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Clinical efficacy and immunological changes subjacent to egg oral immunotherapy

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ABSTRACT

Background: Evidence of the efficacy of food oral immunotherapy (OIT) is not robust enough to change clinical practice from current standard management. Furthermore, the immunologic changes underlying food desensitization are unknown.

Objective: To establish the immunologic basal status and differences between an egg-allergic group of children and a population of nonallergic children and to investigate the safety and efficacy of a specific egg OIT protocol to induce clinical desensitization and the associated immune responses.

Methods: Children with or without egg allergy were recruited. Allergic subjects underwent an OIT protocol based on weekly doses of egg protein and a maintenance phase. Immune profile and changes in all subjects were investigated by measuring T-helper cells types 1 and 2 (T_H1 and T_H2) and T-regulatory cytokines and transcription factors and egg-specific IgE and IgG4 levels.

Results: At baseline, a significantly lower production of ovalbumin-specific interleukin (IL)-10 and tumor necrosis factor- α and a trend toward higher IL-5 and IL-13 were found in allergic children. The egg OIT protocol enabled 60% of them to ingest 32 mL of egg white. Significant increases in egg-specific IgG4 levels and IL-10 production, with a trend toward lower IL-5 and IL-13 and higher tumor necrosis factor- α and interferon- γ levels, and significant decreases in egg-specific IgE concentration were observed.

Conclusion: Egg-allergic individuals display a bias toward T_H2 type cytokine production and decreased T_H1 and IL-10 responses compared with nonallergic individuals. The OIT protocol was safe and effective in inducing egg desensitization, leading to a shift in the immune profile of allergic individuals toward a nonallergic phenotype.

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Introduction

Currently, management of egg allergy is based on the avoidance of egg protein intake. Although most children spontaneously outgrow their egg allergy, up to 15% to 20% remain allergic, and dietetic transgressions that often prove serious can occur.¹ The severity of the reactions usually increases over the years and it becomes more difficult to adhere to the avoidance diet, a situation resulting in school and social problems, with a significant decrease in a patient's quality of life.²

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In recent years, there has been an increasing number of attempts to establish clinical tolerance to egg by specific oral immunotherapy (OIT).³ In OIT protocols, the offending food is administered orally in small doses that are increased slightly.⁴ Although the aim is to establish oral tolerance, understood as the ability to ingest the food without symptoms despite prolonged periods of avoidance or irregular intake, most OIT protocols achieve a desensitization status, in which the patient can ingest the allergen without reaction while continuing to take regular doses of the food.⁵ Desensitization greatly improves a patient's quality of life because the amount of allergen required to trigger reactions is effectively increased compared with the baseline situation, protecting the patient against accidental ingestions.

Egg OIT has shown to be relatively well tolerated and efficient in the induction of desensitization, with a success rate of 50% to 90%. However, although egg OIT efficacy has been suggested, the evidence base is not robust enough to change clinical practice from the current standard management.³ The regimens used in the

Table 1
Egg desensitization protocol^a

Day	EW dilution	Dose (mL)
1	1:10	0.1
8		0.5
15		0.1
22	1:1	0.5
29		1.0
36		2.0
43		4.0
50		8.0
57		12.0
64		16.0
71		20.0
78		24.0
85		28.0
92		32.0

Abbreviation: EW, egg white.

^aOnce patients could tolerate 2 mL of undiluted EW without symptoms, an open challenge with egg-containing baked goods was performed. Similarly, after tolerating 4 mL of EW, patients were challenged with egg-coated foods; boiled egg after 12 mL, French omelet after 20 mL, and fried egg after 32 mL.

different protocols vary from days^{6,7} to years.^{8,9} In all these regimens, the number of adverse reactions recorded is high; however, they are easily controllable, typically involving skin and gastrointestinal symptoms.¹⁰

The exact immunologic changes underlying food desensitization are still unknown, and the conclusions about them extracted from the few reports available are controversial. Results have shown that successful egg desensitization protocols are accompanied by an increase in specific IgG4 levels^{6,8,11}; however the behavior of allergen-specific IgE is highly variable.^{7,8,12,13} Similarly, data related to changes in the cytokine profile associated with egg OIT treatments are thus far sparse and contradictory, with very few studies dealing with the production of specific cytokines after allergen stimulation.^{6,11}

This article presents the results obtained in an open-label study of egg OIT in children with IgE-mediated egg allergy. The first objective was to compare the humoral and cellular responses of an egg-allergic group of patients with a population of nonallergic children of the same age range, with the aim of establishing the basal status and potential differences between groups. Second, the efficacy of a specific

egg OIT protocol to induce clinical desensitization and the associated immune responses was investigated. To evaluate immune changes underlying OIT, a broad panel of cytokines and transcription factors, including T-helper cell types 1 and 2 (T_H1 and T_H2) and T-regulatory (Treg) responses, and egg-specific IgE and IgG4 levels from all the subjects included in the study were measured.

Methods

Ethics Statement

All human samples and procedures were obtained and performed with written consent from next of kin, caretakers, or guardians on behalf of the children involved in the study. The bioethics committees of the Consejo Superior de Investigaciones Científicas and the Hospital Universitario La Paz (Madrid Spain) approved all experiments.

Patient Selection

Subjects were recruited from the Allergology Service at Infanta Sofia Hospital (Madrid, Spain). All children enrolled in the egg-allergic group were patients diagnosed by a compatible clinical history, positive skin prick test reaction (wheal ≥ 3 mm than negative control), and positive egg white (EW), yolk, ovalbumin (OVA), ovomucoid (OM), and lysozyme specific IgE. In addition, all subjects had exhibited a positive reaction during a single-blinded food challenge (SBFC) with commercial pasteurized liquid EW (8.3% protein; Huevos Guillén, Valencia, Spain) the month before beginning the study. A group of nonallergic children with no detectable IgE against a broad panel of the most common allergens also was included in the study.

Egg OIT Protocol

The egg OIT protocol was performed with pasteurized EW as presented in Table 1. The highest tolerated dose during the SBFC was used as the starting dose of the OIT protocol. In this protocol, once the patients could tolerate 2 mL of undiluted EW without symptoms, an open challenge with egg-containing baked goods was performed. Similarly, after tolerating 4 mL of EW, patients were challenged with egg-coated foods; boiled egg after 12 mL, French omelet after 20 mL, and fried egg after 32 mL. Between visits, patients were advised to ingest daily at home the maximum dose

Table 2
Egg-allergic patient's demographics, anamnesis, and response to egg OIT

Patient number	Age (y)	Sex	AA	FA	SBFC symptoms	SBFC dose (mL EW)	Premedication before OIT	OIT symptoms	OIT duration (mo)	Tolerance after OIT (mL EW)
1	12	F	yes	yes	digestive	4.0	yes	yes	12	32
2	5	F	no	yes	skin	0.01	yes	yes	14	32
3	6	M	no	no	skin	4.0	no	no	6.0	32
4	5	M	yes	yes	skin	2.0	no	yes	12	32
5	15	M	yes	yes	anaphylaxis	8.0	no	no	6.0	32
6	12	M	yes	yes	skin	0.1	yes	yes	18	32
7	14	M	yes	yes	skin	0.5	no	no	6.0	32
8	10	M	yes	yes	digestive	0.01	yes	yes	12	32
9	14	F	yes	yes	skin	1.0	yes	yes	12	32
10	11	M	yes	no	skin	0.05	no	no	8.0	32
11	11	F	yes	no	anaphylaxis	0.2	yes	yes	20	32
12	13	M	no	yes	anaphylaxis	0.06	no	no	15	32
13	10	F	yes	no	anaphylaxis	0.1	yes	yes	24	2.0–10
14	9	M	yes	yes	digestive	0.06	yes	yes	18	2.0–10
15	9	M	yes	yes	anaphylaxis	0.001	yes	yes	18	2.0–10
16	14	F	yes	yes	skin	0.01	yes	yes	24	2.0–10
17	15	F	yes	yes	skin	0.1	no	yes	18	<2
18	14	M	yes	no	digestive	8.0	yes	yes	9.0	<2
19	8	F	yes	no	anaphylaxis	1.0	yes	yes	7.0	<2
20	10	F	yes	no	digestive	2.0	no	NA	NA	NA

Abbreviations: AA, allergies to aeroallergens; EW, egg white; F, female; FA, other food allergies; M, male; NA, not applicable; OIT, oral immunotherapy; SBFC, single-blinded food challenge.

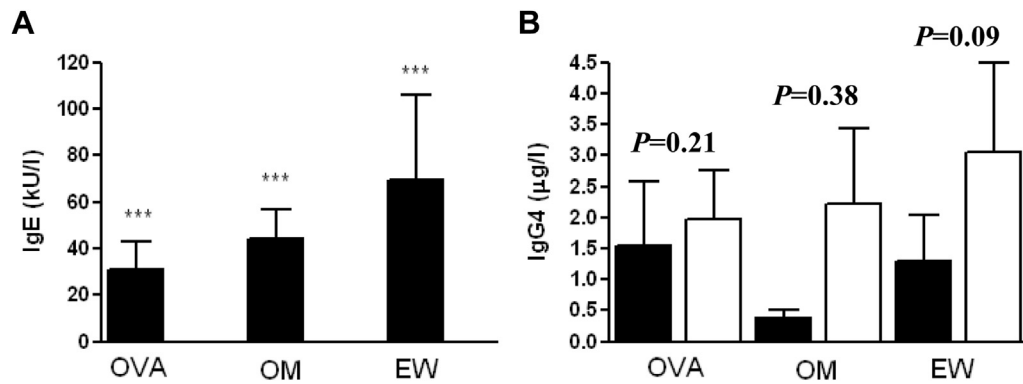


Figure 1. Serum specific (A) IgE (kU/L) and (B) IgG4 (μ g/L) to ovalbumin (OVA), ovomucoid (OM), and egg white (EW) before beginning the oral immunotherapy protocol in egg-allergic children ($n = 20$, black bars) and nonallergic children ($n = 15$, white bars). Bars represent mean $\pm$ SEM. *** $P < .001$.

achieved during their most recent visit to the unit. Premedication with antihistamines was given to those patients who developed adverse reactions during the protocol to control symptoms, if needed. Moderate reactions were pharmacologically treated and the protocol was restarted the following week at the previously tolerated dose. Hence, the length of the protocol underwent step-wise increases depending on the severity of the reactions experienced by each patient. For repeated severe reactions (anaphylaxis), the desensitization protocol was interrupted. Patients were considered to have completed the OIT protocol if they could tolerate 32 mL of pasteurized EW (equivalent to a full EW) in less than 24 months.

Once the patients completed the OIT protocol, they maintained, for 6 months, a daily dose of 16 mL of pasteurized EW at home, except for 2 days per week, when the patients were advised to consume a complete egg (in an omelet or a boiled or fried egg). If, after 6 months, the clinical tolerance was sustained, then the patients were authorized to have an unrestricted diet with the recommendation to eat eggs 2 to 3 times per week.

Measurement of Serum Allergen-Specific IgE and IgG4

Blood samples were withdrawn from the control subjects included in the study and from the allergic patients before and after the OIT protocol was finished, and sera were analyzed for specific IgE and IgG4 levels to OVA, OM, and EW using the Phadia ImmunoCAP System FEIA (Thermo Fisher Scientific, Barcelona, Spain).

In Vitro Stimulation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient separation (Ficoll-Paque PLUS; GE Healthcare, Barcelona, Spain) from heparinized venous blood. The PBMCs (2×10^6 cells/mL) were cultured in vitro for 7 days at 37°C in 5% CO₂ with medium alone (AIM-V, Biowest, Nuaille, France) as a negative control and OVA (200 μ g/mL), and phytohemagglutinin (4 μ g/mL; Sigma, St Louis, Missouri) as positive control. All experiments were performed in 24-well plates (Corning, Corning, New York) with a final volume of 1 mL.

Cytokine Profile Analyses

After 7 days of culture, supernatants were analyzed for levels of interleukin (IL)-5, IL-13, IL-10, interferon- γ (IFN- γ), and tumor necrosis factor- α (TNF- α) by a multiplex bead assay (BD Cytometric Bead Array; BD Biosciences, San Diego, California) according to the manufacturer's instructions. Cytokine secretion was correlated to a standard of each of the human cytokines (0–2,500 pg/mL). The Gallios flow cytometer (Beckman Coulter, Barcelona, Spain) was used to acquire the data, which were analyzed by Beckman Coulter

Kaluza and FCAP Array 3 (BD Biosciences) software. Results are expressed as the amount of each cytokine detected after stimulation with OVA minus the amount detected after stimulation with the negative control.

Quantitative Real-time Polymerase Chain Reaction

Total RNA from the PBMCs cultured for 7 days was extracted using the Total RNA Isolation NucleoSpin RNA II Kit according to the manufacturer's instructions (Macherey-Nagel, Duren, Germany). The RNA template was qualitatively assessed and quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California) and a NanoDrop ND1000 (Thermo Fisher Scientific), respectively. Reverse transcription reactions were performed according to the manufacturer's instructions using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). Quantitative polymerase chain reaction was performed in a ViiA 7 Real-Time PCR System using TaqMan Gene Expression Assay probes (Life Technologies, Carlsbad, California) for genes *GATA3*, *T-bet*, and *FoxP3* according to the manufacturer's instructions. Experimental transcript levels were normalized to those of the hypoxanthine guanine phosphoribosyl transferase (*HPRT*) gene. Data were expressed as relative expression (RQ) values compared with levels measured in the corresponding group by using the $\Delta\Delta$ threshold cycle method of calculation. A minimum RQ value higher than 2 or lower than 0.5 was established as the relevant value.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad, San Diego, California). Data were assessed for normality and equal variation before the appropriate parametric or nonparametric test was applied. Nonparametric Mann-Whitney test or unpaired 2-tailed Student *t* test was used for comparison between groups. Wilcoxon or paired 2-tailed Student *t* test was

Table 3

Cytokine levels after stimulation with ovalbumin of peripheral blood mononuclear cells from egg-allergic children before the egg oral immunotherapy protocol and from nonallergic children^a

Cytokine (pg/mL)	Allergic (n = 20)	Nonallergic (n = 15)	P value
IL-5	38.49 $\pm$ 22.11	8.95 $\pm$ 4.15	.26
IL-13	104.90 $\pm$ 35.00	57.27 $\pm$ 16.23	.27
IFN- γ	22.60 $\pm$ 8.49	82.44 $\pm$ 34.71	.11
TNF- α	96.90 $\pm$ 26.94	409.40 $\pm$ 161.20	<.05
IL-10	120.2 $\pm$ 19.16	337.60 $\pm$ 75.73	<.01

Abbreviations: IFN- γ , interferon- γ ; IL, interleukin; TNF- α , tumor necrosis factor- α .

^aValues are presented as mean $\pm$ SEM.

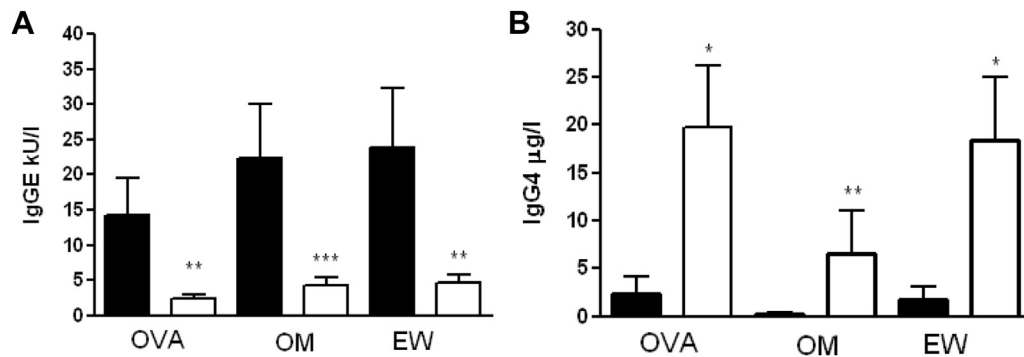


Figure 2. Serum specific (A) IgE (kU/L) and (B) IgG4 (µg/L) to ovalbumin (OVA), ovomucoid (OM), and egg white (EW) before (black bars) and after (white bars) the oral immunotherapy protocol in egg-allergic patients who tolerated 32 mL of egg white (n = 12). Bars represent mean ± SEM. ***P < .001, **P < .01, *P < .05.

used to analyze differences between variables during the OIT protocol. Results are presented as mean ± SEM, unless indicated otherwise. Differences were considered significant at the 95% confidence level.

Results

Baseline Status of Egg-Allergic vs Nonallergic Children

Twenty egg-allergic children (11 boys and 9 girls) 5 to 15 years old (mean 10.8 years) and 15 nonallergic children (7 boys and 8 girls) 5 to 14 years old (mean 8.7 years) were enrolled in the study (Table 2). There were no statistically significant differences in age and sex between groups. All but 3 egg-allergic subjects had allergies to environmental aeroallergens. Also, 65% of them were allergic to foods other than egg (Table 2).

The average dose of pasteurized EW that elicited allergic reactions during the SBFC in the egg-allergic group was 1.56 mL (0.001–8.0 mL), with 45% of patients developing skin-related reactions, 25% developing digestive symptoms, and 30% developing anaphylaxis. None of the recruited children developed respiratory problems during the SBFC (Table 2).

Egg-allergic patients displayed significantly higher levels of OVA-, OM-, and EW-specific IgE than nonallergic subjects (Fig 1A). For IgG4, results showed a trend toward higher concentrations of OM- and EW-specific IgG4 in the nonallergic group compared with the egg-allergic group (Fig 1B).

As presented in Table 3, a trend toward a higher production of T_H2 cytokines (IL-5 and IL-13) after PBMC stimulation with OVA was seen in the egg-allergic patients compared with nonallergic children. In contrast, a decreased T_H1 cytokine concentration (significant for OVA-specific TNF-α) was found in this group of patients. A statistically significant higher production was found for

OVA-specific IL-10 in nonallergic children compared with egg-allergic patients.

Analysis of the expression levels of transcription factors FoxP3, GATA-3, and T-bet between groups showed no apparent differences between them, with RQ values of 1.085 for FoxP3, 0.794 for GATA-3, and 0.949 for T-bet.

OIT Protocol

As presented in Table 2, of the 20 patients enrolled in the study, 12 (patients 1–12) completed the protocol (ie, tolerated the ingestion of 32 mL of pasteurized EW) in an average period of 11.75 months (interval 6–20 months). Fifty percent of this group required premedication with antihistamines and 7 developed symptoms during the protocol. The most common symptoms were mouth itching and lip edema followed by abdominal pain and/or vomiting. After 24 months of treatment, 4 patients (patients 13–16) tolerated 2.0 to 10 mL of pasteurized EW, that is, they could consume egg-containing baked goods and egg-coated foods without anaphylactic reactions. This group of patients had the largest number of adverse reactions during the protocol. In patients 17 to 19, the protocol was stopped after several attempts owing to unacceptable severe adverse effects, including, among others, recurrent anaphylactic manifestations, uvula edema, and severe atopic dermatitis. One patient (number 20) dropped out of the study by parental decision. One year after the OIT protocol was finished, patients 1 to 12 were still tolerant to every egg form without any recorded incidence.

Immunologic Changes During Egg OIT Protocol

All patients who completed the egg OIT protocol (n = 12) had a significant decrease (>5-fold) in their OVA-, OM-, and EW-specific IgE levels (Fig 2A) once the protocol was finished. Furthermore,

Table 4
Cytokine levels after stimulation with ovalbumin of peripheral blood mononuclear cells from egg-allergic patients who tolerated 32 mL of egg white before and after completing the egg OIT protocol and baseline levels for egg-allergic patients who did not successfully complete the protocol^a

Cytokine (pg/mL)	Egg-allergic patients who tolerated 32 mL of egg white before and after OIT (n = 12)			Egg-allergic patients who did not successfully complete OIT (n = 7)	
	Before OIT	After OIT	P value ^b	Before OIT	P value ^c
IL-5	11.93 ± 9.23	3.83 ± 3.22	.42	89.51 ± 59.02	.11
IL-13	79.15 ± 23.93	52.12 ± 21.53	.41	163.70 ± 90.94	.28
IFN-γ	24.50 ± 10.45	53.73 ± 19.33	.20	22.41 ± 17.19	.91
TNF-α	131.70 ± 39.76	277.10 ± 212.90	.51	47.25 ± 26.88	.15
IL-10	97.73 ± 24.03	195.1 ± 38.75	<.05	141.11 ± 30.39	.29

Abbreviations: IFN-γ, interferon-γ; IL, interleukin; OIT, oral immunotherapy; TNF-α, tumor necrosis factor-α.

^aValues are presented as mean ± SEM.

^bBefore vs after OIT in patients who completed the protocol.

^cBefore OIT in patients who completed the protocol vs patients who did not complete the protocol.

a significant increase from baseline was observed in OVA, OM, and EW IgG4 after the egg OIT treatment (Fig 2B).

When comparing cytokine levels before and after the egg OIT protocol in this group of patients ($n = 12$), a trend toward decreased IL-5 and IL-13 production by PBMCs after OVA stimulation and an increase in concentrations of the T_H1 cytokines IFN- γ and TNF- α was found. Furthermore, levels of OVA-specific IL-10 were significantly higher compared with baseline (Table 4).

Transcription factors FoxP3 and T-bet were upregulated in 44.4% and 55.5% of patients (mean of RQ values were 1.57 and 1.76, respectively). However, expression levels of the transcription factors under study did not show adequate levels to consider a relevant genomic variation (data not shown).

Baseline Immunologic Status of Desensitized Egg-Allergic vs Partially Desensitized Patients

When baseline OVA-, OM-, and EW-specific IgE levels were compared between patients successfully desensitized to egg ($n = 12$) and those who did not fully complete the protocol ($n = 7$), egg-protein specific IgE levels were higher in the latter group (14.23 ± 2.51 vs 62.98 ± 29.89 for OVA-specific IgE, $P < .05$; 22.39 ± 7.68 vs 82.53 ± 28.33 for OM-specific IgE, $P = .07$; 23.93 ± 8.30 vs 153.30 ± 99.88 for EW-specific IgE, $P = .07$).

Regarding baseline levels of the different cytokines analyzed, there was a trend toward lower IL-5 and IL-13 and higher TNF- α levels after PBMC stimulation with OVA in the patients who successfully completed the protocol (Table 4).

Discussion

In the present study, the immunologic status of the egg-allergic children enrolled was initially assessed and compared with that of a group of nonallergic children of the same age range. In addition to the presence of egg protein-specific IgE antibodies in plasma, characteristic of the allergic status,¹⁴ PBMCs from egg-allergic patients showed decreased OVA-specific TNF- α and IL-10 production and a trend toward higher IL-5 and IL-13 levels at OVA stimulation. These findings are consistent with other studies demonstrating a bias toward T_H2 -type cytokine production in food-allergic individuals.^{15–18} The authors speculate that the decreased T_H1 and IL-10 responses observed in allergic patients might represent a decrease in T_H1 and IL-10 regulatory cell populations capable of suppressing T_H2 responses. IL-10, in particular, is known to play an important role in the induction and maintenance of antigen-specific T-cell tolerance.¹⁹

The efficiency and safety of the proposed egg OIT protocol were in the range of recent studies by Iacono et al²⁰ and Meglio et al²¹ in which OIT led to the clinical desensitization of 90% and 80% of children, respectively. Owing to moderate side effects, the actual duration of the present desensitization protocol was much longer than originally expected. Similarly, other studies have documented that prolonged treatments enhance the desensitization effect, suggesting that longer treatment courses are more effective and possibly safer.^{21–23} Also, results point out that desensitization periods cannot be planned in advance, making it necessary to individualize the dosing according to each patient's characteristics.¹¹

Spontaneously outgrowing egg allergy in the patients included in the study was very unlikely owing to their high average baseline EW-specific IgE levels and their frequent adverse responses to the therapy.^{24–26} Also, the present protocol cannot confirm that the patients were tolerant to egg, because egg intake was not withdrawn after treatment due to ethical reasons; in a report by Burks et al⁸ in which egg was avoided for 4 to 6 weeks after a desensitization protocol, more than 70% of patients initially desensitized did not pass a subsequent oral food challenge. In any case, it is worth mentioning that all the patients who successfully completed the

present egg OIT protocol could tolerate consuming a complete egg 1 year after the protocol was finished.

The observed changes in antigen-specific IgG4 production in subjects after successful OIT mirror those observed in other food OIT protocols, in which antigen-specific IgG4 increased at the end of immunotherapy.^{6,8,11,21,27,28} Regarding antigen-specific IgE levels, a significant decrease, in accordance with other publications,^{6,7,11,12,20,21} was observed in the present study. However, other studies did not report significant changes in IgE levels during the OIT protocol.^{8,13,28,29} The decrease in the IgE/IgG4 ratio observed during the OIT might be a feature of skewing from allergen-specific T_H2 to Treg predominance. This possibility is supported by the significant increase in IL-10 production by OVA-stimulated PBMCs and by the decrease in IL-5 and IL-13 levels found in patients who could ingest 32 mL of pasteurized EW without developing symptoms (Table 4). Although this study did not measure the populations of egg-specific Treg cells, the role of this cell type in tolerance induction after immunotherapy treatments has been demonstrated.^{27,30} Tregs, through IL-10 secretion, are potent suppressors of total and allergen-specific IgE, whereas they simultaneously increase IgG4 production.^{31,32} The lack of treatment-related changes in the expression of Treg (FoxP3), T_H1 (T-bet), or T_H2 (GATA-3) transcription factors, despite the existence of measurable variations in cytokine production, was reported by Jones et al³³ in their study of peanut OIT.

In the present research, lower baseline antigen-specific IgE levels appeared to be related to successful OIT therapy. A similar observation was made by Vazquez-Ortiz et al³⁴ and García-Rodríguez et al⁷ who found that elevated baseline levels of egg-specific IgE were related to a high probability of discontinuation of the protocol. Furthermore, although not significant, patients able to tolerate 32 mL of pasteurized EW displayed a trend toward lower baseline levels of T_H2 cytokines and an increase in TNF- α secretion after OVA stimulation of PBMCs (Table 4). Along the same lines, IL-4 mRNA has recently been proposed as a possible predictive factor of egg allergy resolution by Sicherer et al.²⁵

Taken together, this report presents an efficient and safe egg OIT protocol characterized by the progressive introduction of egg-containing foods that substantially improve a patient's quality of life. Successful OIT was accompanied by a significant increase in egg-specific IgG4 levels and in IL-10 production by OVA-stimulated PBMCs and by a significant decrease in egg-specific IgE concentration, typical of a nonallergic phenotype. These results highlight the importance of designing individualized protocols, taking into account the baseline egg-specific IgE levels of patients, to achieve a successful desensitization.

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