Statistics

The significance of the differences between groups was analyzed by the Wilcoxon-Mann-Whitney test and the Wilcoxon signed-rank test.^{24,25}

RESULTS

Clinical Features and the Results of DBPCFC

The twenty-four patients were divided into three groups based on the timing between ingestion of the capsules packed with whole egg and onset of allergic symptoms in the DBPCFC. In all five patients with urticaria, immediate allergic symptoms (pruritus, erythema, and urticaria) appeared only within two hours (almost all within 15 minutes) of egg ingestion (patients with immediate allergic symptoms group). These five patients with immediate allergic symptoms ranged in age from 8 months to 3 years (mean 1.75 ± 0.64 years). The five patients with urticaria who were sensitive to egg did not have a present or past history of atopic dermatitis.

In twelve of the 19 patients with atopic dermatitis, non-immediate allergic symptoms (pruritus, erythema, and exacerbation of eczema) appeared more than two hours after egg ingestion (patients with atopic dermatitis group). These twelve patients with atopic dermatitis ranged in age from 10 months to 3 years (mean 1.87 ± 0.27 years).

In the other seven of the 19 patients with atopic dermatitis, both immediate and non-immediate allergic symptoms appeared. Immediate allergic symptoms (pruritus, erythema, and urticaria) appeared within two hours of egg ingestion, and after these symptoms improved, non-immediate allergic symptoms (pruritus, erythema, and exacerbation of eczema) appeared, more than two hours after egg ingestion (patients with atopic dermatitis and immediate allergic symptoms group). These seven egg-sensitive pa-

tients with atopic dermatitis and immediate allergic symptoms ranged in age from 8 months to 8 years (mean 2.69 ± 1.21 years).

The control group consisted of 10 nonatopic healthy children without egg sensitivity, ranging in age from 11 months to 10 years (mean 5.42 ± 1.29 years).

Serum IgE, and RAST Scores

The mean serum IgE value was 1381.2 ± 782.7 U/mL for the patients with immediate allergic symptoms, 89.2 ± 53.1 U/mL for the patients with atopic dermatitis, 195.4 ± 140.2 U/mL for the patients with atopic dermatitis and immediate allergic symptoms, and 77.1 ± 26.7 U/mL for the control group. The mean serum IgE value was significantly higher for the patients with immediate allergic symptoms than for the patients with atopic dermatitis ($P < .05$). The RAST score for egg was positive in all (100%) five patients with immediate allergic symptoms, whereas it was positive in two (16.7%) of the twelve patients with atopic dermatitis, and in five (71.4%) of the seven patients with atopic dermatitis and immediate allergic symptoms (Table 1).

IFN- γ Concentration in Culture Supernatants

The measurement results of IFN- γ activity in culture supernatants of peripheral blood mononuclear cells stimulated with only IL-2 or both ovalbumin and IL-2 after seven days of culture are shown in Table 1. The mean IFN- γ concentration in culture supernatants of only IL-2-stimulated peripheral blood mononuclear cells was 3.60 ± 1.61 IU/mL for the patients with immediate allergic symptoms, 118.07 ± 45.08 IU/mL for the patients with atopic dermatitis, 25.71 ± 16.68 IU/mL for the patients with atopic dermatitis and immediate allergic symptoms, and 13.49 ± 7.82 IU/mL for the control group. The mean IFN- γ concentration in the culture supernatants of only IL-2-stimulated peripheral blood mononuclear cells from the patients with atopic dermatitis was sig-

nificantly higher than that of the control group and the patients with immediate allergic symptoms ($P < .05$). Furthermore, the mean IFN- γ concentration in culture supernatants of both ovalbumin-stimulated and IL-2-stimulated peripheral blood mononuclear cells was 4.84 ± 1.98 IU/mL for the patients with immediate allergic symptoms, 212.71 ± 82.66 IU/mL for the patients with atopic dermatitis, 47.57 ± 20.00 IU/mL for the patients with atopic dermatitis and immediate allergic symptoms, and 33.64 ± 16.18 IU/mL for the control group. The mean IFN- γ concentration in the culture supernatants of both ovalbumin-stimulated and IL-2-stimulated peripheral blood mononuclear cells from the patients with atopic dermatitis was significantly higher than that of the control group and the patients with immediate allergic symptoms ($P < .05$).

Furthermore, we compared IFN- γ production between both ovalbumin-stimulated and IL-2-stimulated peripheral blood mononuclear cells and only IL-2-stimulated peripheral blood mononuclear cells. In general, the IFN- γ concentration tended to be higher in culture supernatants of both ovalbumin-stimulated and IL-2-stimulated peripheral blood mononuclear cells than in that of only IL-2-stimulated peripheral blood mononuclear cells, but the difference was not significant.

IL-4 Concentration in Culture Supernatants

Five patients with immediate allergic symptoms, six patients with atopic dermatitis, and six nonatopic healthy children (control group) were randomly selected and studied for IL-4 production from peripheral blood mononuclear cells stimulated with ovalbumin and IL-2. The measurement results of IL-4 activity in culture supernatants are summarized in Table 2. The mean IL-4 concentration in culture supernatants of only IL-2-stimulated peripheral blood mononuclear cells was 1.74 ± 0.43 pg/mL for the patients with immediate allergic symptoms, $0.88 \pm$

Table 1. Clinical Data, Serum IgE, RAST Scores for Egg, IFN- γ in Culture Supernatants of Only IL-2-Stimulated or Both Ovalbumin-Stimulated and IL-2-Stimulated Peripheral Blood Mononuclear Cells

Subjects	Age	Sex	Serum IgE Levels, U/mL	RAST Scores for Egg	IFN- γ of culture supernatants,* IU/mL	
					(-)*,	Ovalbumin†,
Patients with immediate allergic symptoms group‡						
§I-1	8m	F	911	4	6.6	3.7
I-2	8m	F	154.2	3	0.4	0.1
I-3	1y	M	14.8	3	2.2	2.8
I-4	2y	M	2126.2	3	7.4	9.7
I-5	3y	F	3700	4	1.4	7.9
Patients with atopic dermatitis group‡						
§A-1	10m	F	11	1	213	580
A-2	1y	F	46	3	500	116
A-3	1y	F	17.2	0	17.1	118
A-4	1y	F	34.7	0	39.7	77
A-5	1y	M	23	1	170	670
A-6	1y	M	<5	0	112	720
A-7	1y	M	72.3	0	48	26.2
A-8	2y	M	7.9	0	13.7	22.2
A-9	2y	M	54.8	0	3.3	7.6
A-10	2y	F	26.8	0	23.3	21.5
A-11	3y	M	636.5	3	270	190
A-12	3y	F	140.7	0	6.7	4.1
Patients with atopic dermatitis and immediate allergic symptoms group						
§U-1	8m	M	961	4	6.3	11.1
U-2	1y	M	120	4	3.5	29
U-3	1y	M	35	2	0.7	6.2
U-4	2y	F	42.7	2	57	63
U-5	2y	F	168	4	6.2	99
U-6	5y	F	13	0	0.3	0.7
U-7	8y	F	28	0	106	124
Control group‡						
§C-1	11m	M	62	0	0.6	11.9
C-2	1y	M	65	0	12.7	28
C-3	1y	F	9.8	0	22	20
C-4	2y	M	<5	0	19	50
C-5	2y	F	61	0	3.1	71
C-6	8y	F	16	0	76	154
C-7	8y	F	230	0	0.4	0.1
C-8	8y	F	110	0	0.3	0.2
C-9	9y	F	17	0	0.5	0.3
C-10	10y	M	200	0	0.3	0.9

* (-); culture supernatants of only IL-2-stimulated peripheral blood mononuclear cells.

† Ovalbumin; culture supernatants of both ovalbumin-stimulated and IL-2-stimulated peripheral blood mononuclear cells.

‡ IFN- γ concentration in culture supernatants of only IL-2-stimulated or both ovalbumin-stimulated and IL-2-stimulated peripheral blood mononuclear cells from patients with atopic dermatitis was significantly higher than that of control group and the patients with immediate allergic symptoms ($P < .05$).

§ I = patients with immediate allergic symptoms. A = patients with atopic dermatitis. U = patients with atopic dermatitis and immediate allergic symptoms. C = nonatopic healthy children.

|| There was no significant difference in IFN- γ concentrations between only IL-2-stimulated and both ovalbumin-stimulated and IL-2-stimulated peripheral blood mononuclear cells.

Table 2. IL-4 in Culture Supernatants of Only IL-2-Stimulated or Both Ovalbumin-Stimulated and IL-2-Stimulated Peripheral Blood Mononuclear Cells

Subjects	Serum IgE Levels, U/mL	RAST Scores for Egg	IL-4 of Culture Supernatants,* pg/mL	
			(-)*,	Ovalbumin†,
Patients with immediate allergic symptoms group‡				
§I-1	911	4	0.99	138.05
I-2	154.2	3	0.72	0.83
I-3	14.8	3	2.64	2.75
I-4	2126.2	3	1.98	1.05
I-5	3700	4	2.37	3.63
Patients with atopic dermatitis group‡				
§A-1	11	1	1.63	0.8
A-2	46	3	1.02	1.16
A-3	17.2	0	0.77	0.94
A-4	34.7	0	<0.03	0.33
A-5	23	1	1.02	1.39
A-6	<5	0	0.83	0.45
Control group‡				
§C-1	62	0	0.55	1.62
C-2	65	0	0.65	0.41
C-3	9.8	0	<0.03	0.22
C-4	<5	0	1	0.83
C-5	61	0	1.38	0.74
C-6	16	0	1.27	1.38

* (-); culture supernatants of only IL-2-stimulated peripheral blood mononuclear cells.
† Ovalbumin; culture supernatants of both ovalbumin-stimulated and IL-2-stimulated peripheral blood mononuclear cells.
‡ There was no significant difference between the groups.
§ I = patients with immediate allergic symptoms. A = patients with atopic dermatitis. C = nonatopic healthy children.
|| There was no significant difference in IL-4 concentrations between only IL-2-stimulated and both ovalbumin-stimulated and IL-2-stimulated peripheral blood mononuclear cells.

0.24 pg/mL for the patients with atopic dermatitis, and 0.81 ± 0.23 pg/mL for the control group. The mean IL-4 concentration in culture supernatants of both ovalbumin-stimulated and IL-2-stimulated peripheral blood mononuclear cells was 29.26 ± 30.95 pg/mL for the patients with immediate allergic symptoms, 0.85 ± 0.18 pg/mL for the patients with atopic dermatitis, and 0.87 ± 0.25 pg/mL for the control group. There were no significant differences between the groups. We also compared IL-4 production between both ovalbumin-stimulated and IL-2-stimulated peripheral blood mononuclear cells and only IL-2-stimulated peripheral blood mononuclear cells and found no significant difference.

DISCUSSION

There is evidence that IL-4 and IFN- γ are produced by different subsets of T

cells in mice. Mosmann et al¹⁴⁻¹⁶ reported that mouse helper T-cell clones fall into two main groups (Th1, Th2), defined primarily by differences in the patterns of lymphokines synthesized. Th1 cells produce IFN- γ , IL-2, and lymphotoxin whereas Th2 cells produce IL-4, IL-5, and IL-6.

In humans, a clear functional dichotomy in helper T lymphocytes, as observed in mice, does not seem to be evident. Recently, however, Wierenga et al²⁶ presented evidence of the presence of distinct functional subsets of CD4+ T cells in humans on the basis of IL-4 and IFN- γ production.

Interleukin-4 has multiple biologic activities. For example, IL-4 can induce class II MHC Ag and Fc ϵ RII (CD23) expression on B cells in both humans and mice.²⁷⁻³¹ Furthermore, in both mice and humans, IgE production is promoted by IL-4 and suppressed by

IFN- γ .³²⁻³⁴ thus, Th1 cells induce cell-mediated immunity whereas Th2 cells induce immediate-type reactions.

Wierenga et al²⁶ increased the number of antigen-specific T cells by stimulation with IL-2 and antigen and established antigen-specific T cell clones by the limiting dilution. We tried to increase the number of antigen-specific T cells in peripheral blood mononuclear cells by stimulation with IL-2 and antigen. We measured the IFN- γ and IL-4 concentrations in culture supernatants of thus stimulated peripheral blood mononuclear cells from the patients and compared these with those of nonatopic healthy children.

In this study, the mean IFN- γ concentration in the culture supernatants of both ovalbumin-stimulated and IL-2-stimulated peripheral blood mononuclear cells from the patients with atopic dermatitis was significantly higher

than that of the control group. Furthermore, the mean IFN- γ concentration in the culture supernatants of IL-2-stimulated peripheral blood mononuclear cells in the absence of any antigen from egg-sensitive patients with atopic dermatitis was significantly higher than that of the control group. It is probable that in egg-sensitive atopic dermatitis patients, T cells were already sensitized to food antigen in vivo and the sensitized cells proliferated upon IL-2 stimulation.

Increased IFN- γ production of both ovalbumin-stimulated and IL-2-stimulated peripheral blood mononuclear cells from egg-sensitive patients with atopic dermatitis reflects cell-mediated immunity. Presumably in patients with atopic dermatitis who are sensitive to foods, CD4⁺ T lymphocytes stimulated with food antigens and IL-2 secrete IFN- γ . As a result, cell-mediated immunity may be induced. Further, in patients with atopic dermatitis who have low specific IgE antibody to the food antigen, IgE production by B lymphocytes may be suppressed by IFN- γ and, consequently, cell-mediated immunity may predominantly occur compared with immediate hypersensitivity.

On the other hand, there was no significant difference in the level of IL-4 production from peripheral blood mononuclear cells between the patients with immediate allergic symptoms and the control group, although the patients with immediate allergic symptoms had positive RAST scores. This discrepancy might be due to the short half-life of IL-4 (six minutes), which is locally released and immediately processed by the cells in the cultures as Rousset et al³⁵ reported. A more extensive study is required to elucidate fully the pathogenic mechanism of atopic dermatitis.

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