Immunodeficiency and other clinical immunology

The role of T lymphocytes in patients with food-sensitive atopic dermatitis

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The role of T lymphocytes was assessed in patients with food-sensitive atopic dermatitis (AD). T lymphocytes plus monocytes responded well to ovalbumin or bovine serum albumin (BSA) in children with AD who were sensitive to hen's egg or cow's milk compared with healthy children and children with immediate allergic symptoms who are sensitive to hen's egg or cow's milk. The responding cells were shown to be predominantly CD4+ T lymphocytes. Interleukin-2 activity and interferon-γ concentrations in culture supernatants of ovalbumin-stimulated peripheral blood mononuclear cells (PBMCs) from patients with AD who were sensitive to hen's egg were significantly higher than those of healthy children and patients sensitive to hen's egg with immediate symptoms. Expression of Fcε R II on B lymphocytes in cultures of ovalbumin-stimulated PBMCs from patients with AD was significantly higher than that of healthy children, but it tended to be lower than that of patients with immediate symptoms. These results suggest that, in patients with AD who are food sensitive, CD4+ T lymphocytes stimulated by food antigens secrete lymphokines such as interleukin-2 and interferon-γ that are secreted from TH1 clones in mice, and express Fcε R II on B lymphocyte that is induced by interleukin-4 secreted from TH2 clones in mice. Taken together, cell-mediated immunity may also occur in addition to IgE-mediated hypersensitivity in patients with food-sensitive AD. (J ALLERGY CLIN IMMUNOL 1993;91:658-68.)

Key words: *Atopic dermatitis, food hypersensitivity, CD4+ T lymphocytes, interleukin-2, interferon-γ*

The role of immediate food hypersensitivity in the pathogenesis of atopic dermatitis (AD) is controversial.[1-3] Sampson[1] reported the significance of IgE-mediated food hypersensitivity in patients with AD. However, Wraith et al.[3] reported that the percentage of positive food radioallergosorbent test (RAST) in patients with nonimmediate allergic symptoms after food ingestion (reactions appearing after 1 hour) was lower than that in patients with immediate allergic symptoms after food ingestion (reactions occurring in less than 1 hour). In contrast, from the nature of the cellular infiltrate seen in eczematous lesions, it also appears that some form of cell-mediated immunity may be involved in the pathogenesis of AD.[4] Certainly, many of the symptoms of AD are not only persistent but also late in onset after food ingestion. It was also reported that the histology of an eczem-

Abbreviations used

AD:	Atopic dermatitis
RAST:	Radioallergosorbent test
PBMC:	Peripheral blood mononuclear cell
BSA:	Bovine serum albumin
ConA:	Concanavalin A
rIL-4:	Recombinant interleukin-4
SI:	Stimulation index
CPM:	Counts per minute
IL-2:	Interleukin-2
IL-4:	Interleukin-4
IFN-γ:	Interferon-γ
rIL-2:	Recombinant interleukin-2
PBS:	Phosphate-buffered saline

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atous lesion is more indicative of a type IV cell-mediated response.[5] RAST is a sensitive procedure for the diagnosis of IgE-mediated hypersensitivity but not for other types of hypersensitivity. There have been some reports of allergen-induced proliferation of peripheral blood mononuclear cells (PBMCs) in patients with allergic diseases.[6-8] We previously reported that the proliferative responses of PBMCs to ovalbumin or bovine serum albumin (BSA) in children with AD who are sensitive to hen's egg or cow's milk were significantly higher than those of healthy children and children with hen's egg or cow's milk sensitivity and immediate symptoms, and that, in patients with AD no significant correlations existed between the proliferative responses of PBMCs and the RAST values.[9] In this study it is shown that T lymphocytes respond to food antigens in patients with food-sensitive AD, and the role of T lymphocytes is investigated.

MATERIAL AND METHODS

Subjects

Twenty-five patients with AD, selected as being sensitive to hen's egg or cow's milk on the basis of clinical history and double-blind, placebo-controlled food challenges, were studied. Diagnosis of AD was defined by the criteria of Hanifin.[10] The severity of diseases was graded by the food-challenge symptom scores for skin by Bock et al.[11] Subjects ranged in age from 6 months to 7 years (mean, 2.9 years). In double-blind, placebo-controlled food challenges, each dehydrated powdered food (Meiji Milk Products Co., Ltd., Tokyo, Japan) for testing was placed in white opaque capsules (0.4 gm per capsule). Our first challenge dose was one capsule, and doubled doses were continued every hour until the patient exhibited convincing symptoms or until 16 capsules were ingested as a single challenge dose. For young children, the dehydrated powdered foods were hidden in a broth or juice. We defined the time of reaction as being the time from the first dose of the challenge to the onset of symptoms. Patients were instructed to discontinue all medications for 7 days before and during testing. No patients had been on systemic steroids. The cutaneous manifestations of most patients with AD did not appear sooner than 2 hours after the first dose of the challenge (that is, not until after the second dose, 0.8 gm). The control group included 11 nonatopic healthy children (control group I) ranging in age from 5 months to 7 years (mean, 3.0 years) who were not sensitive to hen's egg or cow's milk, and 15 patients (control group II) ranging in age from 4 months to 7 years (mean, 2.9 years) who had acute urticaria or angioedema (immediate symptoms) that appeared within 1 hour (usually within 15 minutes) of the first dose of the challenge. Blood samples were obtained when diseases were active.

RAST

RAST was performed as recommended by the Phadebas RAST test kit (Pharmacia AB, Uppsala, Sweden).[12] Hen's egg and cow's milk disks were purchased from Pharmacia. RAST results were scored 0 to 4+ by comparison with serially diluted reference sera (Pharmacia) graded A to D (i.e., a serum $<D = 0$; between D and C = 1; and $>A = 4+$). RAST scores of 2+, 3+, and 4+ were recorded as positive.

Cell preparation and separation

PBMCs were isolated from heparinized blood from control donors and patients by gradient centrifugation in Ficoll-Paque (Pharmacia).[13] To remove adherent cells, we incubated 20 to 50 $\times 10^6$ PBMCs in 20 ml of culture medium consisting of RPMI 1640 supplemented with 15% pooled human AB^+ serum, L-glutamine (2 mmol/L), penicillin (100 units/ml), and streptomycin (100 μg/ml) in 75 cm^2 tissue culture flasks (Falcon, Becton Dickinson Labware, Lincoln Park, N.J.) for 2 to 3 hours. Nonadherent cells were recovered, and they were separated into a T-lymphocyte–enriched population and a B-lymphocyte–enriched population by the rosetting method with neuraminidase-treated sheep erythrocytes, after which they were subjected to centrifugation over Ficoll-Paque[13] and by the nylon fiber column method.[14] The rosetted cells were collected from the bottom, recovered by lysis of sheep erythrocytes with isotonic NH_4 Cl/TRIS treatment for 10 minutes at 4° C, passed through the column, and washed with phosphate-buffered saline (PBS). These cells (>98% CD3+) were used as T lymphocytes. Nonrosetted cells were collected from the interface, passed through the column, and washed with PBS. These cells (72% CD20+, 2% CD3+, and 2% CD14+) were used as B lymphocytes. The adherent cells were harvested by a rubber policeman, washed, and used as monocytes. Moreover, to isolate T-lymphocyte populations highly enriched in either CD4 reactive or CD8 reactive cells, the unfractionated T lymphocytes were resuspended at 50 $\times$ 10^6/ml in the culture medium. To 0.2 ml T lymphocytes was added 0.2 ml of OKT4 monoclonal antibody or OKT8 monoclonal antibody (Ortho Pharmaceutical Corp., Raritan, N.J.) at a dilution of 1:250, and the cells were then incubated for 45 minutes at room temperature. After incubation, rabbit complement was added at a final dilution of 1:10 and incubation was further carried out for 1 hour at 37° C.[15] The OKT4-treated population (>90% OKT8+ cells) was used as CD8 T lymphocytes and the OKT8-treated population (>90% OKT4+ cells) was used as CD4 T lymphocytes.

Cell culture

PBMCs, T lymphocytes, B lymphocytes, CD4 T lymphocytes, or CD8 T lymphocytes were suspended to give a density of 10^6/ml in the culture medium. Monocytes were added to T lymphocytes, B lymphocytes, CD4 T lymphocytes, or CD8 T lymphocytes at a final concentration of 10% before cultures were started. Cultures were performed in triplicate at 0.2 ml per well in round-bottomed microtest plates (Nunc, Roskilde, Denmark) with or without various concentrations of food antigens at 37° C for 4 days in a humidified atmosphere containing 5% CO_2.[9] PBMCs (5 $\times$ 10^6/ml) were also cultured at 2 ml per tube in culture

TABLE IA. RAST scores, and maximal proliferative responses of PBMCs and separated cells to food antigens for control group I, control group II, and patients with AD who are sensitive to hen's egg

Subjects* (no. age (yr))	RAST scores for hen's egg (no.)		Maximal proliferative responses to ovalbumin (SI)†				
	Negative	Positive	PBMCs	T lymphocytes + Mo.	B lymphocytes + Mo.	T lymphocytes	B lymphocytes
Control group I (N = 11, 3.0 ± 2.6)	11	0	1.1 ± 0.2 (433.2) [472.2]	1.6 ± 0.3 (452.8) [710.9]	1.2 ± 0.1 (335.1) [403.1]	1.5 ± 0.6 (434.2) [664.0]	1.0 ± 0.2 (355.6) [359.1]
Control group II, sensitive to hen's egg (N = 8, 2.7 ± 2.3)	1	7	1.3 ± 0.3 (430.8) [565.2]	1.7 ± 1.1 (464.7) [785.3]	1.4 ± 0.4 (367.0) [525.0]	1.6 ± 0.4 (449.2) [655.8])	1.1 ± 0.3 (382.6) [409.1]
Patients with AD, sensitive to hen's egg (N = 15, 2.7 ± 1.8)	7	8	3.6 ± 1.2† (415.2) [1519.6]	4.2 ± 1.8§ (430.7) [1851.4]	1.7 ± 0.8 (346.4) [637.0]	1.9 ± 1.0 (388.7) [723.7]	1.2 ± 0.8 (399.3) [507.1]

test tubes with or without food antigens, concanavalin A (ConA, 5 μg/ml), or recombinant interleukin-4 (rIL-4, 200 units/ml; Genzyme, Cambridge, Mass.) for various numbers of days.

Proliferative response

DNA synthesis was measured by adding 0.5 μCi [^{3}II] thymidine (specific activity: 43 Ci/mmol, Amersham, Tokyo, Japan) per well of the microtest plates 4 hours before harvesting onto glass fiber filters. [^{3}H] incorporation was measured by liquid scintillation counting, and the results were expressed as the mean of triplicate. To compare responses between individuals, we expressed the results as stimulation index (SI).

$$SI = \frac{\text{Mean CPM in cultures stimulated with food antigens}}{\text{Mean CPM in unstimulated cultures}}$$

The specificity of proliferative responses of PBMCs to each food antigen was previously confirmed in children with AD. In brief, proliferative responses of PBMCs to ovalbumin (SI 3.09 ± 1.72, mean CPM/mean base CPM 1103.7/351.7) in patients with AD sensitive to hen's egg (n = 23) were significantly ($p < 0.005$ for each) higher than the responses to ovalbumin (SI 1.10 ± 0.33, mean CPM/mean base CPM 352.6/327.8) in patients (n = 15) with AD who were sensitive to cow's milk but not to hen's egg, and the responses to ovalbumin (SI 1.20 ± 0.41, mean CPM/mean base CPM 415.1/360.7) in patients (n = 15) with AD and negative double-blind, placebo-controlled food challenges for hen's egg and cow's milk. Proliferative responses of PBMCs to BSA (SI 3.82 ± 2.31, mean CPM/mean base CPM 1228.0/327.8) in cow's milk-sensitive patients with AD sensitive to cow's milk (n = 15) were significantly ($p < 0.005$ for each) higher than the responses to BSA (SI 1.16 ± 0.35, mean CPM/mean base CPM 415.3/351.7) in patients (n = 23) with AD who were sensitive to hen's egg but not to cow's milk, and the responses to BSA (SI 1.14 ± 0.45, mean CPM/mean base CPM 394.8/360.7) in patients (n = 15) with AD and negative double-blind, placebo-controlled food challenges for hen's egg and cow's milk.

Immunofluorescence analyses

After 5 days of culture, cells were harvested from the culture test tubes, washed, and resuspended in PBS. Phenotyping of the cells was carried out by immunofluorescence assays as described by Bonnefoy et al.[16] Cells (10^6) were stained with fluorescein isothiocyanate-conjugated Leu4 (CD3), Leu3a (CD4), or Leu2a (CD8) (Becton Dickinson Microbiology Systems, Cockeysville, Md.), and were analyzed by means of a fluorescence-activated cell sorter (FACStar; Becton Dickinson, Immunocytometry Systems, San Jose, Calif.). For double fluorescence analysis, cells were collected after 7 days of culture and were incubated simultaneously with fluorescein isothiocyanate-conjugated H107 (Fcε RII/CD23) (Nichirei, Tokyo, Japan) and phycoerythrin-conjugated Leu16 (CD20) (Becton Dickinson). Phycoerythrin was plotted along the y axis, and fluorescein isothiocyanate was plotted along the x axis.

Interleukin-2 (IL-2) assay and interferon-γ (IFN-γ) assay

Culture supernatants in the culture test tubes were spun to remove cells after various numbers of days and were stored frozen at −70° C until assayed. IL-2 activity was assayed by the method described by Gillis and Smith.[17] In brief, each sample was processed for twofold dilutions in culture medium in volumes of 100 μl/well in microtest plates (Nunc). To these, 100 μl/well of 4 × 10^3 CTLL-2 cells (an IL-2 dependent murine line of cytotoxic T lymphocytes) were added. Cells were cultured at 37° C in a humidified atmosphere with 5% CO_2 for 24 hours, and were then pulsed with 0.5 μCi per well [^{3}H] thymidine (specific activity: 43 Ci/mmol, Amersham, Tokyo) and cultured for a further 4 hours before harvest. To allow comparison of

TABLE IB. RAST scores, and maximal proliferative responses of PBMCs and separated cells to food antigens for control group I, control group II and patients with AD who are sensitive to cow's milk

Subjects* (no. age (yr))	RAST scores for cow's milk (no.)		Maximal proliferative responses to BSA (SI)†				
	Negative	Positive	PBMCs	T lympho. + Mo.	B lympho. + Mo.	T lymphocytes	B lymphocytes
Control group I (N = 11, 3.0 ± 2.6)	11	0	1.0 ± 0.6 (433.2) [480.9]	1.5 ± 1.1 (452.8) [534.3]	1.1 ± 0.6 (335.1) [374.6]	1.6 ± 0.6 (434.2) [577.2]	0.9 ± 0.4 (355.6) [329.1]
Control group II, sensitive to cow's milk (N = 7, 3.1 ± 2.9)	0	7	1.5 ± 0.3 (458.7) [711.0]	1.6 ± 0.9 (431.0) [651.0]	1.4 ± 0.2 (300.4) [407.0]	1.4 ± 0.5 (461.2) [641.1]	1.0 ± 0.3 (391.8) [435.0]
Patients with AD, sensitive to cow's milk (N = 10, 3.3 ± 2.2)	7	3	3.0 ± 1.0‖ (449.1) [1342.0]	3.9 ± 0.9¶ (422.2) [1629.7]	1.5 ± 1.1 (358.7) [505.8]	1.7 ± 1.4 (409.7) [708.8]	1.2 ± 0.7 (376.5) [436.7]

Mo, Monocytes.

*Control group I, healthy children; control group II, patients with immediate allergic symptoms. Age distribution (mean ± 1 SD) is also shown.

†SI, Stimulation index; each value is mean ± 1 SD. The number in ( ) is the mean of base cpm in each group, and the number in [ ] is the mean cpm of the proliferative response of PBMCs to ovalbumin or BSA in each group.

The SI value of ‡, §, ‖, or ¶ was significantly higher ($p < 0.005$ for each) than the values of control group I and control group II, respectively. The CPM value of the proliferative response of PBMCs to ovalbumin or BSA in ‡, §, ‖, ¶ was significantly higher ($p < 0.005$ for each) than the values of control group I and control group II, respectively, although there was no significant difference between base CPM value in patients with AD and base CPM values in control group I and control group II, respectively.

results between assays, a dilution curve of recombinant interleukin-2 (rIL-2; Takeda Chemical Industries Ltd., Osaka, Japan) was run in parallel with each assay. IFN-γ concentrations in culture supernatants were measured with use of a commercial solid-phase radioimmunoassay kit (IMRX; Centocor, Inc., Malvern, Pa.).[18] All supernatants were assayed in triplicate. Culture medium for these assays was RPMI 1640 supplemented with 15% heat-inactivated fetal calf serum, L-glutamine (2 mmol/L), penicillin (100 units/ml), and streptomycin (100 μg/ml).

Antigens

Ovalbumin and BSA were purchased from Wako Junyaku Pure Chemical Industries, Inc. (Osaka, Japan) and used in concentrations of 0.25, 2.5, and 25 μg/ml, respectively.

Statistics

The significance of the difference between groups was analyzed by the Wilcoxon-Mann-Whitney test.

RESULTS

RAST scores

The RAST values for hen's egg were positive in seven of eight patients (87.5%) with immediate symptoms (control group II) who were sensitive to hen's egg, whereas values were positive in only 8 of 15 patients (53.3%) with AD who were sensitive to hen's egg (Table IA). The RAST values for cow's milk were positive in all patients tested with immediate symptoms (control group II) who were sensitive to cow's milk, whereas values were positive in only 3 of 10 patients (30.0%) with AD who were sensitive to cow's milk (Table IB).

Proliferative responses of PBMCs and separated cells to food antigens

T lymphocytes of patients with AD who were sensitive to hen's egg or cow's milk showed excellent proliferation to ovalbumin or BSA when cultured with monocytes. In most cases proliferation increased steadily with increasing ovalbumin or BSA dose over the range 0.25 to 25 μg/ml. (Representative dose-response curves are shown in Fig. 1.) As shown in Table I, the maximal proliferative responses of PBMCs or T lymphocytes plus monocytes to ovalbumin or BSA in patients with AD who were sensitive to hen's egg or cow's milk were significantly higher ($p < 0.005$ for each) than those of control groups I and II. T lymphocytes alone did not proliferate in response to ovalbumin or BSA, presumably indicating a requirement for antigen-presenting cells. B lymphocytes showed no significant response to ovalbumin or BSA when cultured with or without monocytes. These results indicate that the responding cells were in the T-lymphocyte population. Moreover, as shown in Table II, in patients with AD who were sensitive to hen's egg, CD4 T lymphocytes showed excellent pro-

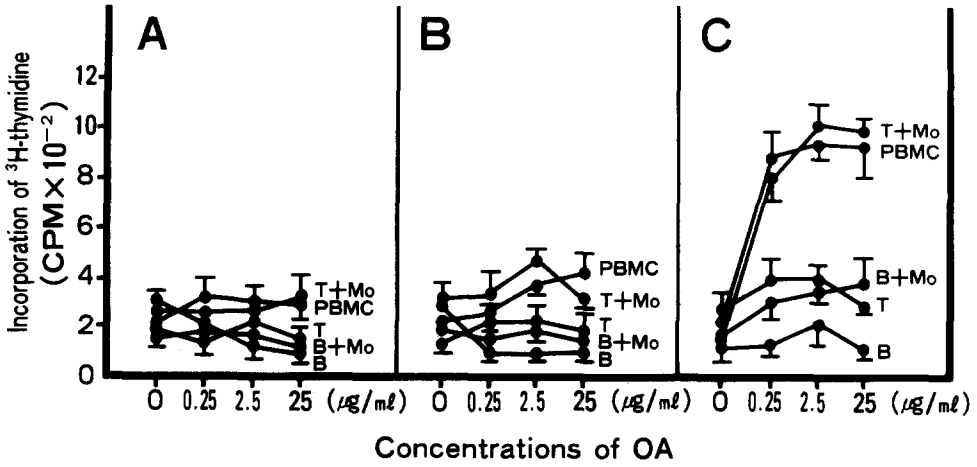

FIG. 1. Proliferative responses of PBMCs, T lymphocytes, B lymphocytes, T lymphocytes + 10% monocytes *(Mo)*, and B lymphocytes + 10% monocytes, stimulated by various concentrations of ovalbumin. **A,** shows the result of a healthy control (control group I). **B,** shows the result of a patient with immediate symptoms who is sensitive to hen's egg (control group II). **C,** shows the result of a patient with AD who is sensitive to hen's egg. Each value is the mean of triplicate, and each *bar* indicates 1 SD.

TABLE II. Proliferative responses of separated T lymphocytes to ovalbumin for patients with AD who are sensitive to hen's egg

Separated T lymphocytes†	No.	Maximal proliferative responses to ovalbumin		
		Base CPM*	CPM*	SI*
T lymphocytes + monocytes	15	430.7 ± 172.0	1851.4 ± 771.3	4.2 ± 1.8
CD4 T lymphocytes + monocytes	15	510.3 ± 251.8‡	1990.2 ± 931.0§	3.8 ± 1.5‖
CD8 T lymphocytes + monocytes	15	504.0 ± 288.4	937.6 ± 657.2	1.8 ± 1.2

*Each value is mean ± 1 SD.
†Separated T lymphocytes (2×10^5/0.2 ml) with 10% monocytes were cultured with various concentrations of ovalbumin.
‡§‖The CPM value of § was significantly higher ($p < 0.005$) than the CPM value of "CD8 T lymphocytes + monocytes," although there was no significant difference between the base CPM value ‡ and the base CPM value of "CD8 T lymphocytes + monocytes."
The SI value of ‖ was significantly higher ($p < 0.005$) than the value of "CD8 T lymphocytes + monocytes."

liferation to ovalbumin when cultured with monocytes, but CD8 T lymphocytes showed little proliferation to ovalbumin when cultured with monocytes.

Surface marker phenotypes of responding cells

Ovalbumin-stimulated PBMCs were collected after 5 days of culture, stained with monoclonal antibodies, and analyzed by FACStar. As shown in Table III, there was an increase in the proportion of CD4+ lymphocytes in responding cultures of patients with AD who were sensitive to hen's egg. There was little or no change in the percentages of subsets on healthy controls (control group I) and patients with immediate symptoms sensitive to hen's egg (control group II). The value when subtracting the percentages of CD4+ lymphocytes in nonstimulated cultures from the percentages of CD4+ lymphocytes in ovalbumin-stimulated cultures on patients with AD was significantly higher than those of control group I and control group II ($p < 0.005$ for each).

IL-2 and IFN-γ production in culture supernatants of ovalbumin-stimulated PBMCs

Culture supernatants from PBMCs stimulated with ovalbumin were tested for IL-2 activity and IFN-γ after 4 days of culture, because maximal levels of IL-2 activity and IFN-γ were seen in culture supernatants after 4 days of culture of ovalbumin-stimulated PBMCs from patients with AD who were sensitive to hen's egg. Maximal levels of IL-2 activity and IFN-γ were seen in culture supernatants of ConA-stimulated PBMCs after 24 hours of culture. The dilution

TABLE III. Surface marker phenotypes of responding cells on control group I, control group II, and patients will AD who are sensitive to hen's egg

	CD3+ lymphocytes (%)[a]		CD4+ lymphocytes (%)[a]			CD8+ lymphocytes (%)[a]		
Patients	**(−)[b]**	**ovalbumin[c]**	**(−)[d]**	**ovalbumin[e]**	**e-d**	**(−)[f]**	**ovalbumin[g]**	**g-f**
Control group I[h]								
C-1	50.6	51.5	39.8	39.9	0.1	17.3	17.1	−0.2
C-2	87.3	80.9	51.3	51.0	−0.3	29.5	28.4	−1.1
C-3	54.4	54.1	36.4	37.8	1.4	17.4	16.1	−1.3
C-4	61.5	62.4	38.1	38.5	0.4	21.3	23.5	2.2
C-5	71.1	68.7	44.1	44.0	−0.1	33.0	31.5	−1.5
C-6	50.5	47.6	36.1	35.2	−0.9	18.3	23.1	4.8
C-7	75.2	75.0	42.3	42.5	0.2	29.7	28.6	−1.1
C-8	80.4	78.1	55.8	55.9	0.1	32.3	29.4	−2.9
C-9	82.3	82.9	48.1	48.9	0.8	36.4	38.2	1.8
C-10	76.6	79.7	37.4	36.7	;0.7	35.5	36.6	1.1
C-11	58.7	59.3	39.5	39.4	−0.1	20.9	21.8	0.9
					0.2 ± 0.5[i]			1.1 ± 2.1[j]
Control group II[k], sensitive to hen's egg								
I-1	78.8	80.6	59.5	63.8	4.3	15.8	15.1	−0.7
I-2	81.6	82.4	45.5	47.4	1.9	34.5	35.2	0.7
I-3	51.4	52.7	39.7	39.5	−0.2	17.2	18.6	1.4
I-4	57.2	58.3	36.3	38.4	2.1	17.4	17.5	0.1
I-5	65.4	68.2	36.1	36.1	0	31.7	30.2	−1.5
I-6	78.8	74.9	59.5	61.8	2.3	15.8	15.1	−0.7
I-7	59.5	59.1	31.3	33.2	1.9	29.5	30.7	1.2
I-8	68.3	71.5	35.8	38.0	2.2	31.1	30.3	−0.8
					2.1 ± 1.1[l]			−0.1 ± 1.2[m]
Patients with AD, sensitive to hen's egg								
1	65.8	68.9	37.2	38.6	1.4	30.3	28.2	−2.1
2	72.3	73.7	46.8	51.7	4.9	35.1	36.4	1.3
3	86.6	89.1	57.0	64.5	7.5	25.7	24.4	−1.3
4	61.0	62.3	34.6	38.3	3.7	21.6	23.8	2.2
5	79.2	81.7	48.3	56.4	8.1	29.5	30.2	0.7
6	87.7	88.4	50.8	52.8	2.0	29.4	27.8	−1.6
7	65.1	67.7	40.5	46.8	6.3	22.3	23.6	1.3
8	71.6	77.3	52.9	60.7	7.8	27.1	26.7	−0.4
9	64.5	66.4	31.8	35.3	3.5	18.9	18.9	0
10	58.1	63.3	38.0	44.4	6.4	26.3	26.7	0.4
11	70.5	73.1	50.8	56.1	5.3	23.1	25.3	2.2
12	66.4	75.0	52.5	59.2	6.7	25.3	25.1	−0.2
13	80.8	82.8	51.8	55.1	3.3	29.2	28.7	−0.5
14	71.2	71.9	42.7	48.8	6.1	30.4	30.2	−0.2
15	59.9	63.4	35.2	42.2	7.0	33.8	32.1	−1.7
					5.3 ± 2.0[n]			0.7 ± 1.2[o]

[a]Percentages in cultured cells.
[b, d, f] Cultures without ovalbumin.
[c, e, g] Cultures with ovalbumin (2.5 μg/ml).
[e-d]Subtracting the percentage in "d" from the percentage in "e."
[g-f]Subtracting the percentage in "f" from the percentage in "g."
[h]Control group I; healthy children.
[k]Control group II; patients with immediate allergic symptoms.
[i, j, l, m, n, o] Mean ± 1 SD; value of "n" was significantly higher than the values of "i" and "l" ($p < 0.005$ for each).

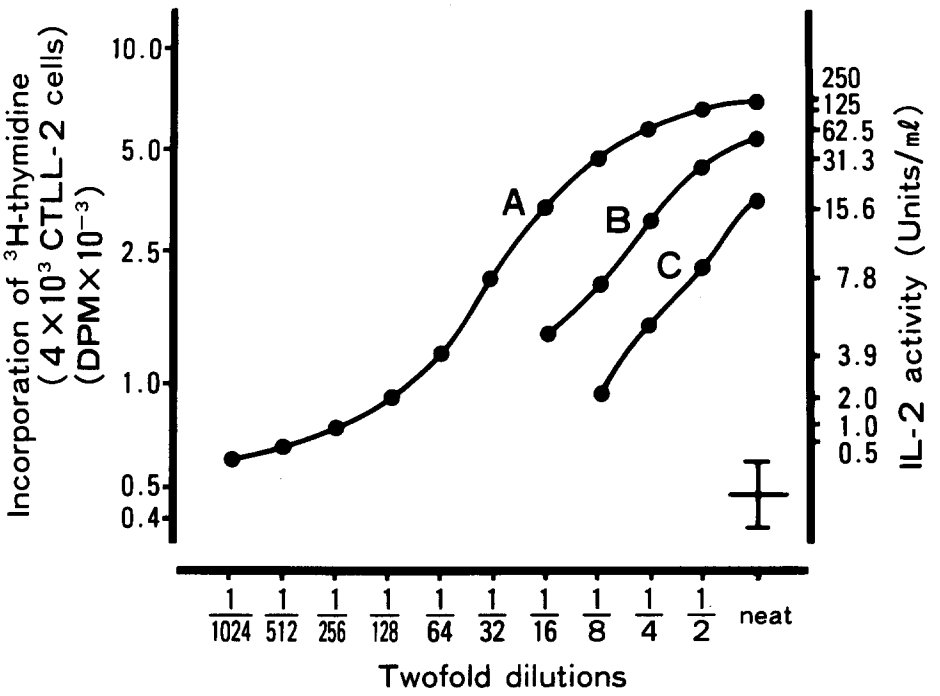

FIG. 2. IL-2 assay: dilution curves of rIL-2 standard **(A)**, 24 hour culture supernatant from a ConA-stimulated PBMCs of a patient with AD sensitive to hen's egg **(B)**, and 4-day culture supernatant from ovalbumin (2.5 μg/ml)-stimulated PBMCs of the patient with AD **(C)**. Each value is the mean in triplicate cultures. Units of IL-2 activity are defined in relation to the rIL-2 standard. *Line* and *bar* indicate mean and 1 SD (n = 12) of the incorporation by CTLL-2 cells cultured without rIL-2.

curves obtained with ConA- and ovalbumin-stimulated PBMC culture supernatants were parallel to those seen with the rIL-2 (Fig. 2). The clinical data, RAST scores for hen's egg, proliferative responses of PBMCs to ovalbumin, IL-2 activity, and IFN-γ concentrations in culture supernatants of ovalbumin-stimulated PBMCs, and expression of Fcε R II on CD20+ cells in ovalbumin-stimulated PBMCs for patients with AD who are sensitive to hen's egg are presented in Table IV. As shown in Table IV and Table V, IL-2 activity and IFN-γ could be detected well in culture supernatants of ovalbumin-stimulated PBMCs from patients with AD who are sensitive to hen's egg. However, they were lower than those in culture supernatants of ConA-stimulated PBMCs (Table V). Small amounts of IL-2 activity and IFN-γ were detected in culture supernatants of ovalbumin-stimulated PBMCs from control group I and control group II. IL-2 activity and IFN-γ concentrations in culture supernatants of ovalbumin-stimulated PBMCs from patients with AD was significantly higher ($p < 0.005$ for each) than those of control group I and control group II, respectively (Table V).

Expression of Fcε R II on cultured cells

Expression of Fcε R II on cultured cells was detected by immunofluorescence studies with use of double-labeling methods (Table IV and Table VI). Expression of Fcε R II on CD20+ cells increased in ovalbumin-stimulated PBMC cultures from patients with AD sensitive to hen's eggs as well as control group II, whereas it was less than that in cultures with rIL-4 (Table VI). Expression of Fcε R II on CD20+ cells in ovalbumin-stimulated PBMC cultures from patients with AD sensitive to hen's egg was significantly greater ($p < 0.005$) than that of control group I, but it tended to be less than that in control group II (Table VI).

DISCUSSION

Concerning the time of reaction in double-blind, placebo-controlled food challenges, we defined the time of reaction as being the time from the first challenge dose to the onset of symptoms. Hill et al.[19] also defined the time of reaction in the same manner. However, Bock et al.[11] and Bock and Atkins,[20] Sampson,[21] Sampson and McCaskill,[22] and Burks et al.[23] defined the time of reaction as being the duration from the last challenge dose to the onset of symptoms. Moreover, our first challenge dose was one capsule (0.4 gm). We continued the doubled doses every hour (1, 2, 4, 8, and 16 capsules) until the patient exhibited convincing symptoms or until 16 capsules of dried food were ingested as a single challenge dose. In their

TABLE IV. Clinical data, RAST scores for hen's egg, proliferative responses of PBMCs to ovalbumin, IL-2 activity and IFN-γ concentrations in culture supernatants of ovalbumin-stimulated PBMCs, and expression of FcεRII/CD23 on CD20+ cells in ovalbumin-stimulated PBMCs for patients with AD who are sensitive to hen's egg

Patients	Age	Severity of AD (other allergic symptoms)	RAST scores for hen's egg	Maximal proliferative responses of PBMCs to ovalbumin (SI)	Culture supernatants of PBMCs*				CD20+ CD23+ cells in cultured cells (%)†	
					IL-2 (units/ml)		IFN-γ (units/ml)			
					(−)	ovalbumin	(−)	ovalbumin	(−)	ovalbumin
1	6 mo	Mild	0	2.34	0.6	6.7	1.9	4.3	1.3	1.8
2	2 yr	Mild	1	2.56	0.2	5.6	1.2	3.8	1.0	2.0
3	4 yr	Mild	2	3.01	0.4	6.1	0.8	3.6	0.8	1.7
4	5 yr	Mild	2	2.31	0.2	3.3	0.9	1.8	0.9	1.4
5	9 mo	Mod. (WH)	0	4.39	0.3	7.5	2.1	7.8	0.7	1.0
6	1 yr	Mod.	3	4.15	0.5	8.4	0.9	2.8	1.9	2.5
7	2 yr	Mod.	2	3.22	0.4	4.6	1.2	2.9	0.7	3.1
8	2 yr	Mod.	0	4.79	0.2	10.2	1.6	8.7	0.9	2.5
9	3 yr	Mod.	3	2.30	0.3	7.9	0.9	3.2	1.1	3.6
10	7 yr	Mod. (BA)	3	2.19	0.6	6.2	0.6	4.8	1.2	3.2
11	9 mo	Sev.	0	6.75	0.7	16.1	1.8	7.9	0.5	1.3
12	2 yr	Sev. (WH)	2	4.38	0.7	12.9	2.2	11.0	0.8	2.0
13	2 yr	Sev.	4	2.86	0.5	8.5	2.1	5.1	0.9	5.1
14	3 yr	Sev.	0	3.92	0.9	8.4	1.5	4.9	1.1	3.1
15	5 yr	Sev. (BA, AR)	0	4.14	0.6	9.9	1.4	8.1	1.0	2.4

Mod., Moderate; *Sev*, severe; *WH*, wheezing; *BA*, bronchial asthma; *AR*, allergic rhinitis.

*(−), Cultures of PBMCs without any antigen or any mitogen for 4 days; ovalbumin, cultures of ovalbumin (2.5 μg/ml)-stimulated PBMCs for 4 days.

†(−), Cultures of PBMCs without any antigen, any mitogen, or rIL-4 for 7 days; ovalbumin, cultures of ovalbumin (2.5 μg/ml)-stimulated PBMCs for 7 days.

studies the allergen doses were increased more rapidly,[21, 23] because up to 8 or 10 gm of dehydrated food was administered over 1 hour. Thus the speed of increasing the dose in our procedure is slower than that in their procedures. For these reasons we consider that our patients with AD seem to exhibit much later onset of clinical responses to food challenges than reported by several other groups.[20, 22, 23]

In our previous study it was shown that the proliferative responses of PBMCs to ovalbumin or BSA in children with AD who are sensitive to hen's egg or cow's milk were significantly higher than those of healthy children and children with immediate symptoms who are sensitive to hen's egg or cow's milk, but that in patients with AD no significant correlations occurred between the proliferative responses of PBMCs and the RAST values.[9] Although BSA is not considered to be a major milk allergen, especially in humoral immunity,[24] proliferative responses of PBMCs to BSA correlated well with the responses to β-lactoglobulin in patients with AD in our study who are sensitive to cow's milk (data not shown). Moreover, BSA is a single protein for which the amino-acid sequence is known.[25] We used BSA as a cow's milk allergen instead of β-lactoglobulin, which has three genetic variants, and casein, which includes some proteins, because we aim to investigate the interaction between T-cell receptors, major histocompatibility complex, and epitopes of the antigen, that is, a single protein, in the responses of CD4 T lymphocytes to the antigens.

In this study we have shown that the proliferative responses of T lymphocytes plus monocytes to ovalbumin or BSA in children with AD who were sensitive to hen's egg or cow's milk were significantly higher than those of healthy children and children with immediate allergic symptoms who are sensitive to hen's egg or cow's milk. Moreover, in patients with AD who were sensitive to hen's egg, CD4 T lymphocytes showed excellent proliferation to ovalbumin when cultured with monocytes, whereas CD8 T lymphocytes showed little proliferation to ovalbumin when cultured with monocytes. The responding cells were also shown, through experiments involving T-lymphocyte phenotypes, to be predominantly CD4+ cells.

Several observations have suggested that the CD4+ helper-inducer T-lymphocyte subset is itself heterogeneous.[26] Mosmann et al.[27] and Mosmann and

TABLE V. IL-2 activity and IFN-γ concentrations in culture supernatants of ovalbumin-stimulated PBMCs for control group I, control group II, and patients with AD who are sensitive to hen's egg

Subjects* (no.)	IL-2 activity in culture supernatants of PBMCs (units/ml)†			IFN-γ concentrations in culture supernatants of PBMCs (units/ml)†		
	(−)	ovalbumin	ConA	(−)	ovalbumin	ConA
Control group I (N = 11)	0.4 ± 0.2	1.0 ± 0.4	24.1 ± 6.0	0.8 ± 0.3	1.3 ± 0.3	21.4 ± 4.4
Control group II, sensitive to hen's egg (N = 8)	0.6 ± 0.2	2.1 ± 1.0	27.2 ± 8.1	0.9 ± 0.6	2.0 ± 0.9	22.4 ± 9.3
Patients with AD, sensitive to hen's egg (N = 15)	0.5 ± 0.2	8.2 ± 3.1‡	27.4 ± 8.6	1.4 ± 0.5	5.4 ± 2.6§	23.4 ± 8.0

*Control group I, healthy children; control group II, patients with immediate allergic symptoms.

†(−), Cultures of PBMCs without any antigen or any mitogen for 4 days; ovalbumin, cultures of ovalbumin (2.5 μg/ml)-stimulated PBMCs for 4 days; ConA, cultures of ConA (5 μg/ml)—stimulated PBMCs for 24 hours; each value is mean ± 1 SD.

‡The value was significantly higher ($p < 0.005$ for each) than the value of control group I or control group II, respectively. The value was also significantly higher ($p < 0.005$) than the value in cultures of PBMCs without any antigen or any mitogen in the same patients with AD.

§The value was significantly higher ($p < 0.005$, for each) than the value of control group I or control group II, respectively. The value was also significantly higher ($p < 0.005$) than the value in cultures of PBMCs without any antigen or any mitogen in the same patients with AD.

TABLE VI. Expression of Fcε RII/CD23 on CD20+ cells when PBMCs were cultured with ovalbumin or rIL-4, for control group I, control group II, and patients with AD who are sensitive to hen's egg

Subjects* (no.)	Percentages of CD20+ CD23+ cells in cultured cells (%)†		
	(−)	ovalbumin	rIL-4
Control group I (N = 11)	0.9 ± 0.4	1.3 ± 0.3	2.2 ± 0.4
Control group II, sensitive to hen's egg (N = 8)	1.6 ± 0.8	3.1 ± 1.0	6.2 ± 2.3
Patients with AD, sensitive to hen's egg (N = 15)	1.0 ± 0.3	2.4 ± 1.0‡	5.1 ± 3.0

*Control group I, healthy children; control group II, patients with immediate allergic symptoms.

†(−), Cultures of PBMCs without any antigen, any mitogen or rIL-4 for 7 days; ovalbumin, cultures of ovalbumin (2.5 μg/ml)-stimulated PBMCs for 7 days; rIL-4, cultures of PBMCs with rIL-4 (200 U/ml) for 7 days; each value is mean ± 1 SD.

‡The value was significantly higher ($p < 0.005$) than the value of control group I, but the value tended to be lower than the value of control group II.

Coffman[28] have reported that mouse helper T-cell clones fall into two main groups (TH1 and TH2), defined primarily by differences in the pattern of lymphokines synthesized. TH1 clones synthesize IL-2, IFN-γ, and lymphotoxin, whereas these lymphokines are not detectably expressed in TH2 clones. Conversely, only TH2 clones synthesize detectable amounts of IL-4 and IL-5. TH1 clones also cause effective delayed type of hypersensitivity reactions. IL-4 causes increased IgE production as well as in-

creased levels of Fcε R II on B lymphocytes.[13] Pene et al.[13] reported that IgE production of human lymphocytes was suppressed by IFN-γ. Paliard et al.[29] reported that CD4+ T-cell clones isolated from human peripheral blood could not be grouped into subsets comparable to the TH1 and TH2 cells in the mouse. Recently, however, Romagnani et al.[30] reported that IFN-γ and IL-4 could be produced by different T-helper cells in human beings, as shown in mice, but they could also be the product of the same T-cell clones. Moreover, Wierenga et al.[31] presented evidence for the presence of distinct functional subsets of CD4+ T cells in human beings on the basis of the production of IL-4 and IFN-γ. In this study IL-2 activity and IFN-γ concentrations in culture supernatants of ovalbumin-stimulated PBMCs from patients sensitive to hen's egg with AD were significantly higher than those of healthy children and patients with immediate symptoms who are sensitive to hen's egg. Expression of Fcε R II on B lymphocytes in ovalbumin-stimulated PBMC cultures from patients sensitive to hen's egg with AD was significantly greater than that of healthy children, but it tended to be less than that in children with immediate symptoms who are sensitive to hen's egg. These results suggest that in patients with AD who are sensitive to foods, the CD4+ T lymphocytes stimulated by food antigens secrete well lymphokines such as IL-2 and IFN-γ that are secreted from TH1 clones in mice, in addition to expression of Fcε R II on B lymphocytes that is induced by IL-4 secreted from TH2 clones in mice, and that, as a result, cell-mediated immunity may also occur in addition to IgE-mediated hypersensitivity. Moreover, in patients with AD who have low specific IgE antibody to the offending food antigen and whose symptoms are late in onset after food ingestion, IgE production of B lymphocytes may be suppressed by IFN-γ, and consequently, cell-mediated immunity may predominantly occur compared with IgE-mediated hypersensitivity. However, it is not clear how cell-mediated immunity is correlated with IgE-mediated reaction in the pathogenesis of AD. Experiments along these lines are now under way.

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Effect of elimination diets on food-specific IgE antibodies and lymphocyte proliferative responses to food antigens in atopic dermatitis patients exhibiting sensitivity to food allergens

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Peripheral blood mononuclear cells (PBMCs) from patients with atopic dermatitis (AD) selected as being sensitive to hen's egg or cow's milk responded to food antigens, ovalbumin, or bovine serum albumin, with significantly enhanced DNA synthesis compared with the DNA synthesis in PBMCs from nonatopic control subjects and food-sensitive patients with immediate symptoms. Patients were treated with elimination diets. Symptoms of AD had been in remission during elimination diets. The levels of specific IgE antibodies to hen's egg or cow's milk decreased during elimination diets in patients with positive radioallergosorbent test (RAST). In patients with negative RAST, specific IgE antibodies remained negative during elimination diets. The proliferative responses of PBMCs to food antigens also decreased during elimination diets in patients with proliferative responses before elimination diets. Taken together, specific IgE antibodies to food antigens are useful indexes of the effect of elimination diets in food-sensitive patients with AD and positive RAST, and proliferative responses of PBMCs to food antigens are useful indexes of the effect of elimination diets in food-sensitive patients with AD and proliferative responses of PBMCs. (J ALLERGY CLIN IMMUNOL 1993;91:668-79.)

Key words: *Atopic dermatitis, elimination diets, food allergy, lymphocyte proliferative responses, specific IgE antibodies*

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