

Characterization of ovomucoid-specific T-cell lines and clones from egg-allergic subjects

Eigenmann PA, Huang S-K, Sampson HA. Characterization of ovomucoid-specific T-cell lines and clones from egg-allergic subjects. *Pediatr Allergy Immunol* 1996; 7: 12–21. © Munksgaard 1996.

In the pathogenesis of allergic reactions, T cells and cytokines play a major role. However, characterizations of food allergen-specific T cells are very limited. In this study, we screened the peripheral blood mononuclear cells (PBMC) of 14 patients for reactivity to ovomucoid (*Gal d I*), the major hen's egg allergen, and ovalbumin (*Gal d II*). Cell lines and clones specific to ovomucoid were generated from PBMC of four egg-allergic subjects, in order to study antigen domain specificity and cell cytokine production profiles. The results demonstrated, firstly, that egg-allergic patients respond to ovomucoid rather than to ovalbumin, and, secondly, that antigen specificity is predominantly directed toward the second and third domains of ovomucoid. The T-cell cytokine message was characterized by reverse transcriptase polymerase chain reaction (RT-PCR). Cell lines and clones from all four patients consistently expressed interleukin (IL)-5, IL-4, IL-13, and interferon-gamma were found to be expressed only by certain lines or clones. This observation suggests a central pathogenic role for IL-5 in food allergy-related symptoms.

**Philippe A. Eigenmann¹,
Shau-Ku Huang², Hugh A. Sampson¹**

Divisions of ¹Pediatric Allergy and Immunology and ²Clinical Immunology, Johns Hopkins University, School of Medicine, Baltimore, MD, USA

Hugh A. Sampson, MD, Division of Pediatric Allergy and Immunology, Johns Hopkins Hospital/CMSC 1102, 600 N. Wolfe St., Baltimore, MD 21287-3923, USA

Key words: cytokines; reverse transcriptase polymerase chain reaction; food hypersensitivity; antigenic determinants; T cells.

Received October 25, 1995
Accepted December 27, 1995

T cells and T-cell cytokines play an important role in the modulation of allergic responses and IgE production. Specific epitope recognition of the corresponding antigen by TCR initiates an immune response and cytokine production. Major antigenic epitopes may be defined by stimulating T cells with short peptide fragments, obtained either by protease cleavage of antigenic proteins, or by generation of overlapping synthetic peptides. Over the last several years, diverse allergen-specific T-cell lines and clones have been extensively studied. For example, ragweed antigen recognition by T cells was defined by Meuer et al. (1), house-dust mite antigen *Der p I* by Lanzavecchia et al. (2), birch pollen antigen *Bet v I* by Ebner et al. (3), and, more recently, phospholipase A₂ antigen from bee venom by Carballido et al. (4). Besides bee venom allergen, most of the cell lines were specific to aeroallergen with symptoms mainly of the respiratory tract.

Peptide-mediated immunomodulation of T-cell responses to dust-mite allergen has been documented. Establishing antigen-specific T-cell lines and clones allows extensive studies of these mechanisms at the molecular level. House-dust-mite-specific T-cell responses could be downregulated by an antigen-blocking peptide binding to selected HLA class II molecules (5). T-cell response to dust-mite aller-

gen was abrogated in nude mice when a peptide corresponding to the major T-cell epitope was administered by nebulization (6). Ultimately, a better understanding of T-cell epitope recognition and modulation may lead to more specific and safer forms of immunotherapy in IgE-mediated allergic disease (7).

Diverse cytokines are secreted by T cells to modulate allergic responses. Interleukin (IL)-4, IL-12, IL-13, and interferon-gamma (IFN- γ) have been shown to be involved in antibody class switching, modulation of antibody secretion, and chemotaxis of inflammatory cells. IL-4 and IL-13 are the principal cytokines promoting B-cell switching to IgE isotype production, and, together with IL-5, are cytokines characteristic of TH2 cells. In contrast, IFN- γ is secreted by TH1 subsets and inhibits switching of B cells to IgE isotype production (8). IL-5 has been found to be expressed in bronchial biopsies of asthmatic patients after antigen challenge (9), and also in skin biopsies from patients with chronic atopic dermatitis lesions (10). These findings have been correlated with an increase in eosinophil numbers, suggesting T-cell involvement in eosinophil recruitment in target organs.

Although T-cell processing of specific aeroallergens such as those of *Dermatophagoides* has been extensively studied and well characterized, only

Table 1. Proliferative response of PBMC in egg-allergic patients (six children and four adults) to ovomucoid and/or ovalbumin

Patients	No antigen (cpm)	Ovomucoid (cpm /SI)	Ovalbumin (cpm /SI)
E. R.	499	1191/2.4	841/1.7
B. P.	2002	23034/ 11.5	7543/3.8
J. S.	2835	8645 / 3	685/0.2
F. S.	209	11269/54	606/2.9
T. Y.	370	1012/2.7	631/1.7
C. C.	2472	9753/ 3.9	264/0.1
T. K.	279	670/2.4	222/10.8
P. B.	479	1920/4	297/0.6
X. M.	506	804/1.6	1261/2.5
J. K.	782	13723/17.5	136/0.2

Ovomucoid vs. ovalbumin: $P = 0.02$.

limited information is available on the T-cell response to food allergens (11). Egg is one of the most common sources of food allergens, and elicits various clinical symptoms (12). In a previous report, ovomucoid (*Gal d I*) was found to be the major hen's egg allergen, since egg-allergic patients diagnosed by double-blind, placebo-controlled food challenge (DBPCFC) had greater skin test reactivity to ovomucoid than to ovalbumin (*Gal d II*), as well as higher levels of ovomucoid-specific IgE in the serum. Furthermore, ovomucoid-specific IgE levels correlated with clinical reactivity to egg, whereas antibodies to ovalbumin, ovotransferrin (*Gal d III*), and lysozyme (*Gal d IV*) did not (13).

In this communication, we report the generation of ovomucoid-specific T-cell lines and clones for studies of antigen specificity, and antigen-driven T-cell cytokine expression in egg-allergic subjects. In an initial screening, peripheral blood mononuclear cells (PBMC) grown from egg-allergic patients were found to be predominantly specific to purified ovomucoid, but not to ovalbumin, providing further support for our previous observations. The CD4⁺ T-cell lines and clones subsequently generated from four egg-allergic subjects showed variable proliferative responses to the ovomucoid do-

mainly, with T cells from one patient consistently proliferating to the second domain, and from a second patient to the third domain. Cytokine profiles of antigen-stimulated T cells determined by reverse transcriptase polymerase chain reaction (RT-PCR) revealed that IL-5 was consistently expressed by all T cells. IL-4, IL-13, and IFN- γ were found to be expressed only by specific lines or clones.

Material and methods

Initial screening for ovalbumin and ovomucoid

PBMC were separated on Ficoll-Hypaque™ (Pharmacia Biotech, Piscataway, NJ, USA, and Sanofi-Wintrop, New York, NY, USA), and prepared as previously described (14). The cells were washed and resuspended at 4×10^6 cells/ml in RPMI 1640 medium with 5% autologous plasma, supplemented with 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin (Gibco Life Technologies, Grand Island, NY, USA). A total of 200000 cells per well was cultured in the presence of either purified grade VII ovomucoid (200 µg), or ovalbumin (260 µg) (Sigma Chemicals, St Louis, MO, USA). Ovalbumin was repurified to remove contaminating ovomucoid, as described previously (13). The purity of both antigens was verified by SDS-PAGE. Both negative controls (culture media only) and positive controls (either PHA and/or tetanus toxoid) were included in each assay. Previous studies showed that proliferation was maximal at 7 days. After this culture time period, the cells were pulsed for 18 h with 1 µCi ^3H -labeled thymidine (Amersham, Arlington Heights, IL, USA), harvested, and counted on a liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA). The results of triplicate test conditions were expressed as an average cpm, and as a stimulation index (SI) ($\text{SI} = \text{cpm with antigen} / \text{cpm without antigen}$). An SI equal to or greater than 2 was considered significant. Statistical analysis was performed by Fisher's exact test.

Table 2. Proliferative response of PBMC from egg-allergic patients to ovomucoid and each of three ovomucoid domains

Patients	No antigen	Ovomucoid (cpm/SI)	OMCHI1 (cpm/SI)	OMCHI2 (cpm/SI)	OMCHI3
E. R.	499	1191/2.4	420/0.8	411/0.8	423 1 0.8
B. P.	2002	23034/11.5	4225 1 2.1	4917/2.5	424412.1
J. S.	2835	8645/3	2006/0.7	259810.9	3032/1.1
N. L.	538	37410.7	24710.5	982/1.8	26310.5
T. Y.	370	1012/2.7	1502/4.1	643/1.7	1513/4.1
C. C.	2472	9753/3.9	ND	96810.4	3746/1.5
J. B.	3607	8788/2.4	7414/2.1	4442 1 1.2	6025/1.7
L. B.	550	87 1 0.2	61 10.1	36710.7	172/0.3
J. C.	2313	16835/7.3	ND	2729 1 1.2	5257 1 2.3
A. G.	919	5849/6.4	ND	1090/1.2	1303/1.4

Results are expressed as cpm or as stimulation indices (SI). ND: not determined.

Patients

Six children (average age 5.6 years; range: 2–16 years) with atopic dermatitis and egg allergy diagnosed by DBPCFC, and four egg-allergic adult patients were screened for antigen specificity to ovalbumin or ovomucoid. Subsequently, cell lines and clones were generated from four adult egg-allergic subjects. All four patients were prick skin test positive and had a history of IgE-mediated reactions to egg, with predominantly upper respiratory and gastrointestinal symptoms; none had active atopic dermatitis. Two were male and two female; three patients were Caucasian and one was Asian. Ovomucoid domain specificity was studied on the cell lines and clones, and on PBMC from 10 egg-allergic children with atopic dermatitis and positive DBPCFC (average age 5.5 years; range: 2–12 years).

Ovomucoid

Ovomucoid (*Gal d I*) is one of the major proteins in hen's egg white. The protein is 186 amino acids long (28 kDa), and is composed of three major domains of similar length, OMCHI1 (residue to 1–68), OMCHI2 (residue to 65–130), and OMCHI3 (residue to 131–186) (15). The three highly purified domains utilized for domain-specificity studies were isolated after trypsin digestion and affinity column separation, as described elsewhere (15). They were graciously provided by M. Lakowski, Purdue Uni-

versity, West Lafayette, IN, USA. There was no cross-contamination in any sample, as confirmed by ELISA using domain-specific monoclonal antibodies.

Establishing T-cell lines and clones

PBMC were isolated on Ficoll-Hypaque, washed three times, and resuspended at 4×10^6 cells/ml in RPMI 1640 medium supplemented with 5% autologous plasma. One milliliter of the cell suspension was added to each well of a 24-well plate, and purified ovomucoid at a concentration of 5 mg/ml, or as otherwise specified, was added. On the second day of culture, 20 U/ml of rIL-2 (Collaborative Biomedical Products, Bedford, MA, USA) was added to the culture media. Ten to 14 days later, the cells were harvested and restimulated with 1 mg/ml of ovomucoid in the presence of autologous irradiated PBMC (3300 rads) at the ratio of one T cell to 10 PBMC or irradiated EBV-induced B cells (4000 rads), established as previously described (16), at the ratio of two B cells to one T cell. Cell medium was supplemented with 20 U/ml of rIL-2, and after two to three cycles of antigen stimulation, 50 U/ml of rIL-4 (Genzyme, Cambridge, MA, USA) was also added.

T-cell blasts were cloned after the first restimulation from the cell lines by limiting dilution in Terasaki cell culture plates (Nunc, Naperville, IL, USA). Culture conditions were set up as 1.2 T cells and 50000 fresh PBMC per microwell, in medium supplemented with 20 IU/ml rIL-2 and 50 IU/ml rIL-4, and 1 mg ovomucoid/ml. Preliminary experiments showed that 1.2 T cells per well was the lowest number to obtain an acceptable yield of growing T-cell clones specific to ovomucoid. After 7 days, dividing cells were transferred into round-bottom, 96-well plates, and after a further restimulation, into 24-well plates. T-cell clones were restimulated and expanded periodically in the presence of ovomucoid, and irradiated PBMC as antigen-presenting cells (APC).

Table 3. Cytokine profiles of T-cell lines and clones determined by RT-PCR

	Cytokine mRNA expression			
	IL-4	IFN- γ	IL-5	IL-13
Patient T. K.				
TK1	+	+	+	–
TK2	–	+	+	+
Patient P. B.				
PB2*	+	+	+	–
PB2*	+	–	+	–
PB2*	+	+	+	+
<i>PB2-3E5†</i>	–	–	+	+
<i>PB2-3E5†</i>	–	–	+	–
<i>PB2-2B3</i>	–	–	+	–
<i>PB2-1B7</i>	–	–	+	–
Patient X. M.				
XM1	–	+	+	+
<i>XM1-2C8</i>	+	+	+	+
Patient J. K.				
JK1	–	+	+	–
<i>JK1-3B4</i>	–	+	+	–

Cell lines are in bold; clones are in bold and italic. *Cytokine expression of PB2 cell line was determined on three occasions at 3-week intervals. †PB2-3E5 cytokine expression was determined on two occasions at 8-week intervals. (–) no band on gel; (+) band present on gel.

Table 4. mRNA signal intensity for β -actin, IL-4, IFN- γ , IL-5, and IL-13 for ovomucoid stimulated (muc) and unstimulated control cells (cntrl) from cell line PB2 and clones PB 2-3E5 and XM 1-2C8, measured by densitometry

	β -actin	IL-4	IFN- γ	IL-5	IL-13
PB2 (muc)	0.63	0.17 (0.26)	0.01 (0.02)	0.65 (1.03)	0.07 (0.11)
(cntrl)	0.60	0.07 (0.11)	–	0.60 (1)	0.26 (0.43)
PB2-3E5 (muc)	0.51	–	–	0.40 (0.8)	0.32 (0.63)
(cntrl)	0.25	–	–	–	–
XM1-2C8 (muc)	0.86	0.4 (0.46)	0.11 (0.13)	0.7 (0.81)	0.4 (0.46)
(cntrl)	0.76	–	–	0.5 (0.65)	0.26 (0.34)

Values in parenthesis represent ratio (message intensity with corresponding cytokine/message intensity with β -actin). (–): no band seen on ethidium-stained gels.

Table 5. IL-4, IFN- γ , IL-5, and IL-13 concentration found in supernatant of cell line JK1 and clone JK1-3B4 by ELISA

	IL-4	IFN- γ	IL-5	IL-13
JK1	0.004	0.7	>500	0.6
JK1-3B4	—	—	>500	2.0

Values for IL-4, IL-5, and IFN- γ are in ng/ml. Values for IL-13 are in pg/ml.

Seven days after restimulation, 2×10^6 cells were harvested, purified on Ficoll-Hypaque gradient, washed twice in RPMI 1640, and suspended in phosphate-buffered saline (PBS) with 1% fetal calf serum. Cells were stained with the following monoclonal antibodies: anti-CD3, anti-CD4, anti-CD8, and anti-CD45RO (Becton Dickinson, San Jose, CA, USA). FACS analysis was performed on a two-channel cell sorter (Epics Profile Coulter, Hialeah, FL, USA).

T-cell antigen specificity

The T-cell antigen specificity was confirmed at each restimulation by determining the proliferative response of T cells to ovomucoid, and to a nonrelevant antigen (short ragweed or tetanus toxoid). Proliferation assays were performed by culturing cells from the lines or clones with fresh, irradiated APC (ratio 1:10) for 5 days with or without antigen, and pulsing with ^3H -labeled thymidine. After 18 h, the cells were harvested, and radioactivity was measured. Results were expressed as counts per minute of triplicate cultures (or duplicate cultures in cases where cell numbers were low). Furthermore, the T-cell lines and clones were stimulated at least once with each of the three purified ovomucoid domains to determine the domain specificity of each line and clone.

Cytokine characterization by RT-PCR

Ten to 14 days after restimulation, cultured cells were harvested, washed twice, and resuspended in culture media. One to two million T cells were incubated for 18 h in the presence of irradiated APC with or without ovomucoid. The cells were then harvested and resuspended in PBS and spun down, and the pellet was frozen at -70°C . RNA was extracted from the cells by adding 1 ml of RNeasy to the cell pellet. Cells were mixed thoroughly, and incubated on ice. After 5 min, 200 μl chloroform was added to each sample on ice for 5 min, and then centrifuged for 20 min at 9170 g. The upper layer containing RNA and chloroform was removed; 200 μl of isopropanol was added to it and the mixture stored for 2 h at -70°C for RNA precipitation. After centrifugation for 20 min at 9170 g, all supernatant was removed, and the

cell pellet was dried and resuspended in 10 μl DEPC-water, and frozen at -70°C . RT-PCR was performed with appropriate primers, as previously described (17). Briefly, RNA was reverse transcribed in the presence of oligo-dT, RNase inhibitor, reverse transcriptase, and dNTP(s) (Perkin Elmer, Branchburg, NJ, USA) on a thermal cycler (PTC-100 Programmable Thermal Controller, MJ Research, Watertown, MA, USA). All PCR amplifications were performed at the exponential phase to avoid saturation of the amplification. PCR was performed in the presence of Taq polymerase (0.5 U/reaction), dNTP(s), and primers: denaturation at 95°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 90 s for 40 cycles.

PCR products were loaded on a 2% gel containing 1% NuSieve Agarose (FMC Bioproducts, Rockland, ME, USA) and 1% Agarose (Sigma, St Louis, MO, USA). The gel was stained with 125 $\mu\text{g}/\text{ml}$ ethidium bromide. Each study condition included an appropriate negative and positive control (amplification for β -actin message, and amplification of internal standards (17) for IL-4 and IFN- γ). The intensity of the bands obtained was subsequently measured by scanning densitometry (Ultrosan, LKB,

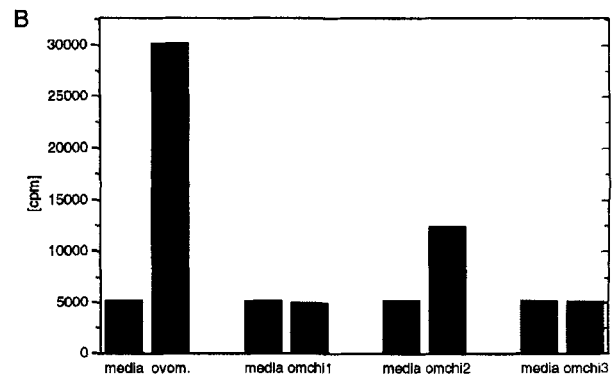
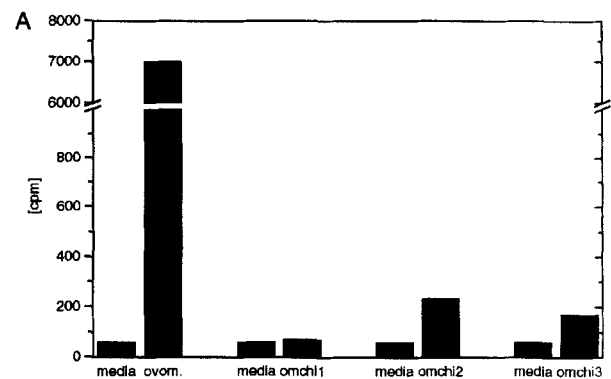


Fig. 1. Proliferation values in counts per minute for cell lines TK1 (A) and TK2 (B).

Broma, Sweden) and compared to the β -actin message.

To confirm that cytokine mRNA expression reflected protein synthesis, cell supernatants were collected 4 days after restimulation and assayed by ELISA for IL-4, IL-5, IL-13, and IFN- γ . With commercially available ELISA kits for IL-4, IL-13, and IFN- γ (Endogen, Cambridge, MA, USA), and for IL-5 (R&D Systems, Minneapolis, MN, USA), supernatants were assayed for cytokines according to the manufacturers' instructions. ODs were read at 490 nm and 650 nm with an automated ELISA plate reader (Molecular Device Corporation, Menlo Park, CA, USA).

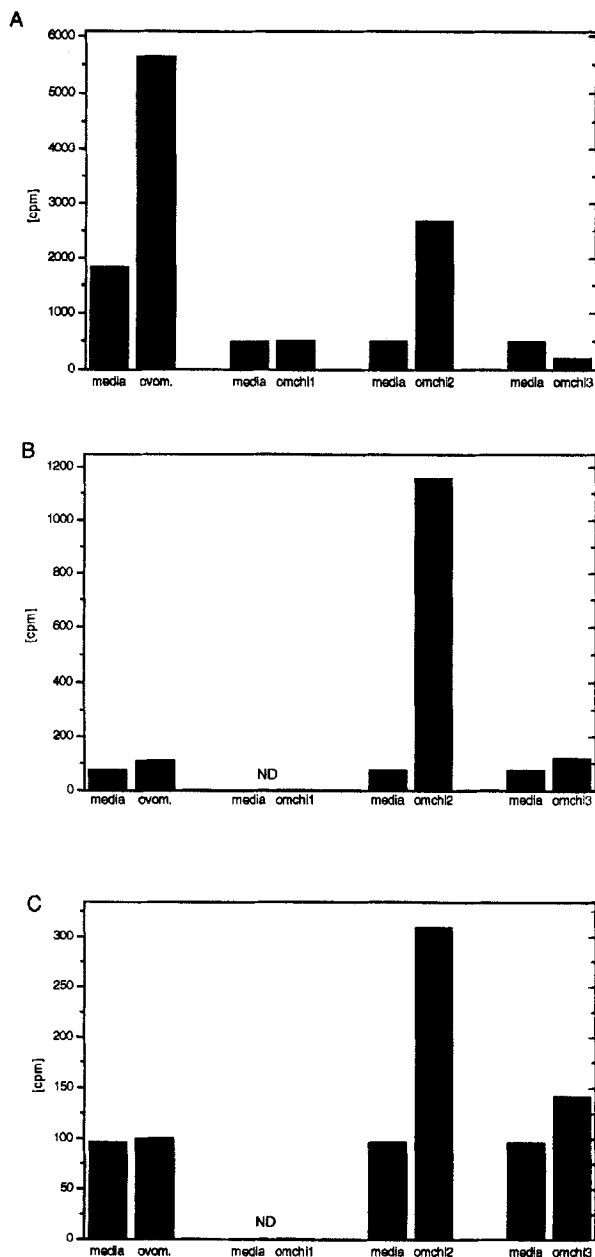


Fig. 2. Proliferation values in counts per minute for cell line PB2 (A), and clones PB2-3E5 (B) and PB2-3E5 (C).

Results

Proliferation to ovomucoid and ovalbumin, and ovomucoid domains

In the 10 egg-allergic patients screened for egg antigen specificity, PBMC had a significantly greater proliferative response to ovomucoid than to ovalbumin ($P < 0.02$), with SIs ranging from 1.6 to 54 for ovomucoid, and from 0.1 to 3.8 for ovalbumin (Table 1). Furthermore, PBMC were screened for ovomucoid domain specificity (Table 2). Domain-induced T-cell proliferation titration studies with three half-logarithmic concentrations from 10 to 100 $\mu\text{g/ml}$ showed optimal T-cell responses with a concentration of 100 $\mu\text{g/ml}$. The reactivity to specific ovomucoid domains was slightly greater than for whole ovomucoid in one patient (T. Y.), who had $\text{SI} = 4.1$ to OMCHI1 and 3, as compared with $\text{SI} = 2.7$ to whole ovomucoid. In other patients, trends could be observed toward domain specificity (OMCHI1 in three patients, OMCHI2 in one patient, and OMCHI3 in three patients).

T-cell lines and clones

In our initial antigen titration studies with four logarithmic ovomucoid concentrations from 10 $\mu\text{g/ml}$ to 10 mg/ml , we found that high concentrations of ovomucoid antigen (1–10 mg/ml) were necessary for appropriate cell growth. Lower ovomucoid concentrations did not induce T-cell growth, and proliferation assay values to ovomucoid were similar to those of cultures without antigen (results not shown). Daily observation of culture conditions in 24-well plates revealed an initial 3–5-day period without apparent T-cell growth, although culture media was supplemented with IL-2 and cells appeared morphologically viable. After this latency period, significant cell growth could be observed with clusters of dividing T cells. Active cell division was still observed in proliferation assays 14 days after restimulation. After further restimulation, proliferation-assay values without antigen were high (cpm of several thousands), confirming ongoing cell division. In addition, after two to three antigen restimulation cycles, a significant slowing of cell division could be observed after restimulation. Different antigen concentrations were added to the cultured cells at subsequent restimulation times but without significant improvement of cell growth. Eventually, the addition of IL-4 (50 U/ml) reversed this process of progressive cell death, and restored appropriate cell division comparable to that observed initially.

Of a total of eight T-cell lines, four were chosen for further cellular characterization. Of these cell lines, two underwent further cloning. Approximately 10 expanding clones were obtained per pro-

cedure. Those with the best cell growth were selected for cytokine-expression studies.

Determination of the T-cell phenotype by flow-cytometry was performed after cell-line specificity for ovomucoid was established. One cell line from each subject was tested. All three cell lines were mainly CD3⁺ cells (98–99%), of which most were CD4⁺ T cells (75–97%). Only a minority of the cells were CD8⁺ T cells (25% to less than 10%). Furthermore, most of the cells (87–99%) were memory T cells (CD 45 R0⁺).

T-cell antigen specificity

All cell lines and clones were found to be ovomucoid-specific, and did not respond to nonrelevant antigens (tetanus toxoid or ragweed, data not shown). When appropriate cell growth was obtained, lymphocyte-proliferation assays were performed to characterize ovomucoid domain specificity. The cell lines from subject T. K. (TK1 and TK2), showed a strong proliferative response to ovomucoid, and a moderate response to OMCHI2 for TK1 and TK2, to OMCHI3 for TK1, but not to

OMCHI1 for either cell line (Fig. 1). The cell line from the second patient, PB2, showed reactivity to ovomucoid and OMCHI2 only. Similarly, the clones derived from this cell line (PB2-2E3 and PB2-3E5) proliferated in response to OMCHI2, but not to OMCHI3. Stimulation indices for OMCHI2 were higher for the PB2-2E3 clone (15.6) than for the cell line (8.4), suggesting that T cells from this patient recognize dominant allergenic epitopes on OMCHI2 (Fig. 2). Cell line XM1, generated from the third subject was found to be specific to ovomucoid; however, neither OMCHI1, OMCHI2, nor OMCHI3 induced proliferation when compared to control conditions. From this experiment, no domain specificity could be established for cell line XM1, or for the cell line derived clone XM1-2C8 (Fig. 3), suggesting a major epitope at the junctional region. T cells from patient J. K. consistently proliferated to ovomucoid with a high SI (line JK1: SI = 139; clone JK1-3B4: SI = 12.7). OMCHI3 induced a significant proliferation for all clones and the JK1 cell line, although OMCHI1 also induced some cell proliferation (Fig. 4).

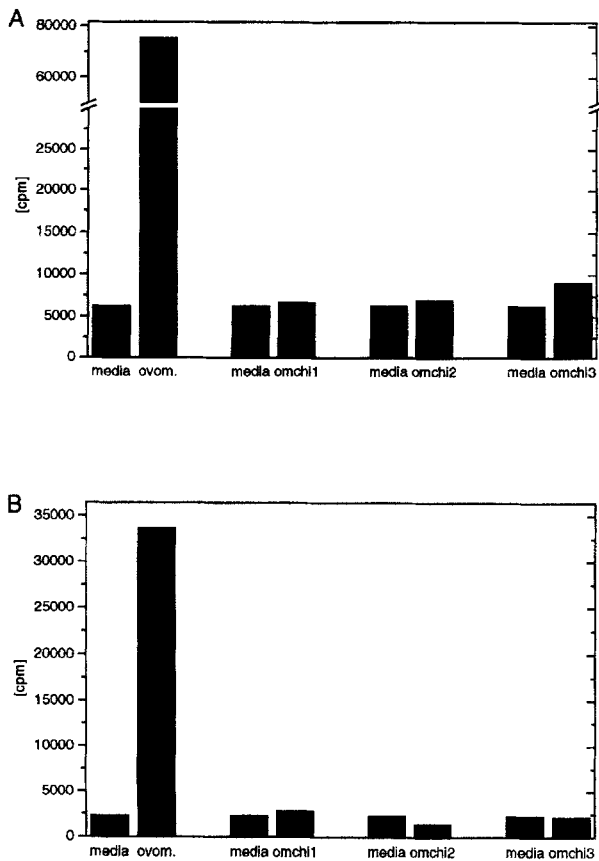


Fig. 3. Proliferation values in counts per minute for cell line XM1 (A) and clone XM1-2C8 (B).

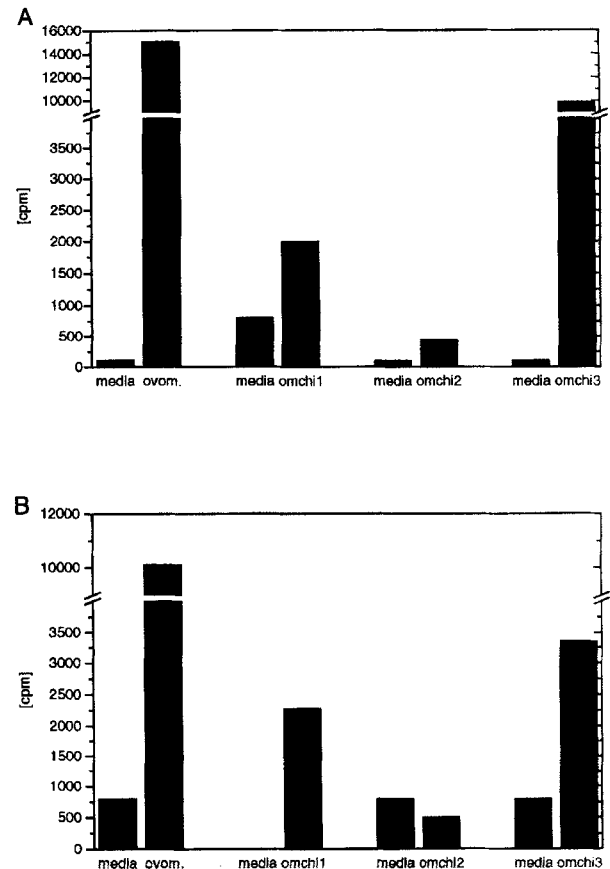


Fig. 4. Proliferation values in counts per minute for cell line JK1 (A) and clone JK1-3B4 (B).

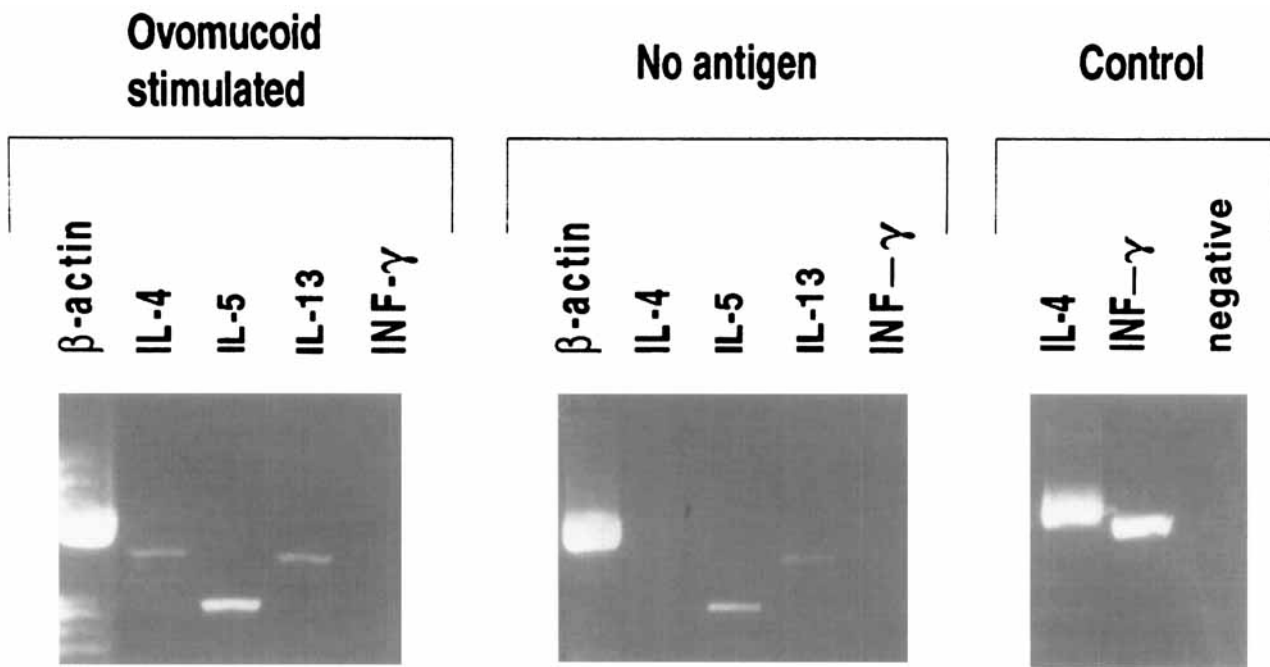


Fig. 5. Cytokine mRNA expression for clone XM1-2C8, stimulated with ovomucoid, and unstimulated.

Cytokine expression characterization by reverse transcription polymerase chain reaction

To examine the cytokine profile of ovomucoid-specific T cells, two culture conditions were set up for each experiment: T cells were restimulated either with or without ovomucoid in the presence of APC. The cells were cultured for various times (12–48 h), and harvested for isolation of RNAs. Although the cytokine message pattern remained similar at all times, RNA obtained after 18 h of antigen stimulation was found to express the highest mRNA signal. No message was found at times between 6 and 12 h.

Cytokine expression for two TK cell lines was characterized (Table 3). TK1 was found to express both IL-4 and IFN- γ . These results suggest that the cell line is composed either of TH0-type cells or of a combination of TH1 and TH2-type cells. In contrast, TK2 was found to express only IFN- γ , and no IL-4. However, mRNA for IL-5 was expressed in both cell lines. IL-13 mRNA could also be detected in cells from TK2. The PB2 cell line was studied for cytokine mRNA at three different times. IL-4 was expressed by the cell line at all three time periods studied. IFN- γ could be detected in two out of three time points. As in TK cell lines, no definite TH cytokine mRNA expression phenotype could be established. Again, IL-5 was expressed consistently by the line and seemed to be the dominant cytokine. Surprisingly, neither of the PB2 clones studied expressed either IL-4 or IFN- γ , but again IL-5 was expressed consistently. The cytokines expressed sug-

gested a TH2-type profile, but without the IL-4 message at detectable levels. IL-13 could be found in conjunction with IL-5 in one clone, again without detectable IL-4. The XM1 cell line was found to express IL-5, IL-4, and IL-13, and its derived clone, XM1-2C8, expressed IL-4, IL-5, IL-13, and IFN- γ . The clone and the cell line from patient J. K. were found to express mRNA for IL-5 and IFN- γ . No IL-4 or IL-13 mRNA could be detected.

For semiquantitation of mRNA expression, the intensity of cytokine mRNA seen on ethidium-stained gels from cell line PB2 and the clones PB2-3E5 and XM1-2C8 was measured by scanning densitometry and compared to the intensity of the β -actin message (Table 4). The greatest intensity was obtained for IL-5 in all cultures. IL-13 was found with a weaker signal in all cultures, while IL-4 was found to be expressed by cell line PB2 and clone XM1-2C8. IFN- γ was found with a weak signal in PB2 and XM1-2C8 cell cultures. Furthermore, bands of lower intensity could be found in control cultures, probably reflecting ongoing proliferation from the previous restimulation.

Supernatants from cell line JK1 and clone JK1-3B4 were assayed by ELISA for IL-4, IL-5, IL-13, and IFN- γ (Table 5). IL-4 and IFN- γ were found in low amounts in the supernatant of the cell line. IL-13 could be measured in both the supernatant of the cell line and the clone. IL-5 was detected in high amounts. These data confirm the predominance of IL-5 secretion for all T cells tested. The occasional discrepancy between cytokine message expression and cytokine secretion may in part be ex-

plained by the time of examination for mRNA expression, which would occur over a limited period, and cytokine secretion by the T cells, which would be cumulative over the course of the culture. However, the consistently high IL-5 secretion pattern indicates that IL-5 may be the dominant cytokine expressed by ovomucoid-specific T cells.

Discussion

In this study, we provide further evidence confirming our previous observations that ovomucoid (*Gal d 1*) is the major egg allergen, and we have generated and characterized ovomucoid-specific cell lines and clones from four egg-allergic subjects. Cell lines from different subjects were found to proliferate differently to various ovomucoid domains, but mostly to OMCHI2 and OMCHI3. Cytokine profile determination by mRNA RT-PCR showed consistent expression of IL-5 by all cell lines or clones, and variable expression of IL-4, IL-13, and IFN- γ , as shown by analysis of cytokine protein in cell supernatants.

Unlike T-cell studies characterizing aeroallergen-specific T-cell lines and clones, only a few abstracts, and one study involving food-antigen-specific T cells have been published to date (11). The authors of the latter study generated an ovalbumin (*Gal d II*)-specific T-cell line from an egg-allergic subject. By performing proliferation assays with several ovalbumin fragments, they found that the ovalbumin peptide 323–339, which is a major T-cell epitope in mice, generated the highest T-cell proliferation in their series of experiments. However, we believe that ovomucoid is the clinically dominant egg allergen in man, since our screening of children with egg allergy by proliferation assays (SI ranging from 1.6 to 54 for ovomucoid and from 0.1 to 3.8 for ovalbumin) supports our previous observation (13).

The growth characteristics of ovomucoid-specific cell lines from four egg-allergic patients appear to differ from those of previously characterized aeroallergen-specific cell lines (18), suggesting the following observations. First, high concentrations of ovomucoid (1–10 mg/ml) are necessary to generate appropriate cell proliferation. In comparison, most aeroallergen-specific cell lines need much lower antigen concentrations for appropriate cell growth (3, 18, 19). Second, the latency period observed between restimulation and formation of cell clusters reflecting T-cell growth suggests that adequate presentation of the antigen to T cells requires a longer time period, since our observations with aeroallergens consistently resulted in active T-cell replication 24–36 h after restimulation, with significant proliferation after 72 h (18). Both of these observa-

tions suggest that ovomucoid processing by APC may differ from the processing of other commonly tested antigens. Ovomucoid is a highly glycosylated protein which is very resistant to digestion, a fact which possibly explains the slower antigen uptake or slower intracellular antigen processing, and the delayed antigen-MHC interaction for T-cell proliferation induction. Furthermore, the ovomucoid-specific cell lines generated could be expanded with IL-2 for a relatively short period of time; after 6–8 weeks (two or three antigen stimulation cycles), the lines often underwent cell death for undetermined reasons, and only the addition of IL-4 could reverse this process. In contrast, antigen-specific T cells are usually grown in the presence of exogenous IL-2 alone and generally can be expanded for a longer time without additional exogenous cytokines (18–20). Overall, our observations suggest a possible difference in antigen processing for ovomucoid, as compared to antigens in aeroallergen-specific T-cell lines previously described (4, 18, 19).

The proliferation-assay data for antigen specificity in our study suggest that different patients recognize different epitopes of ovomucoid. The cell line TK1 responded similarly to OMCHI2 and OMCHI3, suggesting that it contained T cells specific to epitopes in both domains. The cell line TK2 only moderately proliferated to OMCHI2. However, cell line PB2 from another subject expressed a specificity for OMCHI2. Both clones isolated from this cell line (PB2-2E3 and PB2-3E5) also proliferated to OMCHI2. T cells from the third patient (X. M.) did not proliferate specifically to any domain, although a good proliferative response to ovomucoid was observed. This may be explained by modification of epitopes located between two ovomucoid domains. Patient J. K.'s cell line and clone consistently proliferated to OMCHI3, with a partial response also to OMCHI1. Furthermore, PBMC from 10 egg-allergic children with atopic dermatitis diagnosed by double-blind, placebo-controlled food challenges were stimulated with the ovomucoid domains. Two patients responded to OMCHI1, one responded to OMCHI2, three to OMCHI3, and five to none. Taken together, these results indicate that patients recognize different ovomucoid domain specificities. Further efforts to characterize major epitopes of ovomucoid will have to focus on all three regions of the protein with individual patient specificities.

T cells play a key role in allergic diseases by cytokine-modulated IgE synthesis. Although no definite distinct cytokine expression pattern in the ovomucoid-specific cell lines derived from PBMC could be discerned, IL-5 was consistently found in all cell lines and clones, and IL-4, IFN- γ , and IL-13 mRNA were variably expressed. These results suggest that cell lines contain both TH1 and TH2 cells,

or TH0 cells. However, cytokine expression by the clones was predominantly TH2-like, with a consistent IL-5 expression. It has been reported previously that exogenous IL-4 may skew T cells toward TH2-type cytokine expression with predominantly IL-4 secretion (21). This may have influenced our results, but lymphocytes in this study had undergone from two to three restimulation cycles without IL-4, and may have switched from naive T cells to TH1- or TH2-type cells. Furthermore, mRNA cytokine expression of the clones PB2-3E5, PB2-2B3, and PB2-1B7 had been determined with exogenous IL-4 added, and no IL-4-specific message could be found. Further studies will be needed to clarify this issue.

IL-5 is a powerful eosinophil chemotactic and activation agent. It has been demonstrated, together with eosinophil infiltration, in chronic skin lesions of atopic dermatitis patients (10), and in the bronchial mucosa of asthmatic patients, with a direct correlation to eosinophil counts (22). Only a few studies are available on cytokine expression by antigen-specific cell lines and clones. With bee venom phospholipase A₂, the cytokine secretion pattern measured by ELISA has been shown to be antigen-concentration dependent, with low concentrations inducing only IL-4 production, whereas high concentrations induced both IL-4 and IFN- γ (4). Some clones also were found to express IL-5. In another study of ragweed-stimulated PBMC, mRNA amplified by RT-PCR was found to be IL-4 and IL-5 specific (17). The RT-PCR analysis applied in this study is similar to the method used in our experiments, and is the most sensitive procedure to date for cell cytokine-expression analysis. The only other available study with food antigen demonstrated ovalbumin-specific T cells secreting IL-5 only, as measured by ELISA of cell supernatants (11). The absence of IL-4 was not definite, since the sensitivity of the method used was not adequate to detect low-level cytokine synthesis. The consistent finding of IL-5 production by antigen-stimulated T cells in these reports and in our study suggests a predominant role for eosinophils in food-induced T-cell-mediated allergic reactions.

In summary, we established ovomucoid-specific T-cell lines and clones from PBMC. The cell kinetics appeared to differ from those of aeroallergen-specific T cells. This different pattern suggests either distinct antigen-related cell growth, or a phenomenon possibly related to processing by gut-associated lymphoid tissue. Furthermore, T cells from different subjects responded to different ovomucoid domains. T-cell epitope mapping will be necessary to determine which regions are the major epitopes of ovomucoid. Finally, cytokine production determined by mRNA RT-PCR showed a predominance

of IL-5, and variable IL-4 and/or IL-13 and IFN- γ expression. The presence of IL-5 may also suggest T-cell-mediated eosinophil recruitment and activation, although it is not yet well established how circulating eosinophils, or eosinophils in target organs, are specifically involved in food hypersensitivity.

Acknowledgments

P. A. E. is supported by grants from the Swiss National Research Foundation and the Eugenio Litta Foundation. S. K. H. is supported in part by grant no. AI34002 from the NIAID, and H. A. S. is supported in part by grant nos. AI24439 from the NIAID and RR00052 from the DRR, NIH. We would like to thank J. Rowe and D. Ho for excellent technical assistance.

References

1. MEUER SC, COOPER DA, HOGDON JC, et al. Identification of the receptor for antigen and major histocompatibility complex on human inducer T lymphocytes. *Science* 1983; 222: 1239-42.
2. LANZAVECCHIA A, SANTINI P, MAGGI E, et al. *In vivo* selective expansion of allergen specific T cells from atopic patients. *Clin Exp Immunol* 1983; 52: 21-8.
3. EBNER C, SZEPPFALUSI Z, FERREIRA F, et al. Identification of multiple T cell epitopes on *Bet V I*, the major birch pollen allergen, using specific T cell clones and overlapping peptides. *J Immunol* 1993; 150: 1047-54.
4. CARBALLIDO JM, CARBALLIDO-PERRIG N, TERRES G, HEUSSER CH, BLASER K. Bee venom phospholipase A₂-specific T cell clones from human allergic and non-allergic individuals: cytokine patterns change in response to the antigen concentration. *Eur J Immunol* 1992; 22: 1357-63.
5. O'HEHIR RE, BUSCH R, ROTHBARD JB, LAMB JR. An *in vitro* model of peptide-mediated immunomodulation of the human T cell response to *Dermatophagoides* spp (house dust mite). *J Allergy Clin Immunol* 1991; 87: 1120-7.
6. HOYNE GF, O'HEHIR RE, WRAITH DC, THOMAS WR, LAMB JR. Inhibition of T cell and antibody response to house dust mite allergen by inhalation of the dominant T cell epitope in naive and sensitized mice. *J Exp Med* 1993; 178: 1783-8.
7. WALLNER BP, GEFTER ML. Immunotherapy with T-cell-reactive peptides derived from allergens. *Allergy* 1994; 49: 302-8.
8. GEHA RS. Regulation of IgE synthesis in humans. *J Allergy Clin Immunol* 1992; 90: 143-50.
9. BENTLEY AM, MENG Q, ROBINSON DS, HAMID Q, KAY AB, DURHAM SR. Increase in activated T lymphocytes, eosinophils, and cytokine mRNA expression for interleukin-5 and granulocyte/macrophage colony-stimulating factor in bronchial biopsies after allergen inhalation challenge in atopic asthmatics. *Am J Respir Cell Mol Biol* 1993; 8: 35-42.
10. HAMID Q, BOGUNIEWICZ M, LEUNG DYM. Differential *in situ* cytokine gene expression in acute versus chronic atopic dermatitis. *J Clin Invest* 1994; 94: 870-6.
11. SHIMOJO N, KATSUKI T, COLIGAN JE, et al. Identification of the disease-related T cell epitope of ovalbumin and epitope-targeted T cell inactivation in egg allergy. *Int Arch Allergy Appl Immunol* 1994; 105: 155-61.
12. BOCK SA, ATKINS FM. Patterns of food hypersensitivity during sixteen years of double-blind, placebo-controlled food challenges. *J Pediatr* 1990; 117: 561-7.

13. BERNHISEL-BROADBENT J, DINTZIS HM, DINTZIS RZ, SAMPSON HA. Allergenicity and antigenicity of chicken egg ovomucoid (*Gal d* III) compared with ovalbumin (*Gal d* I) in children with egg allergy and in mice. *J Allergy Clin Immunol* 1994; 93: 1047–59.
14. BÖYUM A. Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* 1968; Suppl. 97: 77.
15. KATO I, SCHRODE J, KOHR WJ, LAKOWSKI 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.
16. SUGDEN B, MARK W. Clonal transformation of adult human leukocytes by Epstein-Barr virus. *J Virol* 1977; 23: 503–8.
17. HUANG SK, ESSAYAN DM, KRISHNASWAMY G, et al. Detection of allergen- and mitogen-induced human cytokine transcripts using a competitive polymerase chain reaction. *J Immunol Methods* 1994; 168: 167–81.
18. HUANG SK, ZWOLLO P, MARSH DG. Class II major histocompatibility complex restriction of human T cell response to short ragweed allergen, *Amb a* V. *Eur J Immunol* 1991; 21: 1469–73.
19. O'HEHIR RE, YOUNG DB, KAY AB, LAMB JR. Cloned human T lymphocytes reactive with *Dermatophagoides farinae* (house dust mite): a comparison of T- and B-cell recognition. *Immunology* 1987; 62: 635–40.
20. MOHAPATRA SS, MOHAPATRA S, YANG M, et al. Molecular basis of cross-reactivity among allergen specific human T cells: T-cell receptor V alpha gene usage and epitope structure. *Immunology* 1994; 81: 1520.
21. PALIARD X, DE VRIES JE, SPITS H. Comparison of lymphokine secretion and responsiveness of human T cell clones isolated in IL-4 and in IL-2. *Cell Immunol* 1991; 135: 383–93.
22. HAMID Q, AZZAWI M, YING S, et al. Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma. *J Clin Invest* 1991; 87(5): 1541–6.